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Review Article

Application of Statistical Methodology in Quality Control Functions of the Pharmaceutical Industry

A Survey

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INTRODUCTION

"OF WHAT Significance Statistics" was the title of an editorial by Edward G. Feldmann in the November 1963 issue of the *Journal of Pharmaceutical Sciences* (1). Feldmann recognized the increased use of statistics and commented on the merit of the uses to which statistics were then being applied. This paper, which is limited to the application of statistical methods to pharmaceutical quality control, is intended to amplify that editorial.

There has been a steady increase in the use of statistics in the quality control functions of most of the companies in the pharmaceutical industry over the past 10 years. This is not too well evidenced when examining the literature of the pharmaceutical sciences, but is readily apparent if one examines the technical statistical literature.

The past few years have shown that the analytical controls, and those after-the-fact subjective or inspection-type controls employed during the manufacture of a pharmaceutical product may be about only 10% of the effort used to control a product adequately. The other 90% would consist of the people, material, facilities, building, equipment, product design, procedures and specifications, and documentation under which and by which the product is manufactured.

Statistical methods applied to outgoing material, and control charts on analytical assays are after the fact, that is, after the material or product has been manufactured. Statistical methods applied to filling operations or on-line inspection of products are of much more value since they are controlling the material at the time of manufacture. The 1962 drug amendments to the Food and Drug Act of 1938 acknowledge that it is the in-process controls applied to a product which in effect make a quality product.

Five years ago statistical sampling plans with a sensitivity in the range of 1 to 5% defective

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were considered adequate for inspection of packaging supplies. Now engineers would like 0.1% sensitivity for certain types of defects which interrupt high-speed packaging lines. When problems such as these arise, statistical methods must be modified for a different approach to the problem because the cost of sampling 3000 to 5000 units would be prohibitive, let alone the cost to manufacture certain materials with a value of defectiveness so low.

Mainland (2) poses 2 interesting questions that could be applied to our desire for increased knowledge and perfection: "If the verdict of a test is 'significant' at our predetermined level, what will we do in consequence thereof?" And the second question: "If the verdict of a test is 'not significant' at our predetermined level, what will we do in consequence thereof?" These two questions applied to a series of alternative quality control situations will soon show that the way to produce a product is to design it right and do it right the first time. It is obvious that sampling plans which examine 10 out of a million tablets do not adequately control a product. A sampling plan which weighs 1 out of each 100 tablets as they are produced is controlling the product.

The statistical methods which are discussed in this paper are not discussed from an "after-the-fact" or "during-processing" concept, but it is obvious that the value of statistical methods will be the greatest if directed at the during-processing concept. Statisticians working in the quality control field all find quite a change from dealing with the research aspects of pharmaceuticals. The scientist who is inherently an optimist may accept odds of 5 to 1, the statistician being analytically a pessimist prefers odds of 20 to 1, but the quality control man wants zero defects, unrealistic as this may be.

STATISTICS OF ANALYTICAL PROCEDURES

The accuracy of an analytical procedure can be established by applying it to a product or material containing known amounts of the material to be assayed or by using unknowns and running both the new procedure and another whose accuracy has been established.

Accuracy refers to the closeness of the result to the true chemical content. Precision refers to the agreement of repetitive results with each other (3). The accuracy of an analytical test would be the mean of any repeated results as compared to the amount of material predicted to be in the product. The standard deviation may be estimated from the repetitive assays performed on the product and is a measure of the precision.

Youden (3) describes a simple method of analyzing analytical data by plotting the amount found against the amount taken for analysis. The plot should be a straight line. Figure 1 indicates the types of problems encountered with assays. A constant per cent bias of the slope is an inability to obtain in the amount found the same per cent increase that was in the amount taken. A constant intercept bias is the inability to recover the amount found in proportion to the amount taken.

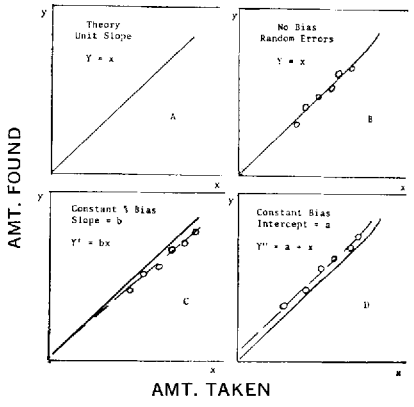

Fig. 1.—Diagram to show the effect of random errors, constant per cent errors, and constant error of analytical results. [Figure 1 of Youden (3).]

Youden describes the many types of problems encountered with the evaluation of new analytical methods including comments on Round Robins. He suggests a half-dozen different materials might be sent to a minimum of 5 different laboratories with requests for only single analytical determinations. He asserts that it is not necessary to ascertain the laboratory's precision because of the large number of results necessary to estimate the precision, the usual faithfulness of deviations from a procedure, and the possibility of censoring the duplicated data.

In a series of papers (4–8), Garrett *et al.* discuss the selection, evaluation, and control of the assay of several pharmaceutical products. Garrett makes use of many statistical techniques in these papers, including control charts, analysis of variance, design of experiments, and degradation rate statistics in addition to the usual statistical tests of significance. Statistical treatment of the data permitted Garrett to determine optimum solvent quantities for separation of the active ingredient, the number of dosage units to assay within a desired confidence limit, the reproducibility of fill weights, the homogeneity of the fill mix, and other relationships between assay economy and product variability. This series of articles provides a good summary of the statistical

methods available to an analytical laboratory in evaluating assay and dosage form variation.

Davies (9) outlines an economical testing program whereby the costs of routine analytical tests are compared to the costs of accepting material which is either above or below stated specifications. His cost for the rejection of acceptable batches (the α-error) is on the basis of reprocessing cost; the cost for accepting unacceptable batches (the β-error) is not quite as easy to quantify.

In the pharmaceutical industry, the probability of wrongly classifying good material is rather low because of the tendency to attempt to test quality into the product. It would be expected that any material to be rejected would be tested sufficiently to prove that point; and so the cost would only be for additional testing. These points are explored further in the paper.

Calder (10) suggests a revised method of calibrating instrumentation relating to spectrochemical analysis where the calibration procedure involves plotting the instrumental response for an element against the concentration of that element. By calculating regression lines using the method of least squares for the response *versus* concentration, only 24 instead of 84 spectra and only 18 hr. instead of 60 hr. were required as compared to the original method.

Wernimont (11) used a computer to compare 16 Beckman spectrophotometers as to (*a*) the variation of the absorbance curve of each spectrophotometer about its own absorbance curve, (*b*) a comparison of the variation of a selected group of spectrophotometers about their average absorbance curve, and (*c*) a comparison of the remaining unselected group of spectrophotometers to the standards and tolerances of the selected group. Spectral analysis was used to analyze the data. The data were explained by the computation of 2 characteristic vectors. The first vector related to the vertical displacement of the absorbance curves and the second vector to the horizontal shift of the curves. Wernimont described a tentative absorbance standard of 60 mg. potassium chromate per liter, together with a procedure to check the Beckman spectrophotometers and a high-speed computer program which will make the necessary transformation to scale multiples of the 2 vectors.

The current growth in the use of statistics as applied to analytical chemistry can be attributed to an increased awareness of the colleges to mathematics in general. The use of automated analyses and computers will ultimately require extensive use of statistical methodology in the modern analytical laboratories.

SAMPLING PLANS

Description of Sampling Plans.—Acceptance sampling, which received its start during World War II, when it was used to pass on the quality of armaments, has been accepted for a large number of uses by the pharmaceutical industry.

An acceptance sampling plan may be described as follows. A company produces a lot of compressed tablets. It obtains a representative sample of tablets from the lot, examines the sample and, based on information obtained from the sample, either accepts the lot as conforming to standards or rejects it. The sampling plan is defined by (*a*) the size of the sample taken and (*b*) the number of defectives or degree of defectiveness allowed in the tablets. Graphically, a sampling plan can be explained by an operating characteristic curve.

Four points are defined on the operating characteristic curve selected for an example by Breunig and King (Fig. 2, *A*). First, a lot of tablets is said, for the sake of illustration, to be satisfactory if it contains only 10% defective tablets (the acceptable quality level, AQL). Second, a lot of tablets is said to be unacceptable if it

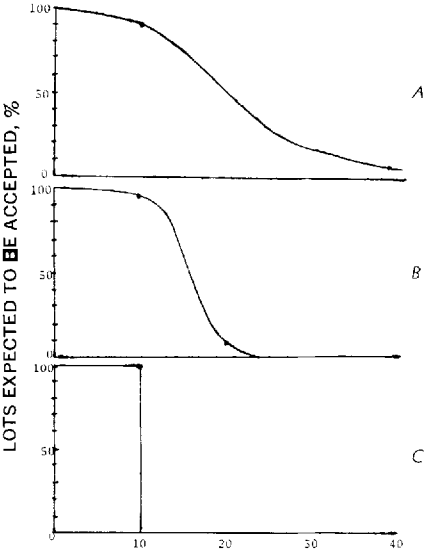

Fig. 2.—Key: *A*, operating characteristic (OC) curve for tablet example from *Reference 15*, p. 11, illustrating AQL = 10%, UQL = 40%, R_p = 10%; R_c = 8%, n = 10 [figure 5 of Breunig and King (12)]; *B*, typical operating characteristic curve from *Reference 15*, p. 27, illustrating AQL = 10%, UQL = 20%, R_p = 5%, R_c = 10%, n = 85 [figure 4 of Breunig and King (12)]; *C*, ideal operating characteristic curve illustrating perfect discrimination but unrealistic stringency [figure 3 of Breunig and King (12)].

contains more than 40% defective tablets (the unacceptable quality level, UQL). Third, there is a certain probability that a lot which in reality is 10% or less defective is observed on test to be more than that with the result that the lot is wrongly rejected. This is the producer's risk or α error. Fourth, there is the probability that a lot which is truly 40% defective is observed on test to be less than that with the result that the lot is accepted. This is the consumer's risk or β error. Once the above four values are selected the sample size is automatically determined.

Figure 2, *B*, shows an operating characteristic curve where the UQL has been decreased to 20% with a sample size of 85 tablets, and Fig. 2, *C*, shows a curve where the AQL and UQL are identical. To obtain the curve shown in Fig. 2, *C*, the entire lot of tablets would have to be examined.

The ability of a sampling plan to discriminate is dependent upon the size of the sample. Sampling plans requiring 10 to 30 samples have relatively low powers of discrimination.

There are two types of sampling plans: attribute and variables. Attribute sampling refers to a zero-one situation where a tablet is either good or bad; variables sampling is based on a continuous distribution of degrees of defectiveness and covers the gray zone between good and bad situations.

Attribute sampling plans require only the counting of the number of defectives found in the sample, or a mean or percentage of defectiveness [100 × (number of defects/sample size)]. The acceptance or rejectance of the lot depends on whether this value is smaller or larger than the one stated by the sampling plan.

Variables sampling plans require the calculation of a mean and standard deviation or range. For example, a sample of 10 tablets is taken which should have a theoretical mean weight of 100 mg. The mean weight of the sample is 94 mg./tablet, with a calculated standard deviation of 2 mg. Using the formula (100 mg. − 94 mg.)/2 mg. = 3.0, and entering a set of tables similar to the normal tables, it is found that the value of 3.0 indicates the material is 1.2% defective, which is acceptable or unacceptable depending on the values of acceptance.

Double sampling refers to the following example situation. A sample of 50 tablets is taken. If it contains 2 or less defectives, the lot is accepted. If it contains more than 4 defectives, it is rejected. If it contains more than 2 defectives but less than 4 defectives, a second sample of size 50 is taken. If in the 2 samples, there are less than 4 defectives, the lot is accepted. Triple and multiple sampling are merely extensions of the above premise.

Sequential analysis of sequential sampling is an extension of multiple sampling developed by Wald during World War II (13). Successive samples are taken based on a varying set of criteria until either a decision to accept or reject is made. There is, of course, the possibility that no decision could ever be made and the samples would stay in the indifference zone indefinitely. The problem is easily resolved, however, by deciding to stop at a given sample size.

For practical purposes the need to design a sampling plan has been eliminated by a series of government-sponsored sampling plans, 2 of which are MIL-STD-105D for attribute single, double, and multiple sampling plans (14); and MIL-STD-414 for variables sampling plans (15). These books have gained acceptance throughout most of United States industry in a manner much like the U.S.P. and N.F. Government contracts for the purchase of pharmaceuticals usually refer to one or both of these books. The obvious advantage of selecting plans from either of these books is communicability and acceptance throughout industry. Hence, there is little or no advantage to specially designed sampling plans

Application of Sampling Plans.—Pharmaceutical products which are solutions are sampled ordinarily with a sample size of 1. This is based on the premise that a solution is a homogeneous mixture in which every milliliter is like every other milliliter. Suspensions are frequently sampled the same way, but this is predicated on a thorough or continuous mixing. It is frequently necessary to take more than 1 sample from a suspension to verify that it is truly homogeneous. If a lack of homogeneity is known to be present, then a suspension could be thought of statistically as resembling a lot of compressed tablets with an infinite population size.

If a drum of a powdered chemical is known to be homogeneous, then for statistical purposes it can be thought of as a solution. Single or duplicate samples then are considered sufficient to obtain a reliable response by an analytical test. If a drum of chemical is known to come from a process where the entire lot of chemical is not blended prior to being filled into drums, there can be no assumption of homogeneity for certain properties of the chemical. For practical purposes an infinite population size could be assumed such as for a lot of tablets. The reliability of 1 or 2 samples from such a drum would be of serious doubt no matter how good the

answers appeared. According to MIL-STD-105D, sample sizes in the range of 2500 may be necessary for attributes, and for MIL-STD-414, sample sizes of 200 may be necessary to obtain adequate representation of an entire production lot. It is obvious that if sample sizes of this size were used, they would be economically impossible to test. The military plans, however, allow the dividing of the production lot under certain conditions into inspection lots (subsets of the production lot) which substantially reduce the astronomic sample sizes mentioned above.

A situation analogous to the sampling of pharmaceuticals in the powder state was studied extensively by Duncan (16–18) under the auspices of the National Plant Food Institute. Four different fertilizers, 3 different sampling instruments, and 3 different laboratories were used in the experiments. It was noted that under certain conditions, 1 of the instruments showed a tendency to take in a higher percentage of larger particles and a lower percentage of smaller particles, which in turn gave a higher assay value. Little or no evidence was found to indicate that instrument sampling differed more on the average than samples obtained by riffling. Relative differences between laboratories were noted along with differences between days from the same laboratory. The official method of the Association of Official Agricultural Chemists for sampling bulk material requires a sample from 10 bags for lot sizes greater than 10 bags. One tube core is removed from each bag. For lot sizes less than 10 bags, at least 10 cores are to be taken, but at least 1 core from each bag. From a blended sample of the 10 cores, a sufficient quantity is taken for analysis. Duncan assumes the running of 2 tests on the samples, no more, no less, in his mathematical model of the sampling and assay procedure.

The work done by Duncan in the fertilizer experiment would lend itself to the formation of an official method of sampling bulk pharmaceutical powders where the mean of the replicate assays is the standard for acceptance.

Duncan (19) devised the operating characteristic curves for fertilizer inspection plans which utilized sample sizes of 20 and 10. Ten samples were suggested for use because of the lower cost of sampling which is comparable to the pharmaceutical problem.

All of the above discussion is useful as long as it can be assumed that the characteristics being sampled are randomly distributed between bags or packages. However, the writer has investigated the problem of powder in barrels from a lot containing varying particle size distributions, although the material was accepted on the basis of its chemical purity. In this case, particle size distribution in certain ranges caused undesirable physical properties in the final product.

For adequate sampling in a situation such as this, reversion to knowledge of how the powder was manufactured is a necessity if homogeneity of a property is known to be a problem. Statistical sampling of the product is no substitute for manufacturing the product by a process known to produce satisfactory characteristics. All that should be necessary is to run an identity test of the material.

Literature on appearance of pharmaceutical dosage forms is rather conspicuous by its absence in the case of sterile types of products. As a result, most manufacturers inspect 100% of all sterile products for appearance-type defects such as particulate matter within the vial. It apparently is not desirable to say that a given lot of ampuls contains particulate matter in 1% of the vials. Still, at the present state of the art, particle-free sterile products are virtually impossible to manufacture and also exceedingly difficult to inspect on an economic basis. Each company, however, must have a set of standards and a method for verifying that the standards are being followed. This represents its level of excellence. Attribute sampling techniques can do this verifying with ease.

Tablets and capsules are frequently 100% inspected. Tablets, however, frequently are produced with a very high degree of excellence and also do not carry with them the problem of sterility and injectable elegance. Sampling plans therefore, work well with tablets, except for the large lot sizes which, depending on how the lot is sampled or divided, may require that large sample sizes be taken.

If tablets are inspected as they come off the compressor and little or no capping is noticed, it is not uncommon to find an unacceptable level of capping a few days later when they are packaged. This, in reality, is saying that a measurement for the potential to cap has not been employed in the inspection process; but in addition it says that the tablets were not manufactured correctly the first time.

What is the proper way to handle a tablet containing a metal chip traced to the feed frame of the compressor? Is the whole lot to be 100% inspected, a portion of the lot 100% inspected, or more samples taken? This is not really a problem in statistics, but in quality control.

The statistics of weight variation have been undergoing a gradual but continuous refinement in the pharmaceutical industry. The following

discussion outlines the various approaches that have been investigated over the past two decades along with the problems associated with the various plans.

The U.S.P. XIV and N.F. IX had the following weight variation test for compressed tablets (20):

"Weigh 20 whole and uncoated tablets and calculate the average weight. When weighed singly, not more than two of the tablets deviate from the average weight by a greater percentage than that shown in the following table, and no tablet deviates by more than double that percentage."

Average Weight	Percentage of Deviation
13 mg. or less	15
More than 13 mg. and including 130 mg.	10
More than 130 mg. and including 324 mg.	7.5
More than 324 mg	5

(The above table has been modified in U.S.P. XVII by eliminating the first weight class.)

Dunnett and Crisafio (21) derived the operating characteristic curves for the above official tablet weight variation method using sample sizes of 10, 20, 50, and 100. It was found that by simulating a batch containing 5% defective tablets (too heavy or too light) the lot would be accepted 93, 95, 98, or 99%, depending on whether a sample size of 10, 20, 50, or 100 was used. A batch containing 20% defective would be accepted, 40, 23, 4, or 0% using the same sample sizes. It was concluded that a sample size of 10 tablets had only meager ability of protection against inferior products. The use of a standard deviation test for 10 and 20 tablets was discussed and also a 2-sample attribute plan requiring a total of 50 tablets. If the first sample of 20 was satisfactory, the remaining were not examined. The operating curve for this was better than the operating characteristic curve for the 10- and 20-tablet standard deviation plans.

Non-normality of tablet weight variation was studied. The higher proportion of non-normal lots found, than was expected, was attributed to (*a*) the incapability of machines to turn out uniform tablets, (*b*) the differences between punches, (*c*) the sampling at various times from the machine. Dunnett concluded that no reasons could be found for the substitution of another mathematical distribution in place of the normal distribution.

Smith (22) suggested as an alternative to the B.P. and U.S.P. methods of weight variation the use of sequential analysis using formulas given by Wald (13). The use of half-defective (tablets deviating by half the specified amount) and double-defective (tablets deviating by double the specified amount) were discussed, and a table for acceptance or rejectance using sequential analysis for half-defective tablets was presented. The advantage of using sequential analysis was in the fewer weighings (about half the number requested by the B.P.) required for a decision on acceptability. Disadvantages cited were the very small sample size which is to represent the lot and the ease of giving exactly 20 tablets to an analyst for weighing.

Green and Knudsen (23) discuss three types of sampling which were applied to samples of 20 dry-filled ampuls. These could be stated as (*a*) the average of the sample shall not be greater than or less than ___, and no individual ampul from the sample shall be greater than or less than ___; (*b*) the average of the sample shall not be greater than or less than ___, (*c*) and no individual ampul from the sample shall be greater than or less than ___. A comparison of the operating characteristic curves for the 3 plans indicates that the average plan, and the combined average and individuals plan are comparable and that both of these are better than the plan dealing with individuals. By analyzing collected data, it was asserted that a plan then suggested by the manufacturers in 1950 and a Canadian tolerance were too tight in the lower weight ranges and too loose in the upper weight ranges.

Breunig and King (12) described the advantages of variables sampling plans as having a greater ability to detect excessive tablet weight variation than the plan employed by the U.S.P. When Breunig and King compared their variables plan to a 2-step attribute sampling plan such as is now in the U.S.P. XVII and N.F. XII for assay variation, they found the variables plan superior for (*a*) cases of excessive variation and (*b*) for excessive variation combined with a shift in the mean and about equal for cases of mean shift alone. Two nonstatistical points were stated by Breunig and King which illustrate how inadequate the use of a statistical sampling plan is without simultaneous human thought. (*a*) A lot is accepted or rejected on the basis of an exceedingly small number of units (10 or 30 out of 1,000,000). As a result, it is not practical to expect a sampling plan to guarantee that all accepted lots will be of acceptable quality, although such a plan may be quite effective in detecting an occasional bad lot when all others are of good quality. (*b*) If many highly defective lots are submitted to such a plan, it is possible that half of them will end up being used by the consumer.

The solution to the above is an adequate quality control system throughout all the various stages of the processing of the product, which assures that the product is made right the first

time. The U.S.P. and N.F. sampling plans then become merely guide rules as to what level of quality is expected of the manufacturer of a pharmaceutical. From an enforcement viewpoint, however, the sampling plan's quality standards must be enforced on a lot-to-lot basis.

Haynes (24) demonstrated by the use of actual data from sterile solid weight variation and by computer simulation that certain suggested attribute plans were more robust than equivalent variables plans. Robustness refers to the problem which causes variables plans to be more susceptible to the effects of non-normal distributions commonly found in small samples (10 to 30 items). Figure 3, *A*, shows a normal distribution of weights as generated by simulation on an IBM 7070 with approximately 5% of the values outside the vertical dotted lines; Fig. 3, *B*, shows a platykurtotic distribution of weights; and Fig. 3, *C*, shows a leptokurtotic distribution of weights.

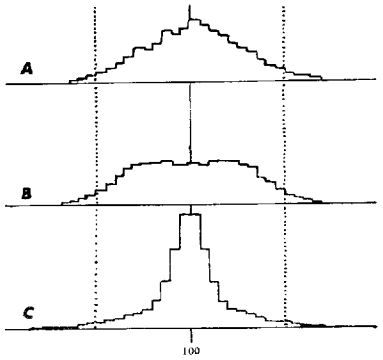

Fig. 3.—Key: *A*, normal weight distribution, quality index 94.5%; *B*, platykurtotic weight distribution, quality index 95.1%; *C*, leptokurtotic weight distribution, quality index 95.0%. [Figures 3, 4, and 5 of Haynes (24).]

Both platykurtosis and leptokurtosis are types of non-normality found in pharmaceutical dosage form weight and assay variation. Paul (25), by using data accumulated for individually assayed compressed tablets, reinforced the work of Haynes that pharmaceuticals were indeed manufactured in non-normal distributions as evidenced by samples of 10, 20, and 30.

In response to a request from the Food and Drug Administration, the Pharmaceutical Manufacturers Association, Quality Control Section, in 1961 set about to collect data for dosage form variation for sterile solids without diluents and for compressed tablets.

By October 1962, two differing plans were advanced by industry statisticians. These were the variables type sampling plan advanced by Breunig and King for compressed tablets and the attribute plan advanced by Haynes.

Although one type of plan was advanced for tablets and another for sterile solids, this was the result of two different committees working on the problem separately. Both tablets and sterile solids were, however, later considered using a single type of sampling plan.

Based on the work of Haynes and other industry statisticians, the P.M.A. Quality Control Section through its subcommittees recommended to the U.S.P. that an attribute type of plan be adopted in the U.S.P. for a selected group of tablets as a test for composition variation.

As a result, the U.S.P. XVII and N.F. XII have adopted the following statement regarding "content uniformity" for certain compressed tablet monographs: "Select a sample of 30 tablets. Assay ten of these individually as directed. The requirements of the test are met if all ten results fall within the limits of 85% and 115% of the average of the tolerances specified in the monograph. If one result falls outside these limits, assay the remaining 20 tablets individually. The requirements are met if not more than one of the 30 results is outside of the limits of 85% and 115%."

An interesting question arises when one asks, "What type of distribution of doses does the consumer have a right to expect when in fact he cannot be given on a repetitive basis exactly 5 gr. of aspirin?" Clearly, a leptokurtotic distribution gives the consumer on the average some of what he expects; likewise, a platykurtotic distribution of values shows a lack of control in a manufacturing process. If a normal distribution or leptokurtotic distribution is suitable for a customer, then is it not consistent to set the bias of the sampling plan against the platykurtotic distribution generated by a poorly controlled process?

Moskalyk *et al.* (26) expressed the observation that dosage variation was greatest in the lightest weight tablets within a batch. Then the question arose concerning whether this could be proved true for all tablets; if it was, a sample of lightweight tablets could be adopted as a control over uniformity of drug dosage.

Haynes (27) also was interested in control of weight variation as a means of controlling drug dosage.

It is clear that what is needed is a non-analytical test which statistically predicts the degree of potency variation between dosage forms. With the advent of the Mettler and Cahn automatic weighing balances, it is not impossible to weigh thousands of tablets, either as they are being processed or as a sample obtained from the finished lot. From the distribution of values obtained from them, a statistical test of normality

could be made. If a statistical technique can then be derived that would allow us to draw conclusions from 3 singly assayed tablets, the 3 lightest, or the heaviest, middle, and lightweight from a random sample of specified size, is it then not possible to predict with an acceptable degree of reliability that the lot conforms to U.S.P. or N.F. standards? Here is an area for some statistical research with great economic rewards.

Although attribute sampling plans are the simplest to use and simplest to enforce legally, variables sampling plans are still the most useful for the internal operations of a pharmaceutical manufacturer. Especially will this be true with the automation of the quality control function of pharmaceutical product manufacture.

The sampling of product container fills by a regulatory agency is properly a sampling technique, although it is routinely controlled by control charts and might logically be discussed under *Control Charts.*

The Department of Agriculture, State of California (28), has now put into effect Article 5 which will attempt to control from a legal viewpoint the concept of filling variation. The size of the sample of consumer size packages required for sampling follows closely the approach used by the Association of Official Agricultural Chemists for sampling bulk material in lot sizes of 10 containers or less. For lot sizes greater than 10 packages, the sample size approximates the square root of the number of packages in the lot. The plan, although not similar to either an attribute sampling plan or a variables plan, contains features of both type plans. The basic feature of Article 5 is that tables are presented for all possible situations which the investigator might meet. Article 5 does not say, however, for example, that a 5-lb. package must contain at least 95% of label claim. It merely says that for a given sample of package weights or fills, the average range and the average error are to be determined. Based on these values, unreasonable weight errors are to be determined, the values of which will determine whether the lot is acceptable or unacceptable.

True statistical sampling has very limited if any application at all in detecting a single foreign label or carton in a lot of labels or cartons. Only 100% nonhuman inspection of the entire lot will give any degree of reliability in detecting a foreign label or carton. Human inspection is second in preference to machine inspection. At the present time, it is better to consider machine inspection as a means of determining the presence of foreign labels, but not as a means of removing them from a lot of labels. It would be preferable to have the inspection machine stop rather than try to remove the offending label. Statistical sampling does, however, work very well in controlling the quality of printed material for poor printing, coloring, centering, etc.

Sampling and inspection of bottles, containers, and cartons has had wide application in the pharmaceutical industry. MIL-STD-105D works very well on this type of application since the vendor should understand the use of this plan.

It is not the statistical aspects of sampling plans which are of major concern in the above applications, but rather the definition of what is a defect. It is possible to have 20 or more types of defects for glass bottles. Are they all to have the same weight as to being called a defect? At what point is a defect called a minor defect, and when does it become a major or critical defect? If the inspection process is human, there is an ever-present tendency to tighter standards. Likewise, if a lot does not quite meet sampling plan standards, there is a tendency to go outside the sampling plan for information as to whether to accept or reject. If this item is needed for production due to low inventories or if the defects all run to 1 type which is not too serious there is a desire to accept the lot. Hence, the most important aspect of designing a sampling plan is a definition of what a defect is or what is to be sampled for. The most important aspect of using a sampling plan is drawing a truly random sample and following and maintaining the definition of a defect.

CONTROL CHARTS

Description of Control Charts.—Control charts are a method of graphing or plotting data in such a manner that a time series of events is summarized. Figure 4 shows a control chart described by Breunig.

Control charts are characterized by a vertical axis which has a scale of a varying measurement such as a mean, range, standard deviation, or fraction defective, and a horizontal axis which is time-oriented. There are frequently 3 horizontal lines on a control chart. The center line is the target value or the historical process average. The upper line is the upper control limit (UCL) which is normally 3 standard deviations above the center line. Likewise, the lower line is the lower control limit (LCL), again 3 standard deviations below the center line. The 6 standard deviation spread between the upper and lower control limits will encompass 99.97% of the values in a distribution with its mean at the

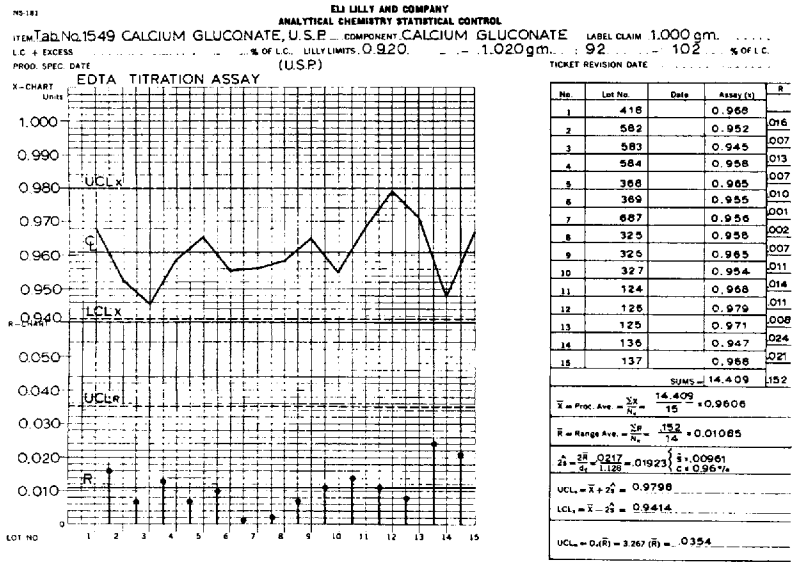

Fig. 4.—A typical analytical laboratory control chart. (Breunig.)

center line or process average. Control charts which are used for plotting sample averages are frequently used in conjunction with a range or standard deviation chart. One chart is used to plot the process average and the other the process variability. If a given value falls outside of the upper or lower control limit, the process is usually adjusted to bring it close to the center line.

It is frequently desirable in pharmaceutical processes to add another set of limits which could be called tolerance limits. If a given value falls outside the tolerance limits, the operation must be halted. If a tablet compressor normally operates under control limits well within the U.S.P. and N.F. specifications, the tolerance limit might well be the equivalent of the U.S.P. and N.F. specifications. This ensures the manufacturer that few, if any, products are manufactured outside of acceptable limits.

There have been many modifications to the structure of control charts, notably the cumulative sum type. However, the use of control limits for decision making is common to all types of them.

Application of Control Charts.—Control charts are primarily used for plotting routine (*a*) analytical or biological assay results or parameters, (*b*) fills of fluid or injectable products, (*c*) weights of tablets or capsules, and (*d*) the percentage or number of defects in a sample of packages emanating from a packaging operation. They are also useful for plotting data gathered at the beginning of a research problem.

Yehle (30) in an investigation of laboratory precision and specification limits used a type of control chart to plot the results of laboratory analyses.

Noel (31) discussed the use of control charts as applied to collecting data about particulate matter in ampuls in order to check the difference between machines and of fill weights. Noel's discussion on control charts was the second of a 5 part series of articles dealing with the use of statistics in the pharmaceutical industry (32–35).

Recently, Breunig (29) discussed the application of statistical control charts to analyses which were run in duplicate on vitamin A palmitate where the problem was *cis–trans* isomerization. Product and analytical control was obtained by purchasing an equilibrium mixture of *cis–trans* vitamin A palmitate. He also discussed the use of control charts for studying tablet weight variation and assay variation prior to introducing single tablet assays.

Brochmann-Hanssen and Medina (36) described the uses of control charts for determining weight variation and composition variation for phenobarbital tablets. Their inspection of the charts indicated that some disruption of uniformity occurred during the compression of the tablets.

One of the problems with control charts for routine applications is the objection of the analyst or operator to writing the results of a test on a control chart since the result is usually first written on another document. Wherever a control chart is a secondary document, resistance to its use is apt to appear. If the control chart can be the primary document, acceptance is made easier.

Breunig suggests as a solution to this problem

an instruction manual, a training course, consultation, and management support. Breunig also suggested that the reason analytical laboratories have not adopted statistical quality control programs is because the U.S.P. and N.F. have their sampling and control procedures "couched" in terms which can be considered obsolete in the light of the knowledge available today (29).

The above statement was in reference to the U.S.P. and N.F. attribute sampling plans for tablet and capsule weight and assay variation, the single assays performed on composite samples, and the associated unrealistic limits specified by the monograph.

EVOP AND ADAPTIVE QUALITY CONTROL: STATISTICS FOR CHEMICAL PROCESS EVALUATION

Quality control whose main forte is controlling new and existing operations, must at times be concerned with improving existing manufacturing operations. After publishing extensively (37–40) in the field of response surface statistics, Box (41) published a paper dealing with a very simple technique called "EVOP" (evolutionary operation) which was useful in finding the optimum operating conditions for a chemical batch process or similar process. His first paper dealt with 2 variables (*e.g.*, concentration and temperature) and 3 variables (*e.g.*, concentration, temperature, and pH) models. Without developing the theory of EVOP, a 2-variable EVOP cycle would consist of 5 experiments performed under the conditions shown in Fig. 5. By some very simple calculations (42), it can be established whether the process is at its optimum or whether the increase of one or both variables in a given direction tends to give a significant increase in response. Usually 3 cycles or 15 experimental units are required to show which variables are significantly influencing the process and in which direction they should be adjusted to maximize the response (yield, impurities, or cost of the process).

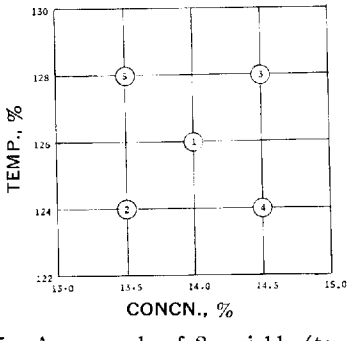

Fig. 5.—An example of 2-variable (temperature and concentration) EVOP cycle. [Figure 4 of Box (41).]

There have been other statistical models, such as adaptive quality control (42), developed for maximizing yield, but these are beyond the scope of this paper.

The point to be made about response surface statistics, as applied to making fine pharmaceutical chemicals or other material, is not with the statistical aspects of the technique, but with the control aspects.

EVOP and similar response surface techniques are difficult for the quality control function of a company to evaluate because they are opposed to the basic control precept of making the product by the same method under the same conditions for every batch. In conclusion, EVOP and related techniques are very useful and powerful statistical tools which definitely should be used on applicable production processes. Nonstatistical problems, however, may play a predominant or controlling part in the extent of the usefulness of these statistical production process optimizing techniques in the pharmaceutical industry.

BIOLOGICAL ASSAYS

Description of Biological Assay.—There are two types of indirect bioassay: one is based on quantitative responses, the other on quantal responses.

An example of a quantitative assay is the guinea pig skin test in which the activity of tuberculin P.P.D. is estimated from the varying size of wheals resulting from the injection of tuberculin-sensitized pigs with graded doses of the antigen.

An example of a quantal response bioassay is a mouse potency test in which antigenicity of a vaccine is measured from the proportion of mice still living after first being injected with the vaccine under test and then being challenged with the live organism against which the vaccine is to protect.

Biological assays can be further divided into two types: analytical dilution assays and comparative assays. In an analytical dilution assay (either quantitative or quantal), both a standard (or reference) and test preparation are considered to consist of an "effective constituent" suspended in an inert diluent (43). Relative potency in such an assay is defined as the ratio of the concentration in an effective dose in a unit amount of the test preparation to that of the standard. It then follows that the postulated relative potency characterizes the two preparations and

not the biological systems which are used to assay them (44).

Assays in which the postulated relative potency is not a constant are often referred to as "comparative" (43). A variable potency may occur in the comparison of a test and standard preparation which are qualitatively different. There are cases where the requirements of an analytical dilution assay are considered to be met and yet the outcome of the assay is far from being analytic because of the unknown extraneous factors which have caused qualitative differences in either preparation. (Analytical dilution assays are usually characterized by the reference and unknown having different slopes.)

In most biological assays, primary concern is directed at estimating the (*a*) effective dose of a test material in terms of the known effect of its constituents and (*b*) precision of that estimate.

Quantal Response Assay.—Finney (45) has pointed out that the ED_{50} (effective dose required to produce a 50% response) may have 1 standard error between determinations by 1 worker, another standard error between different workers using the same apparatus and the same stock of animals in a given laboratory, and still another between determinations in different laboratories. However extensive the experimentation on one population, no statistical analysis can demonstrate the applicability of the conclusion to a different population.

Finney further states that from the basic concept of plot technique, it can be deduced that the magnitude of an assay error is inversely proportional to the degree of correlation among the test subjects. An estimated internal assay error is necessarily an indication of what would be found in repeated tests of the same stock of subjects under the same conditions of testing. This limited interpretation must always be attached to statements about the ED_{50} of unknown material.

Nevertheless, much of the present difficulty seems to be removed by an adoption of comparative experiments (46). The numerous bioassay data which have been available to the writer appear to confirm Finney's statement that a theoretical reason exists for believing a numerical result to be applicable more widely than in the circumstances of the particular experiment that produced it.

The considerations that influence the interpretation of assay validity are of two types: statistical and other. Under statistical considerations, criteria such as linearity and parallelism of the dose–response relationships are included. The nonstatistical considerations include such matters as environmental factors which may have affected the quality of the effective constituent of either preparation. Obviously, both considerations are necessarily integral and eventually lead to a determination of whether the irregular occurrence is merely due to chance.

Such an extraneous factor as a physical disturbance apparently has an effect on the antigenicity of a certain type of vaccine to the extent that the effective constituent in the test material is no longer comparable to the standard. However, this type of deviation from parallelism does not necessarily imply that the assay is invalid (44).

From the realistic point of view, an assay may be considered to be invalid if and only if statistically significant nonlinearity and nonparallelism are accompanied with such factors as inconsistent testing conditions and heterogeneous test subjects which are not randomly allocated to each preparation.

In numerous cases of quantal response assays (6 points with approximately 16 subjects at each point), the test of linearity and parallelism often turns out to be so insensitive that the slope of the dose–response curve for 1 preparation must be at least 2.5 times steeper than that for the other preparation in order for the departure from parallelism to be statistically significant.

In such cases, neither the choice of the form of the transformation of the biological responses nor the arbitrary increase in the number of test subjects would overcome this difficulty. This type of insensitivity evidently stems from the inefficient allocation of dose levels which would be reflected by the insensitive responses.

It has been well-established that the relative potency, slope of the dose–response curve, and assay error represent characteristics of the test material (47, 48). In order for the subjects to be able to produce the true characteristics of the test material, it is a prerequisite for the experimenter to allocate an optimal amount of effective constituent in such a way that the minimum effective dose can be detected with a minimum of error.

Cramer (49) compared methods of fitting the dose–response curve for small samples (3-dose test with 10 subjects at each dose and 5-dose test with 4 subjects at each dose) and found that the probit analysis is superior to the minimum normit χ^2 method with respect to the assay error. This superiority is found to be substantial if the dose levels are poorly placed with respect to the true assay parameters.

The relative merits of the various methods for fitting the dose–response curve would be more meaningful if the existing antigenicity tests (mouse potency tests for influenza vaccine and

pertussis vaccine) are biologically unbiased. For instance, the following question may be asked: Is the antibody level at the time of challenge directly proportional (by an approximately constant factor) to the amount of a vaccine injected at the time of immunization?

It should be pointed out that many statistical analyses of quantal response bioassay are being utilized under the assumption that the reply to the above question is positive. This type of question further complicates the situations in which the valid evaluation of potencies of polyvalent vaccines are required.

Quantitative Bioassay.—The fundamental concepts involved in quantitative bioassay are essentially the same as those discussed under *Quantal Response Assay*. The quantitative assay in general, however, is subject to greater control and in turn requires more elaborate assay plans than the quantal response case.

Some of the antibiotic assays and vitamin assays consist of a number of plates, each plate being regarded as block. The different positions within a plate may have different effects on the response which should be solely attributed to a given concentration of the test material. Each sample should be rotated or allocated at random within a plate, depending on whether the position-to-position difference is systematic across the plates or unique to the individual plates. Both the test and the standard preparations should be represented in each plate, even in the cases where the position difference can be neglected.

It is well known that, when the dose and response are suitably transformed, the mode of the action of organisms can be represented by the monotonic dose–response relationship (either approximately sigmoidal or straight). Whenever a prior knowledge suggests that the dose-response relation for a given test material is approximately sigmoidal or linear, it is advisable to select two reference (standard) concentrations which cover the linear portion of the dose–response curve. The difference in response which is due to the difference in concentration is best detected along the linear portion of the dose–response curve.

The only remaining problem then is to choose target dilutions for the unknown preparation in such a way that at least 1 dilution for the unknown produces the response which stays within the responses produced by the 2 reference concentrations (50).

Another type of quantitative bioassay is that in which the response is measured by an instrument with a response in units of per cent transmission. Because of the possible instrumental drift which may be linear or nonlinear, the time required to process a set of standard and unknown dilutions may be regarded as a block.

There is still another type of quantitative assay in which the potency is measured from time. The activity of prothrombin samples, for example, is estimated from the clotting times for the standard and unknown samples.

Loomis (50) developed an assay method for fibrinolysin which uses a simple formula for estimating activity. His method is based on the assumption that the concentration–time relation for the unknown samples and standard is not only linear but also parallel to each other within the dilution range.

Stone and Bruce (51) reported (based on 250 humans injected intradermally with tuberculin) that there exists a linear relation between the induration–area and the log–dose in constant volume. However, they pointed out that the following questions deserve further clarification. (*a*) Can the form of the dose–response relation of intradermal tests (tuberculin) be extended to other diseases such as diphtheria toxin? (*b*) Is the form of the dose–response relation of intradermal test dependent on experimental technique?

They also presented a method by which the antibody level may be estimated from a knowledge of the dose and concentration of tuberculin used and the resultant area of induration.

The discussion under *Biological Assays* was included in this paper merely to show that it is a part of quality control. The subject alone is so large that a journal, *Biometrics*, is published to keep up with the changes in this one specialized field of statistics.

U.S.P. XVII (54) now recognizes the subject of biologics and biological assay and gives them prominent attention, as can be traced historically from 1950 to 1965.

U.S.P. XIV (20) had a single paragraph dealing with the significance of the standard error in a biological assay. There was no mention of the method of assay for antibiotics; antibiotic products were said to comply with the requirements of the Food and Drug Administration.

U.S.P. XV (52) included a section on the "Design and Analysis of Biological Assays," by C. I. Bliss. In addition, a section was added detailing the methodology for performing assays on the antibiotics included in the U.S.P.

U.S.P. XVI (53) expanded the section on antibiotic assays to show the formula necessary to calculate the slope and potency of the material being assayed. The section on the "Design and Analysis of Biological Assays" was continued.

U.S.P. XVII (54) again expanded the section on antibiotic assays to include some of the tech-

niques of designing antibiotic-type assays. Some revision was made to the section on "Design and Analysis of Biological Assays," including a glossary of symbols used within the section.

In a period of 15 years, the U.S.P. has gone from a book which included no statistical methodology dealing with biological products, to a book which from a legal point of view is an authority on how statistics are to be applied. Included in the section on "Design and Analysis of Biological Assays" is an official method for rejection of outlying or aberrant observations and the replacement of missing values. Likewise, the sections on the combination of independent assays and joint assay of several preparations are of great value to the industry. For the first time, a glossary of statistical symbols with official status is printed for all to use. The writer has a personal preference for a different set of symbols, but the benefits to be gained within the industry from a uniform set of symbols and formulas more than offset any personal feeling.

Beginning in U.S.P. XVI (53), a small section was added called "Biologics," which simply stated that for vaccines, antitoxins, etc., the National Institutes of Health, Division of Biological Standards, controlled the testing requirements and that the actual assay procedure was beyond the scope of the book.

Many methods are commonly used for computing the potency of biological products, some of which are defined adequately by the National Institutes of Health. However, there is a definite need for a specification of the statistical method of choice, regardless of the length of calculation, and very definite rules for the determination of assay validity, combination of independent assays, and joint assay of several preparations.

Bliss' section on the combination of independent assays states that: "additional animals can be added to an insufficiently precise assay until the combined results reduce the confidence interval within the lines specified in the monograph."

Since many biologics are assayed on the basis that the unknown is equal to or greater than the reference standard in potency, it is logical for a manufacturer to make the potency of the vaccine just potent enough to exceed the reference a certain percentage of the time. If the manufacturer performs 4 assays on the unknown and combines their results, which in reality doubles the precision of the assay, this then allows the manufacturer to produce a product whose potency tests greater than the reference about the same percentage of the time, but on a comparison basis does not have to be so excessively potent as material which is only controlled by a single assay.

If the Division of Biological Standards performs a single assay on material which has had multiple assays performed on it by a manufacturer, there is a high probability that the material will fail; hence, the lot is not suitable for distribution. If the Division of Biological Standards reference standard is to be a standard of potency to which a vaccine is to be equated, then the manufacturer should be allowed to use all available statistical methodology to warrant that the product is equal to or greater than the standard.

SUMMARY

Mathematical statistical methods have been found to be useful through all areas of quality control. Statistical sampling plans applied to dosage form variation, by their very controversial nature, have exposed large segments of the pharmaceutical industry to statistics. Analysis of variance, design of experiments, and hypothesis testing have not in themselves found extensive usage in quality control functions because of their research orientedness. Applications using these methods apply and are very effective when dealing with materials which are not routinely meeting their specifications or where specifications are in the process of being developed. Biological assays are now having increased attention paid to their problems, as witnessed by the U.S.P. sections dealing with biologicals.

Most statistical techniques are based on a measure of accuracy (the mean of a set of data) and a measure of variation (the standard deviation). With the advent of the food and drug law amendments of 1962, the increased emphasis on good manufacturing practice, the introduction of high-speed packaging lines, dosage form variation, glass bottle defects, missing bottle labels, and granulation characteristic variation, etc., the concept of statistical variation is no longer welcome to the quality control function. Statistical methodology says there will always be defects. Quality control says there must be zero defects.

The next 15 years will be interesting to see how well the statisticians can adapt themselves to problems implying sample sizes in the 500 to 10,000 range rather than 1 to 1,500.

The statistician and the quality control man must bear in mind the truism, "To attain quality, one must first measure it, and to measure quality, one must establish a standard of rejection." As Youden has pointed out, statistics are the laws of measurement. The new era for quality control statistics may well be in product design, control system design, or quality control simulation—all things to be done before the product is ever manufactured.

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Research Articles

Effects of Potential Inhibitors on Metabolism of Griseofulvin *In Vitro*

By S. A. KAPLAN, S. RIEGELMAN, and K. H. LEE

The inhibitory effects of *p*-ethoxyacetanilide, *p*-methoxybenzylamine, codeine, and SK&F 525-A on the metabolism of griseofulvin were studied in a Krebs-Ringer bicarbonate liver-slice system. This investigation sought to determine the possibility of prolonging the biological activity of griseofulvin.

GRISEOFULVIN, the first available oral antifungal antibiotic, was originally isolated from the mycelium of *Penicillium griseofulvum* by Oxford in 1939 (1). However, it was not until 1958 that Gentles (2) reported that he was able to eradicate experimental ringworm of guinea pigs as a result of oral treatment with griseofulvin. This was followed by the works of Riehl (3, 4), Blank *et al.* (5), and Williams (6), who obtained favorable results in the treatment of superficial fungi infections of man.

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Research Articles

Effects of Potential Inhibitors on Metabolism of Griseofulvin *In Vitro*

By S. A. KAPLAN, S. RIEGELMAN, and K. H. LEE

The inhibitory effects of *p*-ethoxyacetanilide, *p*-methoxybenzylamine, codeine, and SK&F 525-A on the metabolism of griseofulvin were studied in a Krebs-Ringer bicarbonate liver-slice system. This investigation sought to determine the possibility of prolonging the biological activity of griseofulvin.

GRISEOFULVIN, the first available oral antifungal antibiotic, was originally isolated from the mycelium of *Penicillium griseofulvum* by Oxford in 1939 (1). However, it was not until 1958 that Gentles (2) reported that he was able to eradicate experimental ringworm of guinea pigs as a result of oral treatment with griseofulvin. This was followed by the works of Riehl (3, 4), Blank *et al.* (5), and Williams (6), who obtained favorable results in the treatment of superficial fungi infections of man.

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The authors are grateful to Miss Darlene Tanneberg for technical assistance.

It has been shown that griseofulvin localizes in the prekeratin cells (7–9), and that the amount of griseofulvin deposited in the prekeratin cells depends upon the blood levels achieved. Therefore, in treatment it is important that the drug be deposited in the keratin of the hair shaft, skin, or matrix of the nails in fungistatic concentration to keep the organism in check pending removal of the infected tissue and replacement by healthy tissue. Since the turnover rate of these tissues is slow, oral therapy can involve periods of treatment up to 6 months or longer.

The high-dosage regimen required for griseofulvin to exert its therapeutic effect can be attributed to its low water solubility of approximately 1 mg. %, its slow rate solubility, and its relatively rapid rate of metabolism. It is difficult, therefore, to maintain a prolonged high concentration of drug in the blood.

Generally, the duration of drug action after absorption depends upon the rate of metabolism and excretion. Griseofulvin is unique in that it is not excreted intact but apparently only after being metabolized. Barnes *et al.* (10) reported that the major metabolite of griseofulvin in man and rabbits is 6-demethylgriseofulvin. *In vitro* studies by Bedford *et al.* (11) also confirm that griseofulvin is metabolized by the liver and results in the formation of 6-demethylgriseofulvin. Aromatic ethers are known to be metabolized by *O*-dealkylation (12). The authors have chosen several potential inhibitors of griseofulvin. These include *p*-ethoxyacetanilide, *p*-methoxybenzylamine, codeine, and SK&F 525-A. The first three mentioned compounds are aromatic ethers metabolized by oxidative *O*-dealkylation as is griseofulvin, and could therefore act in a competitive manner to inhibit the metabolism of griseofulvin and thereby prolong its biological activity. SK&F 525-A is a well-known nonspecific metabolic inhibitor in animals and affects many *O*-dealkylation reactions.

EXPERIMENTAL

Tissue Preparations.—Livers were obtained from young adult New Zealand white male rabbits of 2.0 to 2.5 Kg. The rabbits were stunned and exsanguinated, and the slices were prepared on a McIlwain mechanical tissue chopper (13) at a thickness of approximately 0.5 mm. at 3° in the cold room. Thirty-milliliter samples containing 10 mcg./ml. griseofulvin in Krebs-Ringer bicarbonate buffer (14) were incubated with 1.5 Gm. of liver slices, with and without the addition of the potential inhibitors at 37°, with shaking under an atmosphere of 5% carbon dioxide in oxygen saturated with water vapor. These samples were incubated for varying time intervals, after which the flasks were removed from the incubator, and supernatants were separated from the tissue slices by decanting. An aliquot of the supernatant was then immediately extracted twice with ethyl ether, evaporated, and reconstituted in ethanol. The griseofulvin was determined by both the spectrophotometric and spectrophotofluorometric methods of analysis. In the ultraviolet the samples were determined in ethanol at 292 mμ, while in the spectrophotofluorometric analysis the samples were activated at 315 mμ, and the maximum fluorescence determined at 450 mμ. SK&F 525-A did not interfere with the ultraviolet analysis of griseofulvin and the samples could be determined directly. However, codeine, *p*-ethoxyacetanilide, and *p*-methoxybenzylamine interfered with the ultraviolet analysis of griseofulvin as they were partially extracted by the ethyl ether along with the griseofulvin. Codeine and *p*-methoxybenzylamine were completely removed by washing the ether extracts with two 25-ml. portions of 1 *N* hydrochloric acid, allowing for the direct ultraviolet analysis of griseofulvin. *p*-Ethoxyacetanilide could not be separated from griseofulvin by extraction and was therefore analyzed by the spectrophotofluorometric method.

Microsomal Preparations.—Twenty-five per cent homogenates of liver were prepared in 1.15% potassium chloride solution by use of a Dounce homogenizer (15) in the cold room at 3°. The tissues were homogenized first with the loose-, then the tight-fitting pestle, and centrifuged at 9000 × *g* for 20 min. The supernatants were decanted and used in the subsequent experiments where each 5.1-ml. sample contained glucose-6-phosphate, 20 μm.; NADP, 0.4 μm.; nicotinamide, 50 μm.; magnesium chloride, 75 μm.; potassium chloride, 1 *M*, 0.1 ml.; griseofulvin in 0.1 *M* phosphate buffer or buffer alone, 1.5 ml.; and enzyme preparation, 1.5 ml. The above samples were incubated at 37° with shaking (110 oscillations per minute) in a Dubnoff metabolic shaker for varying time intervals at which time 2.0-ml. aliquots were removed from the flasks and added to 7.0 ml. of freshly distilled anhydrous ether and analyzed by means of the spectrophotofluorometric assay. The co-factors did not interfere with the analysis of griseofulvin on the Aminco Bowman spectrophotofluorometer.

Standardization of the Microsomal Activity.—*p*-Ethoxyacetanilide has been shown to be metabolized by the microsomal enzyme fraction (12). Therefore, with each determination samples containing *p*-ethoxyacetanilide without the addition of griseofulvin were run to determine the relative activity of the enzyme preparation.

Analysis of 6-Demethylgriseofulvin.—This compound is characterized by its ultraviolet spectrum which has a maximum peak at 293 mμ in acidic ethanolic solution, which shifts to 328 mμ in neutral to alkaline solution. 6-Demethylgriseofulvin is recoverable by extracting the acidified supernatant (pH 1) after removal of the griseofulvin, with two 25-ml. portions of ethyl ether. Other supernatant samples after removal of the griseofulvin were divided into two equal portions. One portion was analyzed for 6-demethylgriseofulvin as described above. The other portion was acidified to pH 4.5 with acetic acid, and incubated with β-glucuronidase at 37°. This system was then extracted as described to determine the amount of free 6-demethylgriseofulvin present in solution.

RESULTS AND DISCUSSION

The per cent of griseofulvin remaining unmetabolized in solution as a result of being incubated with

the Krebs-Ringer bicarbonate rabbit liver slice system has been calculated from 17 individual experiments. The metabolism of griseofulvin appears to follow pseudo first-order kinetics as is determined from the linearity of the semilogarithmic plot seen in Fig. 1. The half-life for the disappearance of griseofulvin has been calculated by the method of least squares to be 53.5 ± 3.8 min. (p = 0.025). Since the metabolism of griseofulvin in this system occurs within defined limits, a control was established to which the inhibitory studies with the potential inhibitors could be compared.

In each of the following experiments rabbit liver slices were incubated with and without the addition of a 100 to 1 molar ratio of inhibitor to griseofulvin. The experiments were repeated at three different levels of griseofulvin concentration, but the system is limited by the low solubility of griseofulvin in water. Figure 2 includes three curves, each of which is from the mean data of a minimum of four experiments and illustrates the pseudo first-order metabolism of griseofulvin and p-methoxybenzylamine with griseofulvin. Figure 3 shows similar experiments utilizing codeine as the potential inhibitor. The compound, SK&F 525-A, is not added to the *in vitro* system but is administered prior to sacrifice and preparation of liver slices. Figure 4 shows the results obtained from animals pretreated 2 hr. prior to sacrifice with 75 mg./Kg. of SK&F 525-A as compared to control animals.

The results indicate that at a molar ratio of 100 to 1, p-ethoxyacetanilide to griseofulvin, the rate of metabolism of griseofulvin is decreased approximately fourfold to give a half-life of 200.6 ± 9.2 min., indicating the rate of metabolism is significantly different in the presence of p-ethoxyacetanilide ($p < 0.01$). p-Methoxybenzylamine decreased the rate of metabolism approximately twofold to 116.5 ± 8.8 min., again indicating a significant difference in the rate of metabolism ($p < 0.001$), codeine with a half-life of 57.4 min. ($p > 0.60$), and SK & F 525-A with a mean half-life of 49.6 min. ($p > 0.80$) showed no inhibitory effects on the metabolism of griseofulvin.

In studying the metabolism of griseofulvin it has been inferred that the metabolism of the drug probably occurs in the microsomal enzyme fraction of the liver. The results in Table I clearly indicate that the microsomal enzymes in the presence of the co-factors of the soluble fraction are responsible for the metabolism of griseofulvin.

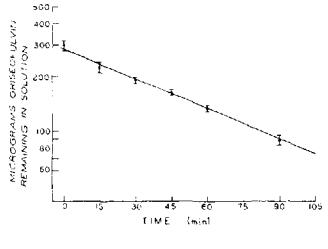

Fig. 1.—Disappearance of griseofulvin from the Krebs-Ringer bicarbonate liver-slice system reported with the 95% confidence intervals.

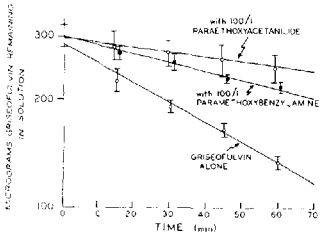

Fig. 2.—Disappearance of griseofulvin in the presence of a 100 M ratio of p-ethoxyacetanilide or p-methoxybenzylamine to griseofulvin with its 95% confidence interval.

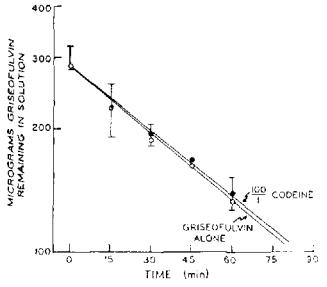

Fig. 3.—Disappearance of griseofulvin in the presence of a 100 M ratio of codeine phosphate to griseofulvin with its 95% confidence interval.

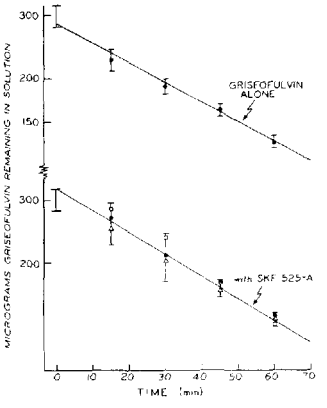

Fig. 4.—Disappearance of griseofulvin as a result of incubating the drug with liver slices pretreated with 75 mg./Kg. of SKF 525-A, reported with its 95% confidence intervals, as compared to that of griseofulvin alone.

TABLE I.—METABOLISM OF GRISEOFULVIN IN THE MICROSOMAL ENZYME SYSTEM

Expt.	Half-Life of Metabolism, min.
1	57.5
2	50.3
3	101.4
4	40.35
5	40.25
6[a]	44.0
7[a]	30.9

[a] Animals starved prior to experimentation.

The results indicate the mean half-lives of experiments *1* through *5* to be 57.9 ± 39.7 min. (p = 0.025).

Starvation is known to lower glycogen levels markedly. Therefore, rabbits were starved for 24 and 38 hr. prior to sacrifice and preparation of the microsomal and soluble fractions of the liver. When the metabolism of griseofulvin was followed as a result of incubation with these glycogen-depleted microsomal enzymes, the results in Table I indicate that starvation did not interfere with or affect the ability of the microsomal enzymes to metabolize griseofulvin. A comparison of the results of the metabolism studies of griseofulvin, to those previously reported by Axelrod for p-ethoxyacetanilide (12) are recorded in Table II.

TABLE II.—COMPARISON OF RESULTS OF METABOLISM STUDIES OF GRISEOFULVIN

	Griseofulvin	*p*-Ethoxyacetanilide
Metabolized by the microsomal system	+	+
Inhibited by SK&F 525–A	−	−
Inhibited by *p*-methoxybenzylamine	+	+
Inhibited by codeine	−	−
Inhibited by *p*-ethoxyacetanilide	+	

Based on the criteria established by Axelrod for differentiating between the two possible *O*-dealkylating enzyme systems, it would appear that griseofulvin is metabolized by the same enzymes that metabolize *p*-ethoxyacetanilide and *p*-methoxybenzylamine rather than by the *O*-dealkylating enzyme system shown to metabolize codeine.

In both the liver slice and the microsomal studies the disappearance of griseofulvin was compared to the appearance of 6-demethylgriseofulvin. Table III presents an analysis of the total recovery of griseofulvin and 6-demethylgriseofulvin.

TABLE III.—PER CENT RECOVERY OF THE TOTAL DOSE OF GRISEOFULVIN AS GRISEOFULVIN AND 6-DEMETHYLGRISEOFULVIN

Time of Incubation, min.[a]	% Recovered as Griseofulvin	% Recovered as 6-Demethylgriseofulvin	Total % Recovery
0	100	0	80.0
15	75.0	2.16	77.16
30	62.7	15.1	77.8
45	54.6	17.4	72.0
60	44.4	31.9	76.3
75	40.0	33.0	73.0

[a] Extrapolated zero-time value.

In no instance was there a material balance since only 75.3 ± 3.8% of the original dose of griseofulvin could be accounted for. The remainder of the dose was sought as a possible glucuronide conjugate of 6-demethylgriseofulvin which would not be extracted prior to hydrolysis. Supernatants were incubated with β-glucuronidase to determine the possibility of glucuronide conjugation.

No real increase in free 6-demethylgriseofulvin was noted as compared to samples which had not been incubated with β-glucuronidase. These results would tend to indicate that 6-demethylgriseofulvin does not form a glucuronide in the rabbit.

It is possible that the glucuronide conjugate does not form due to properties inherent in the 6-demethylgriseofulvin molecule since the 6 pKa has been found to be 4.3. The accepted mechanism for glucuronide conjugation has been postulated as follows (16, 17):

uridine diphosphoglucuronic acid + R − OH

$$\xrightarrow{\text{Walden inversion}} \text{glucuronosyl} - \text{OR} + \text{uridine diphosphate}$$

If this were to occur with 6-demethylgriseofulvin in the body at a pH of 7.3, the metabolite would be in the form of a phenolate anion.

The Walden inversion is a S_N2 displacement reaction. The nucleophile in a S_N2 reaction attacks by using a pair of its own electrons. Therefore, the most effective nucleophile would be one whose attacking atom has the valence electrons most available for coordination. Since this is the criterion for basic strength, it would be reasonable to assume that the strongest base might make the most effective nucleophilic reagent. The phenolate anion is known to be a nucleophilic reagent with reasonable activity. However, basic strength, resonance characteristics, and steric effects may interfere with the relative nucleophilicity of an agent. Perhaps, owing to some adverse nucleophilic properties of 6-demethylgriseofulvin, it might be possible for the metabolite to form a glucuronide conjugate on a purely mechanistic basis.

Dodgson and co-workers (18) have suggested that the ability of a hydroxyl compound to form an ethereal sulfate may depend on its ionization constant. A survey of phenols which are known to conjugate with sulfate has shown that the pKa's of the hydroxyl groups varied from 7 to 10. Anderton *et al.* (19) compared the amount of ethereal sulfate produced with the pKa of the hydroxyl group for a number of compounds. They concluded that the hydroxyl group must have a pKa between 7 and 10 in order to be able to form an ethereal sulfate. Since the pKa of 6-demethylgriseofulvin is 4.3, this factor alone might disqualify possible sulfate conjugation.

It is clear from the above that an explanation for the lack of a material balance cannot be accounted for by presuming conversion of the metabolite to conjugates such as glucuronide or sulfate, and the possibility of the formation of a second metabolite is being investigated.

Further reports will follow on studies of the effect of varying concentration of *p*-ethoxyacetanilide and *p*-methoxybenzylamine to that of griseofulvin and of *in vivo* effects of these inhibitors.

SUMMARY

1. The metabolism of griseofulvin has been demonstrated *in vitro*, using rabbit liver slices respiring in a Krebs-Ringer bicarbonate buffer medium. The metabolism appears to follow pseudo first-order kinetics with a half-life of 53 min.

2. Griseofulvin has been shown to be metabolized also by the microsomal enzymes of the liver in the presence of the co-factors of the soluble reaction, and the metabolism is independent of the glycogen content of the liver.

3. The *in vitro* inhibition of griseofulvin metabolism by *p*-ethoxyacetanilide and *p*-methoxybenzylamine is reported. Evidence is presented for the microsomal enzyme fraction responsible for the *O*-demethylation of griseofulvin.

4. The metabolite, 6-demethylgriseofulvin, as the free phenol has been isolated and quantitated. A glucuronide conjugate has not been found, and reasons for this lack of conjugation are presented.

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Interaction of 8-Hydroxyquinoline Sulfate with Components Present in a Tuberculin PPD Solution I

Binding of 8-Hydroxyquinoline by Polysorbate 80

By S. LANDI and H. R. HELD

This article deals with the interaction between the preservative 8-hydroxyquinoline sulfate (8-HQS) and the surface-active agent polysorbate 80. In buffered solution (pH 7.38) 8-HQS is dissociated to 8-hydroxyquinoline (8-HQ) and H_2SO_4, and it is the base 8-HQ that forms a reversible association with polysorbate 80. The degree of binding of 8-HQ to polysorbate 80 was shown to be a function of the concentration of the nonionic surface-active agent. Polysorbate 80 at low concentration (about 0.005 per cent) has practically no effect on the concentration of 8-HQ in a buffered solution (pH 7.38) as used for preparing dilutions of tuberculin PPD for the intracutaneous method (Mantoux test). 8-HQS, in a buffered solution of pH 3, does not interact with polysorbate 80.

8-HYDROXYQUINOLINE sulfate (8-HQS)[1] is added to tuberculin PPD solutions as an antimicrobial agent. In a previous report (1) the authors described how 8-HQS disappears from these solutions when dispensed in glass vials stoppered with rubber closures and showed that most of the loss of 8-HQS from the solution was caused by sorption of 8-HQ by the rubber closures.

Numerous investigators have shown, by using solubility studies and equilibrium dialysis, that binding of preservatives (3–9) or pharmaceuticals (10–14) with polysorbate 80[2] or other macromolecules takes place.

Tuberculin PPD solutions are used intracutaneously for diagnostic purposes in tuberculosis-prevention programs. Such solutions contain 0.01% 8-HQS added as a preservative and 0.005% polysorbate 80 as a stabilizing agent (2). It was, therefore, of interest to find out if some binding between 8-HQS and polysorbate 80 takes place in the buffer used to prepare these solutions.

The solubility method (3) and the equilibrium dialysis method (3) were used by the authors to determine the compatibility of polysorbate 80 and 8-HQS.

Received September 8, 1965, from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario, Canada.
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[1] Marketed as Chinosol.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.

MATERIALS AND METHODS

Reagents.—8-Hydroxyquinoline sulfate (8-HQS)[1] (Eastman Organic Chemicals, 1776), 8-hydroxyquinoline (8-HQ) (Fisher Scientific Co., 0-261), polysorbate 80 (polyoxyethylene 20 sorbitan monooleate).[2]

Buffer Solution (pH 7.38).—Isotonic phosphate buffered solution (2), pH 7.38 (1.45 Gm. of KH_2PO_4, 7.60 Gm. of Na_2HPO_4, and 4.8 Gm. of NaCl in 1050 ml.). This buffer is also used as diluent for the preparation of tuberculin PPD solutions (Mantoux).

Buffer Solution (pH 3.0).—McIlvaine's buffered solution (15) was prepared by mixing 79.5 ml. of 0.1 *M* citric acid solution and 20.5 ml. of 0.2 *M* Na_2HPO_4 solution.

Dialysis Membranes.—Thin nylon membrane,[3] as recommended by Patel and Kostenbauder (3), was used for dialysis of 8-HQ. A seamless regenerated cellulose tubing, size identity 36/32 (obtained from the Visking Division of Union Carbide, Canada, Ltd.) was used for the dialysis of 8-HQS and of 8-HQ.

Determination of 8-HQ and 8-HQS.—8-HQS, dissolved in buffered solution (pH 7.38), is dissociated in 8-HQ and H_2SO_4. The distinct absorption maximum of 8-HQ in the ultraviolet region at 240 mμ lends itself well to the quantitative deter-

[3] Supplied through the courtesy of Youngs Rubber Corp., New York, N. Y.

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MATERIALS AND METHODS

Reagents.—8-Hydroxyquinoline sulfate (8-HQS)[1] (Eastman Organic Chemicals, 1776), 8-hydroxyquinoline (8-HQ) (Fisher Scientific Co., 0-261), polysorbate 80 (polyoxyethylene 20 sorbitan monooleate).[2]

Buffer Solution (pH 7.38).—Isotonic phosphate buffered solution (2), pH 7.38 (1.45 Gm. of KH_2PO_4, 7.60 Gm. of Na_2HPO_4, and 4.8 Gm. of NaCl in 1050 ml.). This buffer is also used as diluent for the preparation of tuberculin PPD solutions (Mantoux).

Buffer Solution (pH 3.0).—McIlvaine's buffered solution (15) was prepared by mixing 79.5 ml. of 0.1 *M* citric acid solution and 20.5 ml. of 0.2 *M* Na_2HPO_4 solution.

Dialysis Membranes.—Thin nylon membrane,[3] as recommended by Patel and Kostenbauder (3), was used for dialysis of 8-HQ. A seamless regenerated cellulose tubing, size identity 36/32 (obtained from the Visking Division of Union Carbide, Canada, Ltd.) was used for the dialysis of 8-HQS and of 8-HQ.

Determination of 8-HQ and 8-HQS.—8-HQS, dissolved in buffered solution (pH 7.38), is dissociated in 8-HQ and H_2SO_4. The distinct absorption maximum of 8-HQ in the ultraviolet region at 240 mμ lends itself well to the quantitative deter-

[3] Supplied through the courtesy of Youngs Rubber Corp., New York, N. Y.

mination of 8-HQ and herewith to the determination of 8-HQS. The 8-HQS concentration of a solution was determined by measuring its absorbance at 240 mμ after dilution with buffer of pH 7.38, containing 0.005% polysorbate 80 and comparing it with the absorbance at the same wavelength of a standard solution. This method was described previously (1).

The H_2SO_4 liberated from 8-HQS was determined by precipitation and weighing as $BaSO_4$.

Determination of Polysorbate 80.—Polysorbate 80 shows an absorption maximum in the ultraviolet region at 230 mμ. The degree of absorption is too weak to be used for very low concentrations of polysorbate 80 or for higher concentrations of polysorbate 80 in the presence of strong U.V. absorbers like phenolic preservatives, etc. However, for pure aqueous solutions of polysorbate 80, this absorption at 230 mμ can be conveniently used for its determination. The authors determined the polysorbate 80 content by diluting 1 ml. of a pure aqueous solution of polysorbate 80 with 19 ml. of a buffered solution (pH 7.38), and comparing its absorbance at 230 mμ with the absorbance at the same wavelength of a standard solution of polysorbate 80.

Solubility Method.—The solubility of 8-HQS or 8-HQ in polysorbate 80 solutions was evaluated by placing into glass-stoppered weighing bottles 10 ml. of buffered solution containing various amounts of polysorbate 80 and an excess of either 8-HQS (200 mg. of a mixture, consisting of equivalent amounts of 8-HQS and $NaHCO_3$) or of 8-HQ (140 mg.). The bottles were agitated for 3 hr. at 28° then the contents were filtered through Whatman No. 1 filter paper and the 8-HQS or 8-HQ in solution determined spectrophotometrically.

Dialysis Method at pH 7.38.—The dialysis membrane was used in the form of small nylon bags. Into each bag was placed 10 ml. of a buffered solution containing different concentrations of polysorbate 80. Each nylon bag was then placed in a glass-stoppered weighing bottle, containing 10 ml. of buffered solution and 200 mg. of a mixture consisting of equivalent amounts of 8-HQS and $NaHCO_3$. In another experiment the weighing bottles contained 10 ml. of a 0.02% solution of 8-HQS in the same buffer. Each bottle was then stoppered tightly while the end of the nylon bag was protruding to the outside of the bottle, thus providing also a tight closure for the nylon bag. The bottles were then agitated for 4 days at 28°. After this period of time, an equilibrium between the inner and outer solution was reached for each concentration of polysorbate 80 employed, and samples were taken from the inside and outside of the nylon bags to determine the concentration of 8-HQ.

The same type of dialysis as described above was carried out for 2 days using a seamless cellulose tubing instead of the nylon bags.

Dialysis Method at pH 3.—Similar experiments as described were carried out in a solution of approximately pH 3 using as dialysis membrane a seamless cellulose tubing. Into each tubing was placed 10 ml. of an aqueous solution of different concentrations of polysorbate 80. The glass-stoppered weighing bottles contained in one experiment 10 ml. of a 0.2% solution of 8-HQS in H_2O (final pH 3.2), and in another experiment the weighing bottles contained 10 ml. of a 0.02% solution of 8-HQS in buffer (pH 3.0). The bottles were agitated for 2 days at 28°.

RESULTS AND DISCUSSION

Interaction between Polysorbate 80 and 8-HQ at pH 7.38.—When 8-HQS is dissolved in buffered solution (pH 7.38), it is dissociated in 8-HQ and H_2SO_4. This is evidenced from the fact that 8-HQ can be completely extracted with diethylether from this buffered solution into which 8-HQS was added. Further evidence that 8-HQ is present in a buffered solution (pH 7.38) was obtained in a previous article (1), where it was shown that rubber absorbed 8-HQ only while the H_2SO_4 remains entirely in the aqueous solution. Dialysis experiments to be described will show that it is 8-HQ that interacts with polysorbate 80 and not 8-HQS. Nevertheless, since in the literature dealing with tuberculin the concentration of this preservative is given as percentage of 8-HQS, the authors have in the present investigation expressed all measurements in 8-HQS equivalents.

Figure 1 shows that the solubility of 8-HQS or 8-HQ increases linearly with increasing polysorbate 80

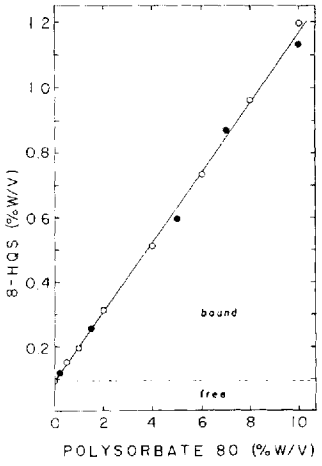

Fig. 1.—Solubility of 8-HQS and of 8-HQ in a buffered solution (pH 7.38, temperature 28°) containing various amounts of polysorbate 80. Key: ○, solubility of 8-HQS; ●, solubility of 8-HQ, plotted in 8-HQS-equivalents.

concentration, and when the solubility of 8-HQ is expressed in 8-HQS equivalents the same slope is obtained for both substances. Because the solubility of free preservative in a buffered solution is constant, as represented by a dashed line in Fig. 1, the increase in solubility of the preservative with increasing polysorbate 80 concentration can be considered to be due to bound preservative. This binding represents a relatively high degree of interaction. Such interaction between polysorbate 80 and other preservatives had been reported previously by several investigators (3–9).

To obtain further evidence of binding between polysorbate 80 and 8-HQ and to obtain data of binding between polysorbate 80 and low concentrations of 8-HQ, the equilibrium dialysis method (3) was used. Figures 2 and 3 show that when the concentration of polysorbate 80 in a buffered solution con-

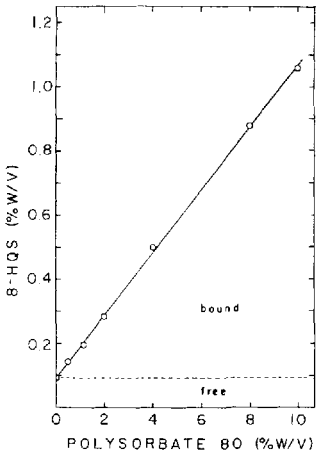

Fig. 2.—Equilibrium dialysis through a nylon bag. Outside the bag was placed a buffered solution (pH 7.38) to which an excess of solid 8-HQS and $NaHCO_3$ was added. Inside the bag was placed a buffered solution (pH 7.38), containing various amounts of polysorbate 80. The total 8-HQ concentration in the dialysis bag is plotted in 8-HQS equivalents *vs.* the polysorbate 80 concentration.

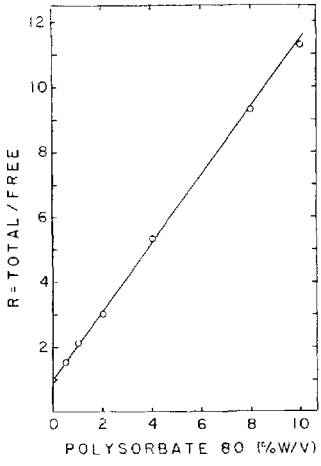

Fig. 3.—Equilibrium dialysis through a nylon bag as in Fig. 2. The binding of 8-HQ (expressed as the ratio R = total/free) is plotted *vs.* the polysorbate 80 concentration.

tained in the nylon bag is increased from 0 to 10% the total concentration of 8-HQ in this solution increases linearly from 0.094 to 1.06%. In this experiment an excess of solid 8-HQS and $NaHCO_3$ was added to the solution outside the dialysis bag. Since at equilibrium the concentration of free preservative is assumed to be approximately the same inside and outside the dialysis bag (12), the increase of 8-HQ with increasing polysorbate 80 concentration (represented by the area above the dashed line in Fig. 2) must correspond very closely to the degree of interaction between polysorbate 80 and 8-HQ. The authors designated this increase in 8-HQ "*bound 8-HQ.*" To test the reversibility of this interaction the contents of the bags were dialyzed against running tap water, and it was found that all 8-HQ (free plus bound) dialyzed out of the bags in approximately 10 days. This shows that the "bound 8-HQ" was reversibly associated to the polysorbate 80.

Figure 2 confirms the results of solubility studies (Fig. 1) and also shows that the concentration of free 8-HQ is independent of the polysorbate 80 concentration when enough preservative is present to form a 8-HQ saturated solution. The free 8-HQ is represented by a dashed line in Figs. 1 and 2. It follows that the ratio between total 8-HQ (bound plus free) and free 8-HQ also increases linearly with increasing polysorbate 80 concentration (Fig. 3). This linearity facilitates the calculation of this ratio for any other concentration of polysorbate 80. Therefore, the results in Table I reveal that, when a concentration of 0.005% polysorbate 80 is present in a buffered solution containing an added amount of 0.094 to 1.06% total 8-HQS, there should be approximately 99.5% of free 8-HQ present. Thus, the loss of free 8-HQS caused by the interaction of 8-HQ with polysorbate 80 should be approximately 0.5%. In order to verify these results for lower concentrations of total 8-HQS (0.0096 to 0.0181%), the dialysis method (3) was also employed by using a concentration of 0.02% 8-HQS in the solution outside the nylon bag. Table II shows that the ratios between total and free 8-HQS are of the same order as those measured for more concentrated solutions of 8-HQS (0.094 to 1.06%, Table I), and that the loss of free 8-HQS caused by the presence of 0.005% polysorbate 80 should also be approximately 0.5%. Therefore, it is obvious that the reversible interaction between 0.005% polysorbate 80 and the 8-HQ liberated from 0.01% 8-HQS, in the buffer solution, as used for the preparation of tuberculin PPD solutions (2, 16), will have practically no effect on the concentration of free 8-HQ and presumably on its antimicrobial activity. That tuberculin PPD solutions containing these concentrations of polysorbate 80 and 8-HQS exhibit antimicrobial activity has been recently demonstrated by Pivnick *et al.* (17).

Further evidence that 8-HQ, and not 8-HQS, interacts with polysorbate 80 was obtained by using a cellulose instead of a nylon membrane. While 8-HQ dialyzed readily through the nylon membrane and reached equilibrium after 4 days, only traces of H_2SO_4 liberated from 8-HQS had passed through the nylon membrane (0.002% w/v inside as compared to 0.35% w/v outside the bag, Table I). It was further ascertained that sulfates like 8-HQS or Na_2SO_4 in buffered solution (pH 7.38) dialyze very slowly, if at all, through a nylon membrane. However, the seamless cellulose tubing was found to be readily permeable to 8-HQ as well as to 8-HQS and Na_2SO_4. Although the cellulose tubing was found to be to some extent permeable to polysorbate 80 (3), as in the case of the nylon membrane, most of the polysorbate 80 was kept inside the bag after 2 days, dialysis. However, considerable water was drawn inside the cellulose tubing from the outside solution changing the concentration of polysorbate 80. For this reason all the concentrations inside (C_i) and outside (C_0) the cellulose tubing were determined after equilibrium was reached. In fact, Table III shows that the binding of 8-HQ by polysorbate 80 is of the same order as previously determined using a

TABLE I.—DEGREE OF BINDING[a] OF 8-HQ BY POLYSORBATE 80 CALCULATED FROM INCREASE OF 8-HQ CONCENTRATION DUE TO PRESENCE OF POLYSORBATE 80 (DIALYSIS METHOD AT pH 7.38, USING A NYLON DIALYSIS BAG)

Concn. Inside Nylon Bag: Polysorbate 80	Concn. Inside Nylon Bag: Total 8-HQ Expressed as 8-HQS Equiv.	Concn. Inside Nylon Bag: Total H_2SO_4	Binding of 8-HQ, Expressed as: Ratio of Total/Free	Binding of 8-HQ, Expressed as: % of Total 8-HQ
% w/v	% w/v	% w/v	...	%
10	1.06	0.002[c]	11.28	91.14
8	0.876	0.002[c]	9.32	89.27
5	0.58[b]	...	6.2[b]	83.87[b]
4	0.500	...	5.32	81.20
2	0.283	...	3.01	66.78
1	0.194	...	2.06	51.46
0.5	0.146	...	1.553	35.61
0.1	0.104[b]	...	1.104[b]	9.42[b]
0.01	0.09498[b]	...	1.0104[b]	1.03[b]
0.005	0.09449[b]	...	1.0052[b]	0.52[b]
0	0.094	...	1	0

[a] After reaching equilibrium. (All concentrations of 8-HQ are expressed in 8-HQS equivalents.) [b] Calculated from slope of curve (total/free *vs.* concentration of polysorbate 80). [c] Total H_2SO_4 liberated from an excess of 8-HQS added to the buffered solution (pH 7.38) outside of the nylon bag was 0.35% w/v.

TABLE II.—DEGREE OF BINDING[a] OF 8-HQ BY POLYSORBATE 80 CALCULATED FROM RATIO C_i/C_0 (DIALYSIS METHOD AT pH 7.38, USING NYLON DIALYSIS BAG)

Concn. in Nylon Bag: Polysorbate 80	Concn. in Nylon Bag: 8-HQ: Total C_i	Concn. in Nylon Bag: 8-HQ: Free C_0	Binding of 8-HQ, Expressed as: Ratio of Total/Free	Binding of 8-HQ, Expressed as: % of Total 8-HQ
% w/v	% w/v	% w/v	...	%
10	0.0181	0.00154	11.75	91.5
5	0.0167	0.00276	6.05	83.5
2	0.0147	0.00482	3.06	67.3
1	0.0135	0.00688	1.96	49.0
0.5	0.0125	0.00764	1.64	38.9
0.1	...	...	1.104[b]	9.42[b]
0.01	...	...	1.0104[b]	1.03[b]
0.005	...	...	1.0052[b]	0.52[b]
0	0.0096	0.0096	1	0

[a] After reaching equilibrium. (All concentrations of 8-HQ are expressed in 8-HQS equivalents.) [b] Calculated from slope of curve (ratio C_i/C_0 *vs.* concentration of polysorbate 80). C_i = concentration C_i inside nylon bag (representing total 8-HQ inside nylon bag). C_0 = concentration C_0 outside nylon bag (representing free 8-HQ outside and inside nylon bag).

TABLE III.—DEGREE OF BINDING[a] OF 8-HQ BY POLYSORBATE 80 CALCULATED FROM RATIO C_i/C_0 (DIALYSIS METHOD AT pH 7.38, USING SEAMLESS CELLULOSE TUBING)

Concn. in Soln. Inside (C_i): Polysorbate 80 C_i	Concn. in Soln. Inside (C_i): 8-HQ C_i	Concn. in Soln. Inside (C_i): H_2SO_4 C_i	Outside (C_0): Polysorbate 80 C_0	Outside (C_0): 8-HQ C_0	Outside (C_0): H_2SO_4 C_0	Retention of Polysorbate 80: Expressed as Ratio C_i/C_0 of: Polysorbate 80 C_i/C_0	Degree of Binding of 8-HQS: Expressed as Ratio C_i/C_0 of: 8-HQ C_i/C_0	Degree of Binding of 8-HQS: Expressed as Ratio C_i/C_0 of: H_2SO_4 C_i/C_0
% w/v	% w/v	% w/v	% w/v	% w/v	% w/v	...	...	...
10	...	...	...	...	...	...	11.3[b]	...
6.30	0.70	0.21	0.115	0.099	0.21	54.8	7.07	1.00
5	...	...	...	...	...	...	6.2[b]	...
3.21	0.462	0.18	0.065	0.097	0.18	49.4	4.76	1.00
1.82	p.331	0.18	0.023	0.100	0.18	79.1	3.31	1.00
1	...	...	...	...	...	...	2.0[b]	...
0.005	...	...	...	...	...	...	1.005[b]	...
0	0.100	0.19	0	0.100	0.19	...	1.00	1.00

[a] After reaching equilibrium. (All concentrations of 8-HQ are expressed in 8-HQS equivalents.) [b] Calculated from slope of curve (ratio C_i/C_0 of 8-HQ *vs.* concentration of polysorbate 80.).

nylon membrane. Example: 10% w/v polysorbate 80, binding of 8-HQ 11.28 (Table I) and 11.30 (Table III); 0.005% w/v polysorbate 80, binding of 8-HQ 1.0052 (Table I) and 1.005 (Table III). Furthermore, when the cellulose tubing was used the final concentration of H_2SO_4 (liberated from 8-HQS) becomes equal on both sides of the dialysis membrane for all the concentrations of polysorbate 80 employed (Table III). This indicates that only 8-HQ and not 8-HQS interacts with polysorbate 80 because, if an appreciable amount of 8-HQS was present at pH 7.38 and did interact with polysorbate 80, the ratio $C_i/C_0 = 1$ of H_2SO_4 (Table III) would become numerically higher than 1.

Interaction between Polysorbate 80 and 8-HQS at pH 3.—Although a diluent at this pH is not used for the preparation of tuberculin PPD solutions, it is, nevertheless, useful to describe briefly the results of a study of a possible interaction between polysorbate 80 and 8-HQS at pH 3. It might con-

tribute to the understanding of the basic mechanism between these two substances in the buffer (pH 7.38) previously employed.

8-HQS, dissolved in buffered solution (pH 3), shows no noticeable dissociation. This was evidenced from the fact that 8-HQ cannot be extracted with diethylether from 8-HQS in solution at pH 3 and that, in contrast to what was found previously using a buffered solution (pH 7.38), pure rubber does not absorb 8-HQ from 8-HQS in a buffered solution (pH 3).

Figure 4 shows the result of an equilibrium dialysis experiment in which seamless cellulose tubing

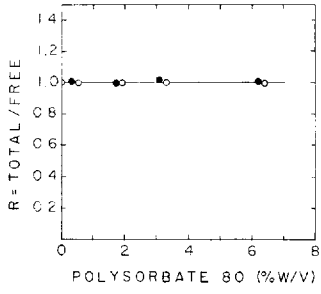

Fig. 4.—Equilibrium dialysis of 8-HQS through seamless cellulose tubing at pH 3. No interaction was detected. Key: O, 0.2% 8-HQS dissolved in H_2O was placed outside the dialysis tubing; ●, 0.02% 8-HQS dissolved in buffered solution (pH 3.0) was placed outside the dialysis tubing.

was used as a dialysis membrane. The ratios of the 8-HQS concentrations inside (C_i) and outside (C_0) of the tubing were plotted *versus* the polysorbate 80 concentration at equilibrium. The final polysorbate 80 concentrations were determined separately by ultraviolet spectrophotometry. The horizontal line (Fig. 4) indicates that there is no interaction between polysorbate 80 and 8-HQS at pH 3.

Interaction of 8-HQ with Other Macromolecules.—The interaction of 8-HQ liberated from 8-HQS with other macromolecules (tuberculoprotein, nucleic acid, and polysaccharide) present in tuberculin PPD solutions is now under investigation and will be reported subsequently.

SUMMARY

1. The interaction of 8-HQS with polysorbate 80 in buffered solutions of pH 7.38 and of pH 3.0 have been studied by means of the solubility method and the equilibrium dialysis method.

2. 8-HQS dissolved in buffered solution (pH 7.38) is dissociated in 8-HQ and H_2SO_4, and it is the base 8-HQ which interacts with polysorbate 80.

3. The degree of binding of 8-HQ by polysorbate 80, expressed as the ratio of total to free 8-HQ, increases linearly with increasing concentration of polysorbate 80.

4. The degree of binding of 8-HQ by polysorbate 80 is the same, regardless of whether 8-HQS or 8-HQ is added to a buffered solution (pH 7.38) containing polysorbate 80.

5. A relatively high degree of interaction has been observed for polysorbate 80 concentrations of 1 to 10%. However, when polysorbate 80 is added at the concentration of 0.005%, as used in tuberculin PPD dilutions for the Mantoux test, practically all 8-HQ (approximately 99.5%) is still present in the free state.

6. The association between 8-HQ and polysorbate 80 is reversible.

7. 8-HQS in a buffered solution of pH 3 does not interact with polysorbate 80.

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Surfactant–Base–Barbiturate Suppositories I

Rectal Absorption in Rabbits

By J. H. FINCHER, D. N. ENTREKIN, and C. W. HARTMAN

A series of petrolatum–paraffin–surfactant suppository bases were tested in rabbits under carefully controlled conditions with a series of barbiturates included. Each surfactant had a different HLB value and each barbiturate had a different distribution coefficient. The depression of the respiratory rate was measured for a period of 2.5 hr. to determine absorption rates. A comparative analysis of the data is presented. The effects of the chemical type of the drug and the surfactant are shown in the comparative studies. Surfactants enhanced the release and absorption of the barbiturate in some bases; however, binding of the drug is suspected in some bases. The relationships of the HLB of the surfactant and the distribution coefficient of the drug to release and absorption are presented. Further work, using different approaches to this problem, is indicated.

THE PRIMARY purpose of this work was to investigate the relationship of some of the physicochemical properties of the drug and the base on the efficacy of base–drug suppository formulations *in vivo*.

The hydrophile–lipophile balance (HLB), as a system of classifying and evaluating surfactants, was proposed and expanded by Griffin (1, 2). The effect of this property has been studied *in vitro* by Spittle and Hartman (3) and Rhyne *et al.* (4). Base composition has been long recognized as having an influence on the therapeutic activity of base–drug combinations (5–9). Emulsification of the base has been shown to effect drug release by many researchers (10–19). Others have shown that the chemical type of the ingredients in a formulation influences the desired clinical response (1, 2, 20). Other factors that have been considered are the distribution coefficient of the drug between the base and body fluids and the pKa of the drug (21–25). While some of the research cited above was conducted *in vivo*, the greater portion was conducted *in vitro*. The need for studies on animals is apparent. For this reason a series of 8 barbiturates were evaluated in a series of petrolatum–paraffin–surfactant suppository bases differing with respect to the emulsifiers which had different HLB values. Since each barbiturate was evaluated under the same conditions, a comparison could be made. A suppository formulation containing an emulsifier and medicament when placed in the rectum produces a complex system indeed. However, it is felt that such a comparative study as described above will reveal some of the major factors to consider when conducting further research intended to place drug diffusion, release, and absorption on a predictable basis.

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EXPERIMENTAL

Preparation of Bases.—Bases were prepared utilizing white petrolatum, white paraffin, polyethylene glycol (PEG), and surfactants. Surfactants with established HLB values (1, 2) were added to obtain a concentration of 10% by weight in each base. To produce anhydrous bases all materials except the emulsifiers were dried to constant weight. The consistency of all bases was standardized by varying the proportion of petrolatum and paraffin, or in the case of the PEG, by varying the proportion of PEGs of different molecular weights. The standard consistency selected was a penetration depth of 60 mm. (±6 mm.), when tested by the U.S.P. method at 25°.

Ten per cent by weight of surfactant was incorporated by fusion methods into lots of petrolatum and paraffin. Portions of these two emulsifier–base mixtures were then combined by trial and error to obtain the desired consistency. An HLB value of 0 was represented by using only petrolatum and paraffin as a base, and a water-soluble base was represented by use of a mixture of PEGs. Base composition and properties are shown in Table I. Table II gives a list of the barbiturates and their chemical properties. Several days before the time of the *in vivo* experiments the barbiturate was incorporated into the various bases using a glass mortar and pestle. The bases were stored in a temperature controlled room (21–23°) until the time of the experiments.

In Vivo Experimental Procedure.—The experimental animals were New Zealand white male rabbits weighing 1.5 to 2.3 Kg.

Seven to ten rabbits were used for each suppository base–barbiturate combination. One rabbit receiving the PEG base plus the drug and one rabbit receiving the petrolatum–paraffin base plus the drug were used as controls. All bases contained 0.0008 mole of the barbiturate per Gm. of suppository, thus regulating the concentration so that 1 Gm. of base would provide a dose of (0.0004 mole/Kg. body weight) for a rabbit weighing 2 Kg. This dose would be lethal to the rabbit if it were available for complete absorption in a short period of time.

A plastic injector having a tube length of 6.35 cm.

TABLE I.—COMPOSITION AND PROPERTIES OF SUPPOSITORY BASES[a]

Base No.	Ingredients	% by Wt.	Consistency in mm. Penetration	HLB Value of the Surfactant
I	Petrolatum	70.0		Approx. zero
	Paraffin (hard)	30.0	59.4	
II	Sorbitan trioleate[b]	10.0		1.8
	Petrolatum	59.4		
	Paraffin (medium)	30.6	62.4	
III	Sorbitan monolaurate[b]	10.0		8.6
	Petrolatum	59.4		
	Paraffin (hard)	30.6	59.1	
IV	Polyoxycthylene sorbitan tristearate[b]	10.0		10.5
	Petrolatum	61.2		
	Paraffin (hard)	28.8	58.5	
V	Polyoxyethylene sorbitan monolaurate[b]	10.0		16.7
	Petrolatum	59.4		
	Paraffin (hard)	30.6	62.8	
VI	Polyoxyethylene lauryl ether[b]	10.0		16.9
	Petrolatum	58.5		
	Paraffin (medium)	31.5	63.2	
VII	Polyoxycthylene monostearate[b]	10.0		17.9
	Petrolatum	59.0		
	Paraffin (medium)	31.0	58.0	
VIII	Polycthylene glycol 1500	98.2		Water soluble
	Polycthylene glycol 4000	1.8	66.8	

[a] Barbiturates were added to these bases. [b] Supplied by Atlas Chemical Co., Wilmington, Del.

TABLE II.—pKa AND DISTRIBUTION COEFFICIENTS[a,b] OF BARBITURATES

Barbiturate			Distribution
Generic Name	Chemical Name	pKa	Coefficient
Allylbarbituric acid	5-Allyl-5-iso-butyl BA[c]	7.86	10.5
Butethal[d]	5-Butyl-5-ethyl BA[c]	8.10	11.7
Pentobarbital	5-Ethyl-5-(1-methyl-butyl) BA	8.17	28.0
Aprobarbital	5-Allyl-5-iso-propyl BA	7.54	4.8
Secobarbital	5-Allyl-5-(1-methyl-butyl) BA		50.0

[a] From *Reference 21.* [b] Chloroform to water. [c] BA, barbituric acid. [d] Supplied by Abbott Laboratories.

and a diameter of 0.63 cm. was used to inject the suppository into the rectum.

All the rabbits used in a given experiment were fasted for 12 to 13 hr. before the time of the studies to render the rectum free of excess fecal matter and bladder free of excess urine. Fasting was begun at 7:30 p.m. (±30 min.) and each experiment was begun at 9:30 a.m. (±30 min.), thus keeping the time of day reasonably uniform. The rabbits were weighed, marked, and tied to a rabbit board (47.0 × 73.7 cm.) at least 45 min. before the injection of the suppository.

After weighing each injector an excess of the base–barbiturate mixture was molded directly into the injector and the exact calculated dose was adjusted by trial and error weighings.

The injector containing the base was then inserted into the rectum 1.3 cm. (indicated by a mark on the plastic tube) using no lubricant except the base itself. The plunger was then used to eject the suppository from the injector into the rectum. Polishing the sharp edge of the injector closed the end just enough to prevent the insertion of the plunger beyond this point, thus geometrically placing the suppository 1.3 cm. into the rectum as measured from the caudal end of the suppository. To prevent expulsion of the suppository a rubber hose clamp was used to hold the anus closed. Each animal was used only one time.

The barbiturates were released from the bases in sufficient quantity to cause marked changes in the respiratory rate. Administration of varying amounts of sodium pentobarbital by intravenous injection showed the change of respiration rate with a change of the dose (Fig. 1).

After administration of the suppository, the respiratory rate per minute was measured at time intervals ranging from 2 to 30 min., depending on the rate of change. Measurement of the respiratory rate was accomplished using a stopwatch and visually counting chest expansions. The respiratory counts were continued through a minimum period of 2.5 hr.

A constant time interval between 50 and 60 min. after rectal administration was arbitrarily selected as the end point for the comparative studies. The lowest respiratory rate within this interval for each

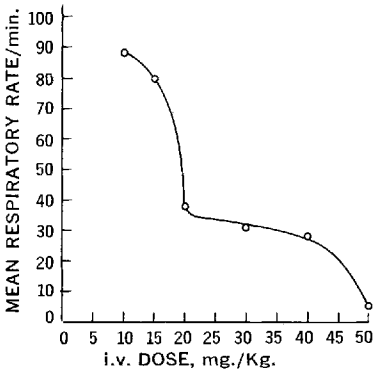

Fig. 1.—Mean respiratory rate as a function of i.v. dose of sodium pentobarbital 10 min. after administration to rabbits

TABLE III.—MEAN RESPIRATORY RATES AND RESULTS OF ERROR CALCULATIONS DETERMINED BETWEEN 50 AND 60 min. AFTER RECTAL ADMINISTRATION OF VARIOUS BASE–BARBITURATE COMBINATIONS TO RABBITS

Base[a]	Barbiturate	Mean Respiratory Rate, 1 min.	Animals, No.	$\pm\sigma m$[b]
I	Pentobarbital	100.3	4	9.8
	Sodium pentobarbital	50.7	3	10.9
	Calcium pentobarbital	105.4	5	15.5
	Butethal	110.0	9	5.5
	Secobarbital	98.5	6	7.2
	Allylbarbituric acid	108.0	8	7.1
	Aprobarbital	97.7	7	14.2
II	Pentobarbital	92.5	7	7.9
	Sodium pentobarbital	18.0	2	6.0
	Calcium pentobarbital	49.6	7	3.2
	Butethal	54.5	8	4.0
	Secobarbital	40.0	8	2.6
	Allylbarbituric acid	50.4	7	3.8
	Aprobarbital	43.3	8	2.5
III	Pentobarbital	128.8	7	17.73
	Sodium pentobarbital	106.9	2	6.0
	Calcium pentobarbital	96.8	7	15.35
	Butethal	97.0	6	12.4
	Secobarbital	71.3	7	5.8
	Allylbarbituric acid	77.3	8	9.8
	Aprobarbital	93.3	8	8.2
IV	Pentobarbital	105.1	7	11.9
	Sodium pentobarbital	64.0	3	7.4
	Calcium pentobarbital	64.1	7	8.5
	Butethal	128.8	6	6.7
	Secobarbital	65.5	8	5.7
	Allylbarbituric acid	99.4	8	8.1
	Aprobarbital	75.4	8	4.4
V	Pentobarbital	94.8	8	9.9
	Sodium pentobarbital	54.0	2	10.0
	Calcium pentobarbital	64.2	6	8.6
	Butethal	111.1	7	6.3
	Secobarbital	87.9	8	8.2
	Allylbarbituric acid	79.8	6	10.7
	Aprobarbital	80.3	7	16.9
VI	Pentobarbital	98.3	7	9.1
	Sodium pentobarbital	59.0	2	5.0
	Calcium pentobarbital	62.8	8	8.6
	Butethal	49.4	10	5.0
	Secobarbital	30.9	9	6.1
	Allylbarbituric acid	98.4	9	8.0
	Aprobarbital	66.8	8	6.53
VII	Pentobarbital	69.3	7	9.4
	Sodium pentobarbital	33.6	5	2.3
	Calcium pentobarbital	61.9	7	7.7
	Butethal	84.9	10	7.2
	Secobarbital	45.1	7	5.0
	Allylbarbituric acid	62.6	9	5.3
	Aprobarbital	47.8	10	4.2
VIII	Pentobarbital	14.3	4	3.9
	Sodium pentobarbital	0	6	0
	Calcium pentobarbital	0	6	0
	Butethal	32.4	8	2.8
	Secobarbital	23.4	7	6.0
	Allylbarbituric acid	37.3	8	3.3
	Aprobarbital	15.8	8	2.8

[a] See Table I for composition. [b] σm = standard error of the mean.

of the animals tested with a given base–drug formulation was used to evaluate the base. The mean respiratory rate was calculated, and from this the individual standard errors of the mean (σm) were determined. Appropriate t tests were performed for the data where quantitative comparisons of data are presented (26).

RESULTS AND DISCUSSION

The comparative results of the surfactant–base–barbiturate studies are summarized in Table III. The availability of pentobarbital is much less than that of calcium and sodium pentobarbital in all bases used with the exception of the petrolatum–paraffin base containing calcium pentobarbital (Fig. 2). This is in partial agreement with Riegelman and Crowell (17), who found the absorption of anions to be enhanced by the presence of surfactants while the absorption of an undissociated molecule was markedly retarded. The solubility of the sodium and calcium salts of pentobarbital in normal rectal fluids is probably much greater than the solubility of pentobarbital. The drug molecule having the

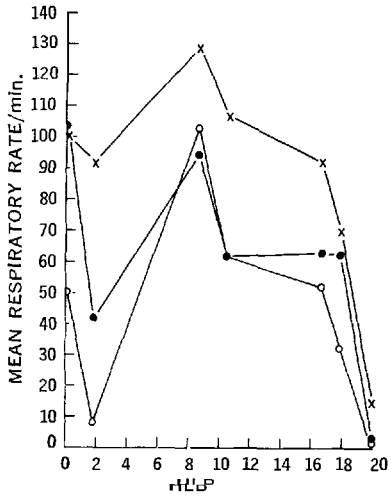

Fig. 2.—Mean respiratory rate as a function of the surfactant HLB value in the base. Comparison of a relatively undissociated barbituric acid to its partial or completely dissociated salts. Key: ×, pentobarbital; ●, calcium pentobarbital; ○, sodium pentobarbital. Statistical analysis: test of significance of difference of means nearest each other (t test) at the respective HLB values. Data are obtained from Table III. The probability of error (p) in the statement that the two means are different is tabulated as follows: × and ●, p <0.4> 0.35, 0 (HLB value); ● and ○, p <0.01> 0.005, 0 (HLB value); × and ●, p <0.005, 1.8 (HLB value); ● and ○, p <0.025> 0.01, 1.8 (HLB value); × and ○, p <0.15> 0.1, 8.6 (HLB value); ○ and ●, p <0.35> 0.20, 8.6 (HLB value); × and ●, p <0.01> 0.005, 10.5 (HLB value); ○ and ●, p ≃100, 10.5 (HLB value); × and ●, p <0.025> 0.01, 16.7 (HLB value); ● and ○, p <0.35> 0.2, 16.7 (HLB value); × and ●, p <0.35> 0.2, 17.9 (HLB value); ○ and ●, p <0.005, 17.9 (HLB value); × and ●, p <0.005, 20 (HLB value); ● and ○, p ≃100, 20 (HLB value). (The symbols consist of drugs compared.)

greater solubility would then be more available for absorption through the rectal membrane, if the drug were released from the base. Binding or complexing is suspected in all forms of pentobarbital, as well as of other barbiturates, used in the sorbitan monolaurate base (Table III).

In an effort to determine the effect of the distribution coefficient (DC) of the drug on release and absorption of barbiturates from suppositories after rectal administration, barbituric acids having varying DCs were chosen (Table II) and studied in bases containing different surfactants. Graphical presentation of the mean respiratory rate as a function of DC (chloroform/water) shows that those surfactants with a close chemical similarity (polyoxyethylene sorbitan tristearate and polyoxyethylene monostearate) yielded curves with approximately the same shape (Fig. 3). Sorbitan trioleate and sorbitan monolaurate yielded similar shaped curves (Fig. 4). When comparing Fig. 3 (polyoxyethylene sorbitan tristearate and polyoxyethylene monostearate) with Fig. 4 (sorbitan monolaurate and sorbitan trioleate), representing a more varied chemical structure, this similarity in the shape of the curves does not exist.

To study the effect of the HLB of the surfactants on release and absorption of barbiturates from the suppositories, surfactants with HLB values covering a large part of the HLB scale were utilized (Table II). A plot of the mean respiratory rate as a function of HLB of the surfactant shows that the two barbituric acids, aprobarbital and secobarbital, which have almost identical chemical structures, exhibited curves very similar in shape, and the degree of respiratory depression at the various HLB values was also similar (Fig. 5). Aprobarbital (5-allyl-5-isopropylbarbituric acid) and secobarbital (5-allyl-5[1-methyl butyl]barbituric acid) differ by 2 carbon atoms in the side chain. When comparing the results obtained with pentobarbital with

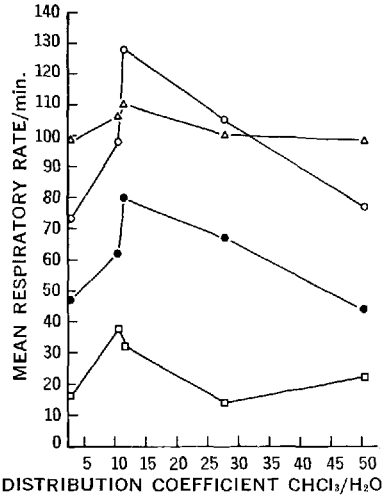

Fig. 3.—The mean respiratory rate as a function of the distribution coefficient of barbiturates. Comparison of surfactant chemical type. Key: ○, polyoxyethylene sorbitan tristearate; ●, polyoxyethylene monostearate; △, petrolatum–paraffin; □, polyethylene glycol.

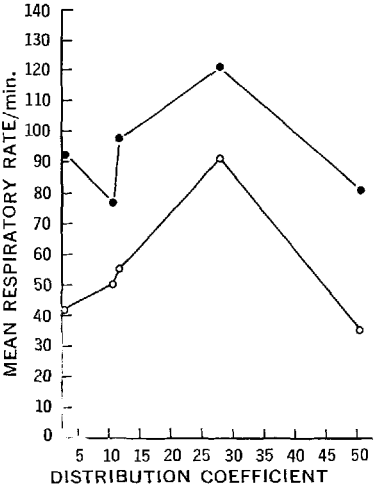

Fig. 4.—The mean respiratory rate as a function of distribution coefficient of the barbiturates. Comparison of surfactant chemical type. Key: ●, sorbitan monolaurate; ○, sorbitan trioleate.

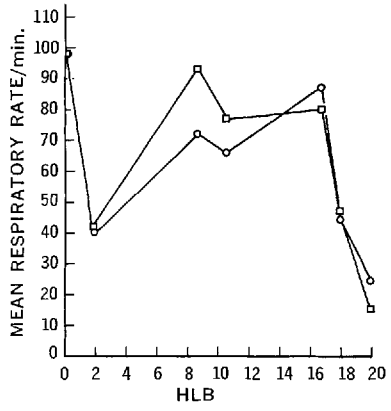

Fig. 5.—The mean respiratory rate as a function of the HLB of the surfactant in the base. Comparison of the chemical type of the barbiturates. Key: □, 5-allyl-5-isopropyl barbituric acid; O, 5-allyl-5-(1-methylbutyl) barbituric acid. Null hypothesis: the mean respiratory rates at each HLB value are different. Accept the null hypothesis if our probability of error is equal to or less than 0.2; reject the null hypothesis if our probability of error is greater than 0.2. Result: HLB value 0, reject; 1.8, reject; 8.6, accept; 10.5, accept; 16.9, reject; 17.9, reject; 20, reject.

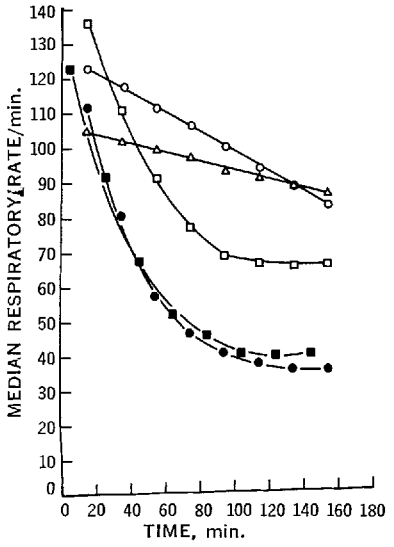

Fig. 6.—The median respiratory rate as a function of time using various bases containing surfactants with different HLB values and 5-ethyl-5-butyl-barbituric acid. Key: O, polyoxyethylene sorbitan monolaurate; □, polyoxyethylene stearate; △, sorbitan monolaurate; ■, sorbitan trioleate; ●, polyoxyethylene lauryl ether.

those obtained with secobarbital and aprobarbital (Figs. 2 and 5), this closeness in structure does not seem significant. The influence of the HLB of the surfactant, however, is not conclusive.

Since the HLB of a surfactant largely determines the type of emulsion formed and the degree of emulsification (27), and since some emulsification probably takes place in the rectum, when using suppository bases which contain surfactants, the effect on drug release at different HLB values may be quite significant. The predicted points of inflection are: (*a*) the point at which the most stable water-in-oil emulsion is formed, (*b*) the inversion point (point of changing from water-in-oil to an oil-in-water emulsion or vice-versa), and (*c*) the point at which the most stable oil-in-water emulsion is formed. The relative solubilities of the drug in the oil and water phases (distribution coefficient) would determine if these inflection points occur at a maximum or minimum biological response.

The change of the median respiratory rate in rabbits as a function of time and the type surfactant used in the bases is exemplified in Fig. 6. It is interesting to note that the laurates[1] exhibited linear relationships, whereas the oleate, stearate, and lauryl ether exhibited similarly shaped curves. The effect of similar chemical types of the surfactant on absorption is obvious here.

The change of the median respiratory rate of rabbits with time using various barbiturates in a base containing a polyoxyethylene lauryl ether[2] are exemplified in Fig. 7.

A complete analysis of the kinetic data will be presented for publication in the near future.

[1] Marketed as Tween 20 and Span 20 by Atlas Chemical Industries, Wilmington, Del.

[2] Marketed as Brij 35 by Atlas Chemical Industries, Inc., Wilmington, Del.

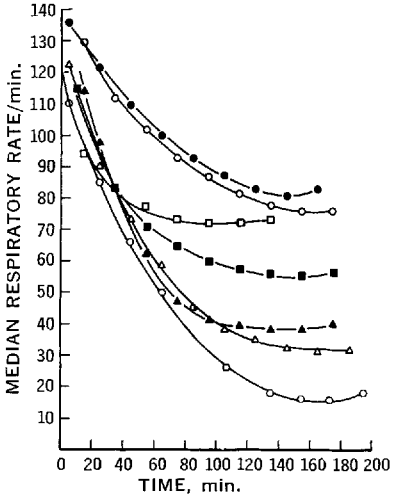

Fig. 7.—The median respiratory rate as a function of time using various barbituric acids in polyoxyethylene lauryl ether base (VI). Key: ●, 5-allyl-5-isobutyl barbituric acid; O, 5-ethyl-5-(1-methyl butyl) barbituric acid; □, 5-allyl-5-isopropyl barbituric acid; ■, calcium 5-ethyl-5-(1-methylbutyl) barbituric acid; ▲, 5-butyl-5-ethyl barbituric acid; △, sodium 5-ethyl-5-(1-methylbutyl) barbituric acid; O, 5-allyl-5-(1-methylbutyl) barbituric acid.

SUMMARY AND CONCLUSIONS

1. The absorption of barbiturates from eight suppository bases, which differed only by including surfactants with different HLB values, has been determined. Seven different barbiturates were used in

the suppositories which were administered to rabbits.

2. The salts of pentobarbital are more available for absorption from most bases containing surfactants based on the results of this study.

3. The relationship between the distribution coefficient of the drug and the HLB value of the surfactants used in the bases and their combined effects on absorption are inconclusive.

4. The chemical type of the surfactant and drug greatly influences the degree of release or absorption of barbiturates from suppositories in rabbits.

5. The addition of a surfactant to a base in most cases affects the availability of the drug from the base to the tissues. Complexation or binding may be one major factor causing these marked changes.

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Condensation of Aldoses and Their Aldehydo Derivatives with Compounds of the Type 1,3-Cyclohexanedione

Synthesis of 2,2-Aldosylidene-bis-[5-(*p*-hydroxyphenyl)-4,6-dicarbethoxy-1,3-cyclohexanedione] and 2,2-Aldosylidene-bis-[5,5-dimethyl-1,3-cyclohexanedione] and Derivatives

By PHILIPPOS E. PAPADAKIS

Glycoaldehyde, glyoxal, *dl*-glyceraldehyde, D-arabinose, D-glucose, and D-mannose react with compounds of the 1,3-cyclohexanedione type to form 2,2-aldosylidene-bis-1,3-cyclohexanedione derivatives.

THE PREPARATION of various 2,2-alkylidene or arylidene bis[5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione] by the condensation of an aliphatic or an aromatic aldehyde with 5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione or its derivatives was reported in a previous publication (1). It was also shown in that report that 5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione could condense with 1,2-acetone-D-xylotrihydroxyglutaric dialdehyde to give [5,5-bis-<2′,2′-{5′,5′-(*p*-hydroxyphenyl)-cyclohexanedione-1′, 3′}>-1,2-isopropylidene-5-desoxy-D-xylofuranose]$_2$ monohydrate. This suggests that the aldehydo form of aldoses and of metabolic products of carbohydrates having a carbonyl group may condense with cyclic 1,3-diketones to form bis-derivatives. Such reactions should be of scientific and pharmacological interest (9).

In the present work 5-(*p*-hydroxyphenyl)-4,6-dicarbethoxycylohexane-1,3-dione was condensed with each of the following carbohydrates (or derivatives) to give bis-derivatives which may be represented by the general formula (I): glycolaldehyde, glyoxal, *dl*-glyceraldehyde, L-

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By PHILIPPOS E. PAPADAKIS

Glycoaldehyde, glyoxal, *dl*-glyceraldehyde, D-arabinose, D-glucose, and D-mannose react with compounds of the 1,3-cyclohexanedione type to form 2,2-aldosylidene-bis-1,3-cyclohexanedione derivatives.

THE PREPARATION of various 2,2-alkylidene or arylidene bis[5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione] by the condensation of an aliphatic or an aromatic aldehyde with 5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione or its derivatives was reported in a previous publication (1). It was also shown in that report that 5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione could condense with 1,2-acetone-D-xylotrihydroxyglutaric dialdehyde to give [5,5-bis-<2′,2′-{5′,5′-(*p*-hydroxyphenyl)-cyclohexanedione-1′, 3′}>-1,2-isopropylidene-5-desoxy-D-xylofuranose]$_2$ monohydrate. This suggests that the aldehydo form of aldoses and of metabolic products of carbohydrates having a carbonyl group may condense with cyclic 1,3-diketones to form bis-derivatives. Such reactions should be of scientific and pharmacological interest (9).

In the present work 5-(*p*-hydroxyphenyl)-4,6-dicarbethoxycylohexane-1,3-dione was condensed with each of the following carbohydrates (or derivatives) to give bis-derivatives which may be represented by the general formula (I): glycolaldehyde, glyoxal, *dl*-glyceraldehyde, L-

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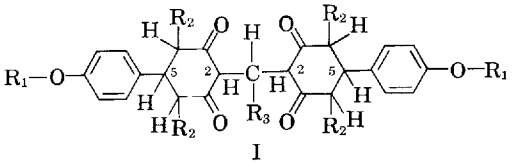
arabinose, 4,6-*O*-benzylidene-D-glucose, D-mannose, and D-galactaric dialdehyde.



I

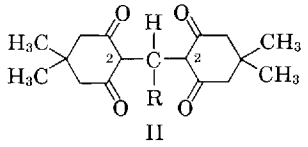
R_1 = H in experiments *1,2,3,5,7;* $-CH_3$ in *4* and $-COCH_3$ in *6* and *8*. R_2 = $-COOC_2H_5$ in *1, 2,3,5,6,7,8* and H in *4*. The value of R_3 is shown in Table I.

The method of preparation described under *Experimental* is similar to that used by Horning and Horning (2) with some modifications (1).

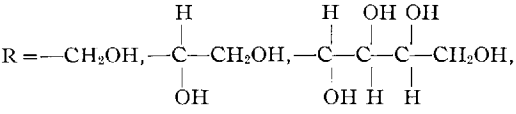
Other β-diketones are known to condense with aldehydes to give bis-derivatives (3–6).

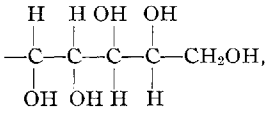
Vorlander (7) treated dimethone (5,5-dimethylcyclohexane-1,3-dione) with each of the following: glycolaldehyde, glyoxal, and *dl*-glyceraldehyde and obtained (bis-derivatives) anhydrides. His method consisted in dissolving the diketone with each of the respective substances and allowing it to stand at room temperature several hours whereby a precipitate was formed which was purified and analyzed. He failed to obtain precipitates with tetroses and higher aldoses. Bourne *et al.* (8) have reported that, under the conditions which they used, D-xylose does not react with dimedone.

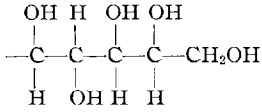
Fletcher (9) and co-workers found that: "D-xylose and dimedone containing a trace of quinoline mutarotates slowly over an extended period and yields a crystalline product which has the analysis of xylose-dimethone anhydride. A crystalline benzoate has also been obtained." In the present work aldosylidene-bis-dimedone derivatives were obtained, general formula (II), by condensing dimedone with each of the following carbohydrates in 75% methanol containing 2 drops of piperidine: glycolaldehyde, *dl*-glyceraldehyde, D-arabinose, D-mannose, and D-glucose. (*Experiments A–E.*)



II

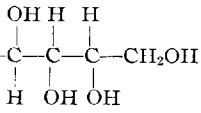
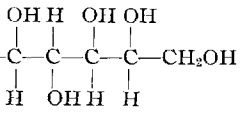
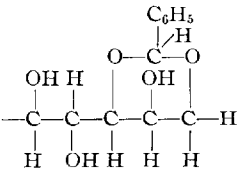
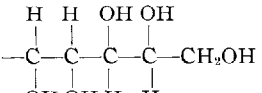
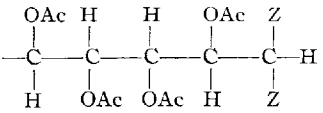


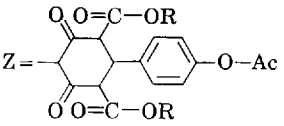




The results of the experiments *A* and *B* indicate that glycolaldehyde and glyceraldehyde react with dimedone to form (bis-derivatives)

TABLE I.—VALUE OF R_3

Expt.	R_3	Expt.	R_3
1	$-CH_2OH$		
2	$-CHO$		
3	$-CHOHCH_2OH$		
4			
5			
		6	
		7	
		8	



anhydrides. This is in agreement with Vorlander's work. Experiments *C*, *D*, and *E* indicate that contrary to Vorlander's findings the dimedone can condense with pentoses and hexoses. Analogous reactions have already been shown in Table I of this report where 5-(*p*-hydroxyphenyl)-4,6-dicarbethoxy-1,3-cyclohexanedione reacted with pentoses and hexoses to form bis-derivatives. (Experiments *6–8*.)

EXPERIMENTAL

5 - (*p* - Acetoxyphenyl) - 4,6 - dicarbethoxy - 1,3-cyclohexanedione, 1, 3, and 5-(*p*-hydroxyphenyl)-1,3-cyclohexanedione and related materials were prepared according to methods previously described (10).

METHOD

The method used for the condensation of the aldehydoform of the sugars to form aldosylidene-bis-derivatives with the above cyclic 1,3-diketones and with dimedone (type formula I and II) is similar to that used previously by the present author (1). In a general way, the molar proportions of the diketone to the aldose were 2:1. The materials were dissolved in hot 75% methyl alcohol, 1 or 2 drops of piperidine were added, and the mixture refluxed 2 hr. and then allowed to stand 3 days at room temperature, and in some cases, in the refrigerator.

Besides this method of procedure, experiments *5* and *7* were repeated and modified. After the reagents and solvents were mixed, they were allowed to stand at room temperature; compound *5* for 6 months and compound 7 for 13.5 months.

After evaporation of the solvents under reduced pressure using a water bath, the residue was purified by recrystallization from 50% methanol and in some cases from boiling distilled water. Details of purification varied, depending on the solubilities of the reagents and products in the different solvents.

1.—**2,2 - (2′ - Hydroxyethylidene) - bis - [5 - (p-hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione].**—Glycolaldehyde, 60 Gm., 5-(*p*-hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3-cyclohexanedione, 6.96 Gm., and 1 drop of piperidine were dissolved and refluxed in 75% methanol for 1 to 2 hr. The mixture was allowed to stay at room temperature 3 days. The solution was concentrated by evaporation on a water bath to an oily liquid which solidified upon addition of water. It was recrystallized from 75% methanol. It softens at 120° to a waxy consistency; it melts and decomposes at 143°. The bubbles formed are opalescent but clear at 156°.

Anal.—Calcd. for $C_{35}H_{42}O_{15} \cdot 1/2\ H_2O$: C, 61.03; H, 5.61. Found: C, 60.81; H, 5.74.

2.—**2,2 - Formalformylidene - bis - [5 - (p - hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione].**—To 6.96 Gm. of 5-(*p*-hydroxyphenyl)-4,6-dicarbethoxy-1,3-cyclohexanedione dissolved in hot 75% methanol, 2 ml. of 30% glyoxal and 1 drop of piperidine were added. The mixture was refluxed 0.5 hr., then cooled and allowed to stand in the refrigerator 3 days. A precipitate was formed which was processed. It was recrystallized from 75% methanol, m.p. 145°, dec. 156°. This compound is analogous to the bis-derivative of glyoxal with dimedone (5,5-dimethyl-1,3-cyclohexanedione) obtained by Vorlander.

Anal.—Calcd. for $C_{38}H_{40}O_{15} \cdot H_2O$: C, 60.41; H, 5.60. Found: C, 60.07; H, 5.45.

3.—**2,2 - (2′,3′ - Dihydroxypropylidene) - bis-[5 - (p - hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3-cyclohexanedione].**—To 2.32 Gm. of 5-(*p*-hydroxyphenyl)-4,6-dicarbethoxycyclohexane-1,3-dione dissolved in 75% hot methanol, 0.30 Gm. of *dl*-glyceraldehyde and 1 drop of piperidine were added with stirring. The mixture was refluxed 2 hr., then the solvents were evaporated and the residue recrystallized from hot distilled water. It melted at 90° and decomposed at 110°.

Anal.—Calcd. for $C_{39}H_{44}O_{16}$: C, 60.93; H, 5.76. Found: C, 61.17; H, 5.70.

The above product treated with phenylhydrazine gave an orange-red precipitate, a small part of which dissolved in ether. The residue was dissolved in alcohol and reprecipitated with distilled water, m.p. 161–163°.

Anal.—Calcd. for $C_{51}H_{56}N_4O_{14}$: N, 5.90. Found: N, 6.27.

From the ether solution after evaporation of the solvent, the small amount of material obtained was dissolved in alcohol and reprecipitated with distilled water, m.p. 129°.

Anal.—Calcd. for $C_{57}H_{60}N_6 \cdot 3H_2O$: N, 7.68. Found: N, 7.66.

4.—**2,2 - (L - Arabosylidene) - bis - [5 - (p-methoxyphenyl) - 1,3 - cyclohexanedione].**—5-(*p*-Methoxyphenyl)-1,3-cyclohexanedione, 4.36 Gm., L-arabinose, 1.5 Gm., and 2 drops of piperidine were dissolved in 75% methanol. The mixture was refluxed for 1 hr., then allowed to stand 3 days at room temperature. After evaporation of the solvents, the residue was recrystallized from boiling distilled water. At 138° it changed to a waxy consistency and melted and decomposed at 140°.

Anal.—Calcd. for $C_{31}H_{35}O_{10} \cdot 1/2\ H_2O$: C, 64.75; H, 6.29. Found: C, 64.76; H, 6.30.

5.—**2,2 - (D - Glucosylidene) - bis - [5 - (p-hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione].**—5 - (*p* - Hydroxyphenyl) - 4,6 - dicarbethoxy-1,3-cyclohexanedione, 6.96 Gm. (0.02 mole), and dextrose, 1.80 Gm. (0.01 mole), were placed in a ground-stoppered bottle with 200 ml. of 75% methanol and 3 drops of piperidine, and the mixture was allowed to stand 7 months in a cupboard at room temperature. Then the solvents were evaporated in a rotating evaporator under reduced pressure using a water bath. The solid residue was recrystallized from boiling water, m.p. 187–188°. The product was dried under reduced pressure at 100°. Yield, 67%. $[\alpha]_D^{20°} = +11.65$ (c 3.0896; acetone).

Anal.—Calcd. for $C_{42}H_{50}O_{19} - 2H_2O$: C, 61.30; H, 5.63. Found: C, 61.49; H, 5.70.

6.—**2,2 - (4′,6′ - O - Benzylidene - glucosylidene)-bis - [5 - (p - acetoxyphenyl) - 4,6 - dicarbethoxy-1,3 - cyclohexanedione].**—5 - (*p* - Acetoxyphenyl)-4,6-dicarbethoxy-1,3-cyclohexanedione, 7.8 Gm., and 2.68 Gm. of 4,6-*O*-benzylideneglucose (11) and 2 drops of piperidine were dissolved in 75% methanol and refluxed for 2 hr. The solvents were distilled off under reduced pressure and the product recrystallized from methyl alcohol, m.p. 168°.

Anal.—Calcd. for $C_{53}H_{58}O_{21}$: C, 61.73; H, 5.67. Found: C, 61.45; H, 5.74.

A phenylhydrazine derivative of the product above was formed in the usual way. It was dissolved in alcohol and reprecipitated with distilled water, m.p. 121°.

Anal.—Calcd. for $C_{74}H_{82}N_8O_{17}\cdot 2H_2O$: C, 64.79; H, 6.03; N, 7.85. Found: C, 64.40; H, 6.19; N, 7.59.

7.—**2,2 - (D - Mannosylidene) - bis - [5 - (p-hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione] - $2H_2O$.**—5 - (*p* - Hydroxphenyl)-4,6-dicarbethoxy-1,3-cyclohexanedione, 6.96 Gm. (0.02 mole), and mannose, 1.80 Gm. (0.01 mole), were placed in a ground-stoppered bottle with 200 ml. of 75% methanol and 3 drops of piperidine. The mixture was allowed to stand 13.5 months at room temperature in a closed cupboard. After the solvents were evaporated under reduced pressure using a water bath, the solid residue was recrystallized from boiling water. The product was dried at 100° under reduced pressure, m.p. 180°. $[\alpha]_D^{18°} = 0$ (c 1.328; acetone).

Anal.—Calcd. for $C_{42}H_{50}O_{19} - 2H_2O$: C, 61.30; H, 5.63. Found: C, 61.37; H, 5.97. C, 61.13; H, 5.98. C, 61.04; H, 5.82.

8.—**2,2 and 2,2-(Galactar-di-ylidenetetraacetate)-tetrakis - [5 - (p - hydroxyphenyl) - 4,6 - dicarbethoxy-1,3-cyclohexanedione].**—Tetraacetyl galactaricdialdehyde, 0.346 Gm., and 1.392 Gm. of 5-(*p* - acetoxyphenyl) - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione and 1 drop of piperidine were dissolved in 75% hot methanol and allowed to stand at room temperature. After 2 days the solvents were evaporated under reduced pressure and the product recrystallized from methanol, m.p. 184°.

Anal.—Calcd. for $C_{94}H_{102}O_{40}$: C, 60.31; H, 5.49. Found: C, 60.41; H, 5.94.

Aldosylidene-bis-dimedone Derivatives

2,2 - (2′ - Hydroxyethylidene) - bis - [5,5 - dimethyl - 1,3 - cyclohexanedione] - H_2O.—*Experiment A.*—Dimedone, 2.33 Gm. (0.0166 mole), glycolaldehyde, 0.50 Gm. (0.0083 mole), 15 ml. of methanol, and 5 ml. of water and 1 drop of piperidine were refluxed for 2 hr. and allowed to stand at room temperature 3 days. The solution was concentrated and cooled. The crystals formed were separated and recrystallized from 50% methanol, m.p. 233°. (Vorlander found m.p. 227°.)

Anal.—Calcd. for $C_{18}H_{26}O_5 - H_2O$: C, 71.02; H, 7.97. Found: C, 70.73; H, 7.92.

2,2 - (2′,3′ - Dihydroxypropylidene) - bis - [5,5-dimethyl - 1,3 - cyclohexanedione] - H_2O.—*Experiment B.*—Dimedone, 1.4 Gm. (0.01 mole), *dl*-glyceraldehyde, 0.45 Gm. (0.005 mole), 15 ml. of CH_3OH, 5 ml. of water, and 1 drop of piperidine were heated at refluxing temperature for 1 hr. and allowed to stand at room temperature for 3 days. The solution was concentrated to 7 ml. and cooled. The crystals formed were recrystallized from 50% methanol, m.p. 209°. (Vorlander found m.p. 197°.)

Anal.—Calcd. for $C_{19}H_{28}O_6 - H_2O$: C, 68.26; H, 7.83. Found: C, 68.17; H, 7.50.

2,2 - (D - Arabosylidene) - bis - [5,5 - dimethyl-1,3-cyclohexanedione].—*Experiment C.*—Dimedone, 2.80 Gm. (0.02 mole), D-arabinose, 1.5 Gm. (0.01 mole), 15 ml. of methanol, 5 ml. of water, and 1 drop of piperidine were placed in a ground-stoppered flask and allowed to stand for 1 week. Then the solution was refluxed 1 hr. and concentrated almost to dryness. The residue, after cooling, was stirred with ether. The ether was decanted and the residue stirred with ethanol and filtered. The white residue was recrystallized from boiling distilled water, m.p. 146°. $[\alpha]_D^{21°} = -274.9°$ (c 1.98; water).

Anal.—Calcd. for $C_{21}H_{32}O_8$: C, 61.14; H, 7.82. Found: C, 61.05; H, 7.81.

2,2 - (D - Mannosylidene) - bis - [5,5 - dimethyl-1,3-cyclohexanedione] · $2H_2O$.—*Experiment D.*—Dimedone, 5.60 Gm. (0.04 mole), and D-mannose, 3.60 Gm. (0.02 mole), were dissolved in 50 ml. of 70% methanol containing 2 drops of piperidine. The solution was refluxed 2 hr. and allowed to stand at room temperature 3 days. The solvents were evaporated under reduced pressure to a thick jelly-looking material which was soluble in alcohol but insoluble in ether. On stirring, it became like taffy. The material was dried on a porous plate and recrystallized from boiling water. It sinters at 87°, froths at 90°, and becomes clear at 112°.

Anal.—Calcd. for $C_{22}H_{34}O_9\cdot 2H_2O$: C, 55.21; H, 8.00. Found: C, 54.76; H, 8.18.

2,2 - (D - Glucosylidene) - bis - [5,5 - dimethyl-1,3 - cyclohexanedione] - H_2O.—*Experiment E.*—Dimedone, 5.60 Gm. (0.04 mole), and D-glucose, 3.60 Gm. (0.02 mole), were dissolved in 50 ml. of 75% methanol containing 2 drops of piperidine. The solution was refluxed 2 hr., then it was allowed to stand at room temperature for 2 weeks. The solution was concentrated under reduced pressure on a water bath. Alcohol and benzene were added for azeotropic distillation. After partial distillation, the solution was cooled, ether was added, and the precipitate formed was filtered, washed with ether, and dried, m.p. 189°, dec. 190°. Yield, 62%. $[\alpha]_D^{21°} = 173.58°$ (c, 4.01536; water).

Anal.—Calcd. for $C_{22}H_{34}O_9 - H_2O$: C, 62.07; H, 7.59. Found: C, 61.90; H, 7.67. C, 62.11; H, 7.78.

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Interaction of Pharmaceuticals with Schardinger Dextrins VI

Interactions of β-Cyclodextrin, Sodium Deoxycholate, and Deoxycholic Acid with Amines and Pharmaceutical Agents

By JOHN L. LACH and WAYNE A. PAULI*

Data are presented for the interactions of β-cyclodextrin and sodium deoxycholate with 11 pharmaceutical agents in aqueous solution. Three of the compounds were also studied as to possible reactivity with deoxycholic acid. Interactions were observed with all of the systems studied. Similar solubility isotherms were obtained for the three pharmaceuticals when interacted with both deoxycholic acid and its sodium salt indicating possible similarities in the reaction mechanisms for these two complexing agents. The larger molecules showed a lower degree of interaction with both β-cyclodextrin and sodium deoxycholate, indicating the importance of molecular size and structure for optimum reactivity. Stoichiometries could be determined for several of the interactions due to the presence of plateau regions in their solubility isotherms. Formation constants and free energies of formation are also reported for a number of the insoluble complexes.

THE IMPORTANCE of molecular complex formation in pharmaceutical formulation is certainly evident. Applications of this type of interaction can be made in the area of drug solubilization, stabilization, etc. The problem of stabilization is of particular interest in that numerous attempts have been made to retard degradation of pharmaceutical compounds. These include pH considerations, changes in solvent systems, antioxidants, and complex salt formation. The use of molecular complex formation in this area represents a relatively new approach to this problem of stability (1–5). Complexation by means of inclusion formation has been used successfully in the stabilization of labile drugs such as benzocaine, vitamin A, and various fatty acids (6, 7). This mode of protection for an unstable molecule offers definite advantages in that the entire molecule can be shielded from its surroundings. It has also been recognized that complexes not only influence the stability and appearance of pharmaceutical preparations, but may also exert some influence on the pharmacological and even biochemical mechanisms by which they operate.

The previous paper of this series dealt with a study of the interactions of β-cyclodextrin with a series of phenyl-substituted carboxylic acids in an effort to explain more clearly the nature of cyclodextrin interaction in aqueous solution. It was the objective of this study to investigate and compare the interaction tendencies of various medicinal agents with both β-cyclodextrin and deoxycholic acid, as only a limited number of these interactions have been reported in the literature. The solubilizing effect of sodium deoxycholate on these drugs was also studied with the hope of understanding more fully the mechanism responsible for this phenomenon.

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Previous paper: Pauli, W. A., and Lach, J. L., *J. Pharm. Sci.*, **54**, 1745(1965).

EXPERIMENTAL

Reagents.—β-Cyclodextrin $[\alpha]_D^{25°}$ in water = + 162.5 ±0.5; lidocaine,[1] m.p. 68–69°; adiphenine hydrochloride,[2] m.p. 113–114°; antazoline hydrochloride,[3] m.p. 234–236°; methapyrilene hydrochloride,[4] m.p. 161–163°; tripelennamine hydrochloride,[5] m.p. 189–192°; testosterone, m.p. 155–156°; morphine, m.p. 250°; procaine hydrochloride, m.p. 154–156°; meperidine hydrochloride, m.p. 187–189°; cortisone acetate, m.p. 235–238°; reserpine, m.p. 257–260°; sodium deoxycholate, analytical grade; deoxycholic acid, m.p. 171–172°; 1 *N* standard sodium hydroxide solution.

Apparatus.—The same apparatus described in the previous paper of this series was used in this study with the following addition: radiometer pH meter, type PHM4C.

Procedures.—The β-cyclodextrin was prepared by the procedure used previously in these laboratories (8). Complex formation was studied by means of the solubility method of Higuchi and Lach (9). The experimental procedures were similar to those outlined in the previous paper of this series, except for the following modifications.

With a few of the drugs, the free bases were formed in the reaction vials from the corresponding hydrochloride salts by the addition of a sufficient amount

[1] Marketed as Xylocaine by Astra Pharmaceutical Products, Inc., Worcester, Mass.

[2] Marketed as Trasentine by Ciba Pharmaceutical Products, Inc., Summit, N. J.

[3] Marketed as Antistine by Ciba Pharmaceutical Products, Inc., Summit, N. J.

[4] Marketed as Histadyl by Eli Lilly & Co., Indianapolis, Ind.

[5] Marketed as Pyribenzamine by Ciba Pharmaceutical Products, Inc., Summit, N. J.

of standard sodium hydroxide solution. A 10% excess of the standard solution was added to insure complete neutralization of the salts. In these particular systems, the amount of distilled water was reduced to compensate for the volume of standard base solution. When deoxycholic acid was used as the complexing agent, it was necessary to employ hydroalcoholic systems because of the acids limited solubility in water. The drugs were analyzed spectrophotometrically at the following wavelengths: lidocaine, 262.5 mμ; adiphenine, 258 mμ; antazoline 242 mμ; methapyrilene, 239 mμ; tripelennamine, 245 mμ; testosterone, 238 mμ; morphine, 285 mμ; procaine, 289 mμ; meperidine, 257 mμ; cortisone acetate, 238 mμ; reserpine, 268 mμ. Because of the concentrations used and corresponding dilutions prior to spectrophotometric analysis, no interference in the absorption characteristics of the compounds tested was observed due to the presence of the various complexing agents. The pH of each reaction vial was determined following its analysis, and no appreciable pH changes were observed in any of the systems investigated.

RESULTS AND DISCUSSION

Solubility isotherms representing the interactions of various drugs with β-cyclodextrin, deoxycholic acid, and sodium deoxycholate are shown in Figs. 1–12. Although several of the amines studied with these complexing agents are known to be relatively unstable at high pH, no appreciable degradation was observed for any of the compounds during the course of this investigation. Definite interactions were observed with all of the systems studied. Linear plots, indicating a first-order dependence of the interactions on the complexing agent concentration, were obtained in most cases. Smaller slopes were generally observed for interactions of both β-cyclodextrin and sodium deoxycholate with larger and thus more sterically hindered molecules. This finding is in agreement with results reported in an earlier article in this series (10). It should be pointed out here that definite relationships between interaction slopes and relative reactivities exist only if the stoichiometries of the various systems are the same. Since stoichiometries could not be evaluated for a number of these interactions due to the nature of the solubility isotherms, the comparison of slopes must be viewed with some reservation.

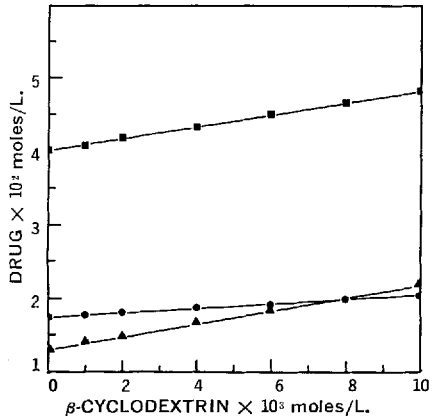

Fig. 1.—Interactions of procaine (■), lidocaine (●), and meperidine (▲) with β-cyclodextrin at 30°.

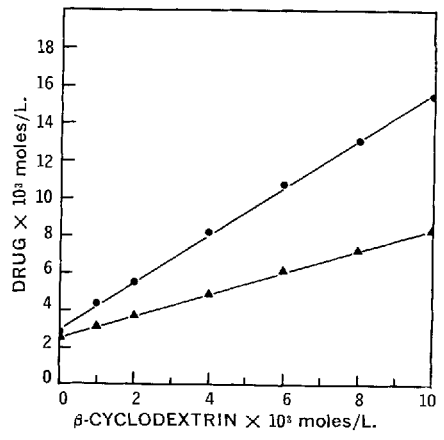

Fig. 2.—Interactions of antazoline (●) and methapyrilene (▲) with β-cyclodextrin at 30°.

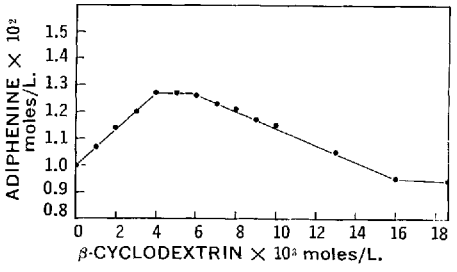

Fig. 3.—Interaction of adiphenine with β-cyclodextrin at 30°.

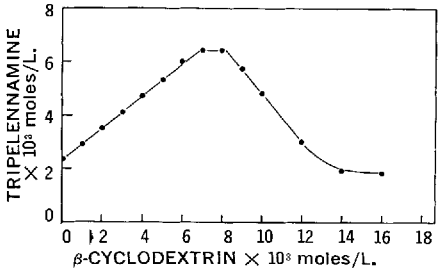

Fig. 4.—Interaction of tripelennamine with β-cyclodextrin at 30°.

Because of the hydroalcoholic systems employed for the deoxycholic acid studies, necessitated by the limited solubility of this acid in water, it is rather difficult to compare the interaction tendencies of this complexing agent with those of sodium deoxycholate and β-cyclodextrin. However, certain similarities were observed in the shapes of the solubility isotherms for both deoxycholic acid and its sodium salt.

β-Cyclodextrin Interactions.—A structural comparison of the compounds interacted with β-cyclodextrin reveals some interesting relationships. Referring to Table I, it can be observed that larger slopes are found for systems composed of β-cyclo-

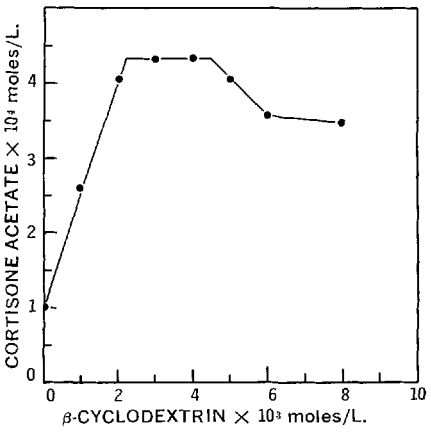

Fig. 5.—Interaction of cortisone acetate with β-cyclodextrin at 30°.

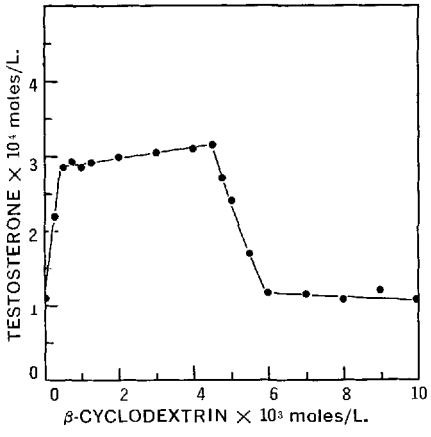

Fig. 6.—Interaction of testosterone with β-cyclodextrin at 30°.

Table I.—Slopes of Isotherms of Interactions of 11 Pharmaceutical Compounds with β-Cyclodextrin

Antazoline	1.25
Meperidine	0.88
Procaine	0.83
Adiphenine	0.68
Tripelennamine	0.60
Methapyrilene	0.59
Testosterone	0.44
Lidocaine	0.30
Morphine[a]	0.24
Cortisone acetate	0.15
Reserpine	0.001

[a] Data reported in *Reference 10.*

dextrin and small or medium-sized guest molecules containing one or more branched ring systems.

This increased reactivity resulting from the presence of a branched ring system might be attributed to the availability of a ring for either total or partial inclusion by the cyclodextrin. Therefore, inclusion, in combination with other attractive forces, would be expected to enhance the reactive tendency of a particular guest compound with β-cyclodextrin. If ring availability were assumed to be the primary factor for interaction with β-cyclodextrin, similar reactivities would be expected with both antazoline and adiphenine, since both molecules contain several branched phenyl groups. On comparison of the slopes for these compounds, listed in Table I, it can be seen that the slope representing the antazoline interaction is almost double that for the adiphenine system, again pointing toward a complex mechanism for these interactions. A smaller slope than would be expected for the interaction between lidocaine and β-cyclodextrin might be attributed to steric hindrance resulting from the multiple methyl group substitution on the phenyl ring. The bulky multi-ringed molecules including morphine, cortisone acetate, and reserpine show extremely small interaction slopes with β-cyclodextrin indicating only limited interactions for these systems. This would be expected due to the extreme size of the molecules and the resulting steric interference that would oppose their proper combination or fit with the cyclodextrin structure. It becomes obvious for molecules of this size, that interactions with β-cyclodextrin could not possibly occur by means of complete or true inclusion formation. The presence of reactive functional groups in these large molecules, capable of bonding with or of being partially included by the cyclodextrin, could be responsible for the observed interactions. Cortisone acetate and testosterone, because of structural similarity, would be expected to interact similarly with β-cyclodextrin. But on a comparison of the slopes for these interactions in Table I, a marked difference is observed with testosterone possessing the greater slope. As indicated previously, molecules of this size would obviously be too large to allow complete inclusion within the cyclodextrin cavity and therefore the mechanism might involve either interaction of or the enclosure of a functional group of the steroid by the cyclodextrin. The hydroxyl group in the C-17 position is relatively open and free in the testosterone structure, while the same group in the cortisone acetate molecule is in close proximity to a rather bulky side chain also at the C-17 position. Interference introduced by this group in the cortisone acetate structure could partially explain the decreased reactivity of this compound with β-cyclodextrin. Again, as emphasized previously, these interactions are, as expected, quite complex due to the size and complexity of the interacting species, and therefore it is quite conceivable that a number of factors are responsible for the net interactions observed.

Stoichiometries were calculated for those systems characterized by the presence of plateau regions in their solubility isotherms. These values, along with stoichiometries determined from an analysis of the isolated complexes, are found in Table II.

The formation constants, calculated in a manner

Table II.—Stoichiometries of the Drug–β-Cyclodextrin Complexes

Compd.	From Phase Diagram Drug–BCD	Analysis of Isolated Complex Drug–BCD
Cortisone acetate	1.98:1.00	2.05:1.00
Testosterone	2.03:1.00	2.06:1.00
Tripelennamine	1.01:1.00	1.08:1.00
Adiphenine	1.01:1.00	1.04:1.00

TABLE III.—FORMATION CONSTANTS AND FREE ENERGIES OF FORMATION OF DRUG–β-CYCLODEXTRIN COMPLEXES AT 30°

Compd.	K_f	K_f[a]	$F°$ (cal./mole)
Cortisone acetate	8.1×10^6	...	−9578
Testosterone	2.3×10^7	...	−10209
Tripelennamine	6.5×10^2	6.5×10^2	−3900
Adiphenine	2.1×10^2	2.1×10^2	−3220

[a] Values determined by the method of Thoma and Stewart for 1:1 complexes only.

analogous to those employed in previous studies (9), and the corresponding free energies of formation for these insoluble complexes are listed in Table III. A method proposed by Thoma and Stewart (11), involving a mathematically derived expression for the calculation of formation constants of 1:1 complexes, was applied to some of our interactions in an effort to check our calculations. These results were in good agreement with the formation constants determined from the solubility isotherms.

The magnitude of the apparent formation constants and free energies of formation observed for these insoluble complexes is indicative of the relative high degree of interaction. Formation constants for other systems involving β-cyclodextrin have been reported in the range of 10^2 to 10^4 (12, 13), again indicating the existence of very stable systems. These formation constants are quite high compared to the values of 1 to 100 reported in pharmaceutical interaction studies with complexing agents such as caffeine (9, 14). Thus interactions involving more than hydrogen bonding or dipole-dipole attractions are suspected, and a combination of these plus inclusion formation is a definite possibility.

Deoxycholic Acid and Sodium Deoxycholate Interactions.—The same compounds studied with β-cyclodextrin were also interacted with sodium deoxycholate in an attempt to clarify further the mechanism by which this agent exerts its solubilizing effects. Interactions were observed with all of the compounds and are represented by Figs. 7–10. No

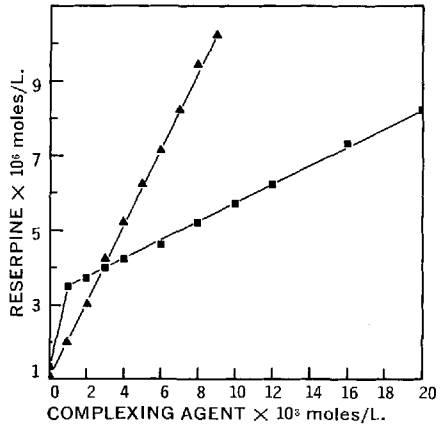

Fig. 7.—Interactions of reserpine with β-cyclodextrin (▲) and sodium deoxycholate (■) at 30°.

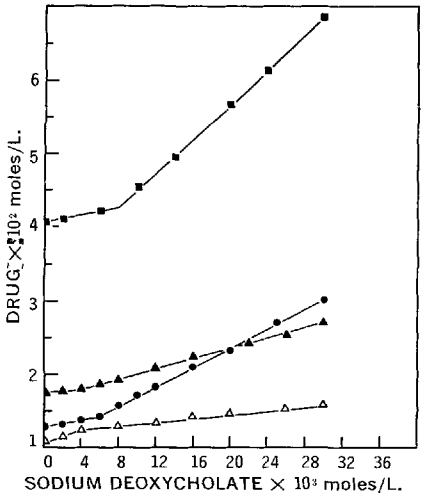

Fig. 8.—Interactions of procaine (■), lidocaine (▲), meperidine (●), and adiphenine (△) with sodium deoxycholate at 30°.

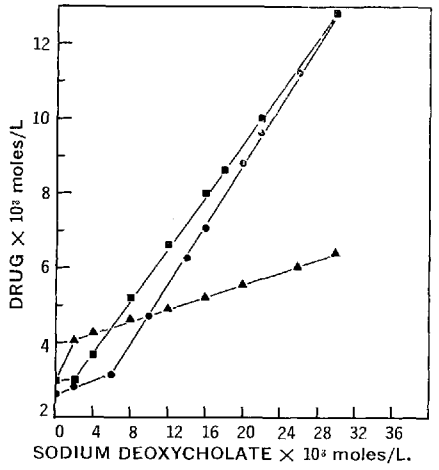

Fig. 9.—Interactions of methapyrilene (■), tripelennamine (●), and antazoline (▲) with sodium deoxycholate at 30°.

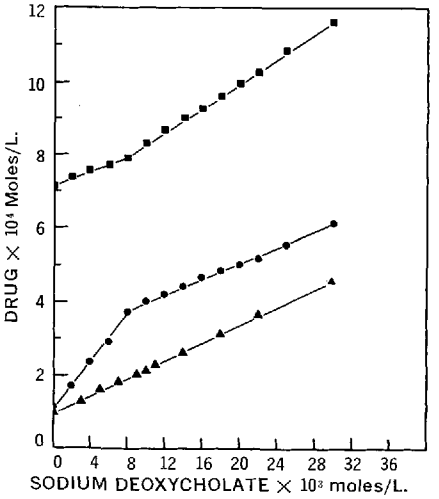

Fig. 10.—Interactions of morphine (■), testosterone (●), and cortisone acetate (▲), with sodium deoxycholate at 30°.

TABLE IV.—SLOPES[a] OF ISOTHERMS OF INTERACTIONS OF 11 PHARMACEUTICAL COMPOUNDS WITH SODIUM DEOXYCHOLATE

Procaine	1.18
Meperidine	0.66
Tripelennamine	0.40
Lidocaine	0.36
Methapyrilene	0.35
Adiphenine	0.13
Antazoline	0.08
Morphine	0.02
Cortisone acetate	0.01
Testosterone	0.01
Reserpine	0.00025

[a] The second slope was used when multislope isotherms were present.

plateau regions were found in the solubility isotherms for these interactions which prevented the determination of the exact stoichiometric relationships and the corresponding thermodynamic values. Slopes for interactions of these compounds with sodium deoxycholate are found in Table IV.

In general, as was true for the β-cyclodextrin interactions, the smallest compounds are seen to exhibit the greatest interaction slopes with sodium deoxycholate. This could either be due to the small size of the molecules or to their greater solubility, as one property is a corollary of the other. In most cases the interactions with β-cyclodextrin yielded larger slopes than the corresponding interactions with sodium deoxycholate, indicating a greater degree of complex formation with the cyclodextrin and possibly a different mechanism. Although there are certain similarities in the reaction isotherms observed, it should be pointed out that β-cyclodextrin is known to form monomolecular inclusion compounds, while a polymolecular mechanism, necessary in the build-up of the channel-like host structure, is essential for choleic acid inclusion formation.

Systems containing the amine bases and either of the complexing agents were quite alkaline (pH 9–12) due to the nature of the guest compounds themselves and to the excess sodium hydroxide added to insure complete liberation of the bases from their corresponding salts. While varying concentrations of sodium deoxycholate would be expected to have a measurable effect on the pH of neutral or acidic systems, no effect was observed in these investigations because of the elevated pH conditions employed. The pH changes observed in less basic systems ranged from 0.1–0.2 pH units. Even though this basic environment would not be expected to have any appreciable effect on β-cyclodextrin, it would markedly suppress hydrolysis of sodium deoxycholate resulting in a predominance of the deoxycholate anion in solution. Also in this pH range, the amine bases would exist as the nonprotonated form, and, therefore, the observed interactions would presumably be between the deoxycholate anion and the free bases. An acid–base reaction between a few of the strongly basic amines and deoxycholic acid in solution might be expected, but at this pH, the probability of an interaction of this type between the nonprotonated amine and the deoxycholate anion is small.

Multislope interaction isotherms obtained for a number of the sodium deoxycholate systems indicate the complexity of these molecular associations. At the present time, the popular theory is that sodium deoxycholate, acting as an anionic surfactant, exerts its solubilizing effects through micelle formation. There has been considerable evidence that lends support to this proposed mechanism (15–17). However, owing to the inability of isolating the reaction products for structural examination, the possibility of a mechanism similar to that for deoxycholic acid, involving channel-like inclusion formation, cannot be ruled out. Deoxycholic acid is believed to bond intermolecularly through the hydroxyl groups in the 3 and 12 positions forming a channel-like host structure. It is quite conceivable that the anion of this acid could assume a similar structure resulting in inclusion formation of the guest amines. Assuming that the carboxyl group of deoxycholic acid is also an active site for the intermolecular bonding necessary in the build-up of the host structure, one might expect a lesser degree of inclusion formation with the anion than with the undissociated acid. The complexity of these interactions could certainly point toward a mechanism composed of both inclusion formation and micellar solubilization. This combination might well account for the different slopes observed in the interaction isotherms for a number of the interactions that could also represent the formation of higher order complexes.

In an attempt to elucidate further the complex nature of sodium deoxycholate's mechanism of interaction, three of the compounds previously studied with this complexing agent were also interacted with deoxycholic acid. The interactions observed between each of the three compounds and deoxycholic acid are represented in Figs. 11 and 12. Hydroalcoholic systems were used for these interactions because of the acid's limited solubility in water. It is interesting to note that in these alcoholic systems, deoxycholic acid would exist almost entirely as the free acid, and, therefore, the concept of micellar solubilization would appear to be minimized as a possible mechanism since it has been

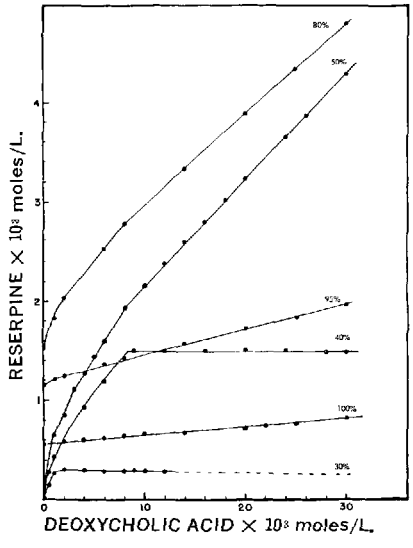

Fig. 11.—Interaction of reserpine with deoxycholic acid at 30° in hydroalcoholic systems.

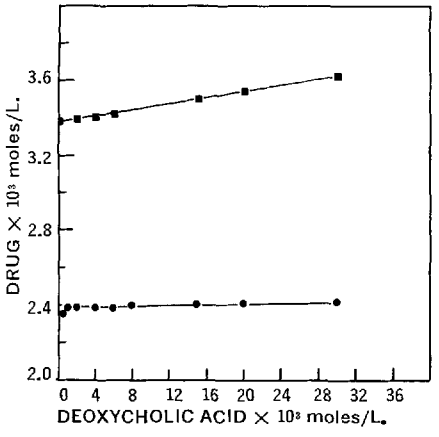

Fig. 12.—Interactions of cortisone acetate (■) (50% EtOH system) and testosterone (●) (30% EtOH system) with deoxycholic acid at 30°.

known for some time that micelles do not readily form in alcoholic solvents. A comparison of the slopes for these systems with the corresponding slopes for the sodium deoxycholate interactions is difficult because of the different solvent systems employed. However, the shapes of the solubility isotherms representing interactions of both the acid and its sodium salt do show certain similarities which might indicate a common mechanism for these complexing agents. Because of the unique multislope isotherm representing the reserpine–deoxycholic acid interaction in a 50% hydroalcoholic solvent, it was felt that a study of this system in solvents of varying alcoholic strength would be interesting, and might possibly yield a plateau region permitting the determination of the stoichiometry for this system. Therefore, this particular interaction was examined in alcoholic systems ranging from 0–100% ethanol. The corresponding isotherms representing the different alcoholic strengths are found in Fig. 11. As would be expected, the solubility pattern of the resulting complexes parallels the alcoholic concentration up to 80% ethanol, but a marked decrease in the interaction slopes is observed at higher alcoholic levels. The presence of deoxycholic acid is seen to exert a pronounced effect on the solubility of reserpine at the lower alcoholic concentrations employed over that of its solubility in the same alcoholic solutions without the deoxycholic acid. In the 50% alcoholic system, formation of the complex is seen to result in almost a fourfold increase in the solubility of reserpine. At lower alcoholic concentrations, the solubility of the complex appears to lie midway between the individual solubilities of reserpine and deoxycholic acid as would be expected. However, the marked decrease in solubility of the reserpine complex and interaction in 95% and absolute ethanol appears to parallel the unexpected decrease in solubility of the uncomplexed reserpine at these solvent concentrations. The solubility profiles of reserpine and deoxycholic acid in various hydroalcoholic systems are shown in Fig. 13. While deoxycholic acid is shown to be infinitely soluble at higher alcoholic concentrations, the solubility profile of reserpine is seen to possess a maximum in approximately 80% ethanol. Solubility studies involving a number of medicinal agents have pointed out the presence of such a maximum solubility in various cosolvent mixtures (18, 19), suggesting that a definite correlation exists between the solubility of the solute and the dielectric constant of the solvent system employed. The solubility of reserpine in these cosolvent systems involves solute-solvent and solvent-solvent associations, and since complexation of reserpine with deoxycholic acid would be in competition with these various associations, it becomes obvious that the mechanism involved in reserpine–deoxycholic acid interaction in these systems is complex. Since it is known that stable deoxycholic acid–alcohol complexes exist (20), the association between the deoxycholic acid and alcohol at these high alcoholic concentrations must be much greater than that for the reserpine–deoxycholic acid interaction. This and the decreased solubility of reserpine in high alcoholic concentrations could account in part for the marked decrease in the observed interaction between reserpine and deoxycholic acid.

The plateau region obtained in the 40% plot found in Fig. 11, representing the solubility limit of deoxycholic acid at this alcoholic strength, made possible the calculation of the apparent stoichiometry that was found to be 1:1. The multislope isotherms observed for the interaction in these alcohol–water systems (*e.g.*, 50%) could represent the successive formation of a series of higher order complexes in solution and certainly illustrate the complexity of this interaction. This was also mentioned as a possible explanation for similar multislope systems observed in many of the sodium deoxycholate interactions, although in these systems, micelle formation is also a definite possibility. It is interesting to note that the stoichiometries determined from the isolation and subsequent analysis of deoxycholic acid complexes prepared from saturated alcoholic solutions are usually of higher order (6:1, 8:1, etc.), while stoichiometries obtained from the solubility isotherms (1:1 for the reserpine–deoxycholic acid complex) are much lower. Such differences in the reported stoichiometries of deoxycholic acid interactions indicate that the complexes formed can be

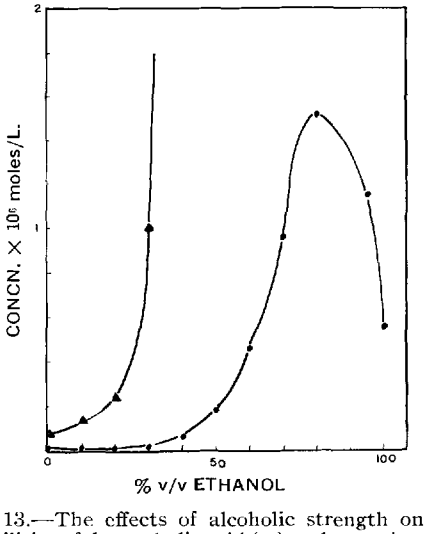

Fig. 13.—The effects of alcoholic strength on the solubilities of deoxycholic acid (▲) and reserpine (●).

greatly influenced by the type of solvent system employed (20).

CONCLUSION

Both β-cyclodextrin and sodium deoxycholate are shown to interact with a variety of pharmaceutical compounds. While varying concentrations of sodium deoxycholate would be expected to have a measurable effect on the pH of systems at lower pH levels, no effect was observed in these investigations because of the elevated pH conditions employed. In general, greater complexing activity is observed between the smaller guest molecules and both complexing agents, illustrating the importance of molecular size and structure in these interactions. Compounds known to be too large for complete inclusion, such as morphine and reserpine, are seen to interact with β-cyclodextrin. The presence of certain functional groups in these large molecules, capable of interacting with or of being partially included by the cyclodextrin, could be responsible for the observed interactions. The high-formation constants determined for some of the β-cyclodextrin interactions indicate the formation of extremely stable complexes. Pure inclusion is described as an association taking place without intermolecular bonding between the guest and host components. Although in aqueous solution, the net interactions could result from both inclusion and intermolecular forces, particularly hydrogen bonding.

Similarities in the shapes of solubility isotherms obtained for interactions of both deoxycholic acid and its sodium salt with several pharmaceutical compounds could indicate similar mechanisms for these two complexing agents. Even though sodium deoxycholate, an anionic surfactant, is currently thought to exert its solubility effects through micelle formation, the possibility of total or even partial inclusion formation by this agent cannot be ignored. Multislopes obtained in the solubility isotherms for many of the sodium deoxycholate interactions could indicate a complex mechanism consisting of both micellar solubilization and inclusion formation, or they might represent the presence of higher order complexes. The complexity of these interactions is clearly shown in the studies dealing with the reserpine–deoxycholic acid interaction in various alcoholic solutions.

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Synthesis of *N*-Substituted Phenethylamines and Corresponding Cyclohexyl Analogs

Preliminary Evaluation as Bronchodilators

By JOHN B. DATA, MARTIN O. SKIBBE, T. LAMAR KERLEY, and LAWRENCE C. WEAVER

A series of *N*-substituted phenethylamines and their corresponding cyclohexyl analogs were prepared and tested pharmacologically for their effects on the duration of hexobarbital anesthesia in mice, and systemic blood pressure and bronchodilatory activity in dogs. Methods for the preparation of these compounds are described, and procedures used in pharmacological testing are indicated and the biological results tabulated. There were no consistent or appreciable bronchodilatory effects observed.

EPINEPHRINE (I) and isoproterenol (II) represent two potent and useful bronchodilators containing the catechol nucleus. Other structurally related substances which have been rated (1–3) as relatively potent bronchodilators are levarterenol (III), 3,4-dihydroxyephedrine (IV), and equine (V). A recently introduced adrenergic substance used in the management of bronchial asthma is the potent inhibitor, protokylol (VI).

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greatly influenced by the type of solvent system employed (20).

CONCLUSION

Both β-cyclodextrin and sodium deoxycholate are shown to interact with a variety of pharmaceutical compounds. While varying concentrations of sodium deoxycholate would be expected to have a measurable effect on the pH of systems at lower pH levels, no effect was observed in these investigations because of the elevated pH conditions employed. In general, greater complexing activity is observed between the smaller guest molecules and both complexing agents, illustrating the importance of molecular size and structure in these interactions. Compounds known to be too large for complete inclusion, such as morphine and reserpine, are seen to interact with β-cyclodextrin. The presence of certain functional groups in these large molecules, capable of interacting with or of being partially included by the cyclodextrin, could be responsible for the observed interactions. The high-formation constants determined for some of the β-cyclodextrin interactions indicate the formation of extremely stable complexes. Pure inclusion is described as an association taking place without intermolecular bonding between the guest and host components. Although in aqueous solution, the net interactions could result from both inclusion and intermolecular forces, particularly hydrogen bonding.

Similarities in the shapes of solubility isotherms obtained for interactions of both deoxycholic acid and its sodium salt with several pharmaceutical compounds could indicate similar mechanisms for these two complexing agents. Even though sodium deoxycholate, an anionic surfactant, is currently thought to exert its solubility effects through micelle formation, the possibility of total or even partial inclusion formation by this agent cannot be ignored. Multislopes obtained in the solubility isotherms for many of the sodium deoxycholate interactions could indicate a complex mechanism consisting of both micellar solubilization and inclusion formation, or they might represent the presence of higher order complexes. The complexity of these interactions is clearly shown in the studies dealing with the reserpine–deoxycholic acid interaction in various alcoholic solutions.

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Synthesis of *N*-Substituted Phenethylamines and Corresponding Cyclohexyl Analogs

Preliminary Evaluation as Bronchodilators

By JOHN B. DATA, MARTIN O. SKIBBE, T. LAMAR KERLEY, and LAWRENCE C. WEAVER

A series of *N*-substituted phenethylamines and their corresponding cyclohexyl analogs were prepared and tested pharmacologically for their effects on the duration of hexobarbital anesthesia in mice, and systemic blood pressure and bronchodilatory activity in dogs. Methods for the preparation of these compounds are described, and procedures used in pharmacological testing are indicated and the biological results tabulated. There were no consistent or appreciable bronchodilatory effects observed.

EPINEPHRINE (I) and isoproterenol (II) represent two potent and useful bronchodilators containing the catechol nucleus. Other structurally related substances which have been rated (1–3) as relatively potent bronchodilators are levarterenol (III), 3,4-dihydroxyephedrine (IV), and equine (V). A recently introduced adrenergic substance used in the management of bronchial asthma is the potent inhibitor, protokylol (VI).

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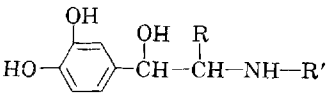
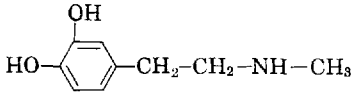


I, R is H; R′ is CH_3
II, R is H; R′ is $(CH_3)_2CH$
III, R is H; R′ is H
IV, R is CH_3; R′ is CH_3



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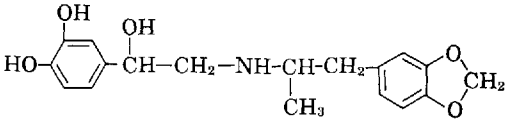
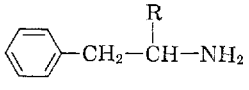


VI

These examples support the conclusion of Tainter and his co-workers (1, 2) and of Biel *et al.* (4) that the catechol nucleus appears to be essential for a high degree of bronchodilation efficiency.

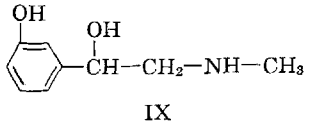
DISCUSSION

Mono-hydroxy and mono-methoxy derivatives of phenethylamine (VII) and 2-phenylisopropylamine (VIII) exhibit this same type of activity, although quantitatively not as great.

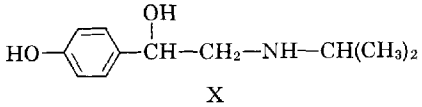


VII, R is H
VIII, R is CH_3

While phenylephrine (IX) has been reported as a fair (3) and a poor (2) bronchodilator, Konzett (5) reported that *p*-hydroxy-*α*-(isopropylaminomethyl)-benzyl alcohol (X) possesses marked activity; the corresponding *N*-methyl derivative is less active. Corrigan *et al.* reported (6) on the activity of additional hydroxy compounds in which the *N*-isopropyl group of X is replaced by other alkyl substituents. These in general were found to be active.

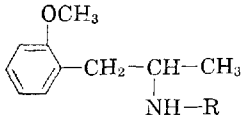


IX



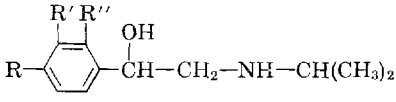
X

All possible mono-hydroxy derivatives of 2-phenyl-isopropylamine have been evaluated (7) for bronchial activity, but these isomers have shown no appreciable response; the corresponding mono-methoxy derivatives were tested by the same investigators, who concluded that the more active compounds were the methoxy derivatives. 2-(*o*-Methoxyphenyl)-isopropylamine (XI), the *N*-methyl, *N,N*-dimethyl, and *N*-benzyl derivatives have been shown (8) to possess a very high degree of specific bronchodilatory activity with little or no pressor effect. These latter studies may have been responsible for the introduction of the orally active and useful medicament, methoxyphenamine (XII).



XI, R is H
XII, R is CH_3

The quantitative difference in the ability of the hydroxyl and the methoxyl group to supply electrons to the conjugated ring system (9) suggests a means for correlating bronchial activity to chemical structure. The finding that isoprophenamine (XIII) is a potent bronchodilator (10) and, in addition has other physiological properties (11) qualitatively similar to methoxyphenamine, suggests that the chloro group, too, can be considered along with the hydroxyl and the methoxyl; while the chloro group is an electron withdrawing group, it can also release electrons to the conjugated ring system. This idea can be extended to include compounds which have two such groups as in the case of isoproterenol and dichloroisoproterenol (XIV), even though the latter is known to block the action of the former (12). While the exact role each group plays in broncho-



XIII, R is H; R′ is H; R″ is Cl
XIV, R is Cl; R′ is Cl; R″ is H

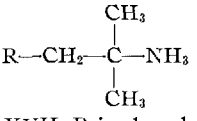
dilatory effect is unknown, the strength of the bond between the drug and receptor due to the magnitude of electron density in the phenyl ring because of the presence of these groups may be very important to explain and/or correlate the effect of a drug. Although from the standpoint of satisfying structural requirements for the same receptor site the chloro, the hydroxyl, or the methoxyl group may or may not be required, differences in pharmacological response may be due basically to difference in electron density created by these substituents. For example, isoproterenol and dichloroisoproterenol very likely possess the same structural requirements for the same *β*-receptor, even though the nature of the ring substituents are different. The difference in the ability of the chloro and the hydroxyl group to supply electrons to the phenyl ring would be evident in rates of release of the drug–receptor complex in a reversible reaction. Thus, the bond strength attributed to the difference in electron density can account, at least in part, for difference in pharmacological behavior of isoproterenol and dichloroisoproterenol. The reported pharmacology for these compounds bear out some of these points. For instance, Moran and Perkins (13) indicate that dichloroisoproterenol may be involved in an equilibrium type of blockade since the block can be reversed by excess of certain amines. Powell and Slater (14) report data which strongly support the

suggestion that the same β-receptors are involved, and they explain that the brief effects of isoproterenol are due to a rapid bio-transformation and/or weak bonding between drug and receptor; for dichloroisoproterenol they suggest the drug–receptor complex to be fairly stable.

Various types of substituents attached to the amino nitrogen have received considerable attention in the search for effective bronchodilators. Simple aliphatic groups from one to five carbons (4–6, 15–21), cycloalkyls (4, 15–17, 19), aralkyls (4, 18–20, 22), and cycloalkylalkyls (4) have been evaluated. The *N*-isopropyl derivative has been suggested for optimal activity in one series of compounds (16), and the *N*-butyl was found to be the most active in a second series (17). There does not appear to be any structural specificity for activity. These examples and the studies cited suffice to suggest that the kind of substitutions on the amino group is not crucial.

Effects of other substituents on the ethyl or isopropyl moiety of VII and VIII other than the amino group is not well documented. Studies designed to evaluate the importance of the hydroxyl group β to the amino are lacking. Seemingly, the literature records only three like pairs of compounds evaluated for their bronchial activity in which the β-OH group is the only variable. The first pair is epinephrine and equine; Pedden *et al.* (2) have rated these as excellent bronchodilators. The second pair is 2-phenylisopropylamine and α-(1-aminoethyl)benzyl alcohol (XV), which are reported (1) to act predominately as bronchoconstrictors, although Alles and Prinzmetal (23) reported slight bronchodilation for 2-phenylisopropylamine. The third pair is isoproterenol and *N*-isopropyl-3,4-dihydroxyphenethylamine (XVI); Siegmund *et al.* (21) indicate that the absence of a β-OH group in XVI causes very marked reduction in activity between this last pair of compounds. While these three pairs do not clearly indicate the effect of the β-OH group, Siegmund and his co-workers (21) concluded from their studies that the β-OH group is essential for bronchial activity.

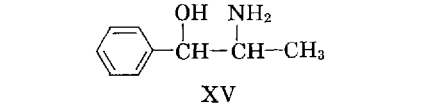
The lack of information on the effect of other substituents on the propyl moiety of 2-phenylisopropylamine prompted the authors to prepare and biologically evaluate as a matter of interest a series of *N*-substituted derivatives of α,α-dimethylphenethylamine (XVII) and their corresponding cyclohexyl derivatives (XVIII).



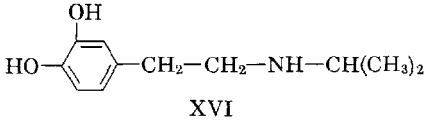
XVII, R is phenyl
XVIII, R is cyclohexyl

Except for α,α-dimethylphenethylamine (18) no one seems to have reported on the broncho-spastic relieving properties of these compounds. The only closely related study among the cyclohexylethylamines is the work of Lands *et al.* (24), who evaluated the spasmolytic activity of such compounds on isolated guinea pig ileum.

The α,α-dimethylphenethylamines and their corresponding cyclohexyl analogs were prepared by reducing with lithium aluminum hydride the formamides obtained by the Ritter reaction from 1,1-dimethyl-2-phenylethanol or its cyclohexyl analog and a nitrile as described under *Experimental.* (Scheme I.)



XV



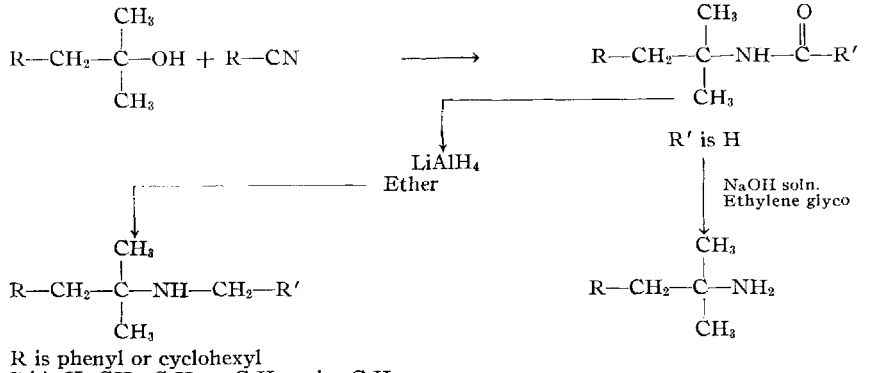
XVI

EXPERIMENTAL

All melting points are uncorrected. Chlorine was determined by the method of Blicke and Zienty (25), and the carbon and hydrogen analysis was performed by Galbraith Laboratories, Inc., Knoxville, Tenn. No attempt was made to improve yields obtained from the first trial.

Preparation of 1,1-Dimethyl-2-phenylethanol.—This product was prepared by the Grignard reaction for the synthesis of tertiary alcohols from esters.

Ethyl phenylacetate, 75.0 Gm. (0.455 mole), was added during 0.5 hr. to 600 ml. of an ethereal solution of methylmagnesium iodide prepared in the usual way from 24.3 Gm. (1.0 Gm. atom) of mag-



R is phenyl or cyclohexyl
R′ is H, CH_3, C_2H_5, *n*-C_3H_7 or iso-C_3H_7

Scheme I

nesium turnings and 150.0 Gm. (1.06 moles) of methyl iodide. The mixture was then refluxed for 3 hr., cooled, and poured onto about 300 Gm. of cracked ice, and then decomposed by the slow addition of 300 ml. of hydrochloric acid (10%). The organic layer was separated from the aqueous portion, which was then extracted 3 times with 100-ml. portions of ether. The combined ethereal extracts were washed successively with 100 ml. of sodium carbonate (5%) and 100 ml. of water. The ethereal solution was then dried over anhydrous potassium carbonate, filtered, the solvent removed under reduced pressure and the residue fractionated to give 59.2 Gm. (86.5%) of product distilling at 100–104° (14 mm.). [Reported (26) b.p. 103–105° (10 mm.).]

Preparation of 1,1-Dimethyl-2-cyclohexylethanol.—This product was prepared by the Grignard reaction for the preparation of tertiary alcohols from ketones.

The Grignard reagent was prepared in the usual fashion from 4.86 Gm. (0.2 Gm. atom) of magnesium and 35.4 Gm. (0.2 mole) of cyclohexylmethyl bromide dissolved in about 150 ml. of ether. To the reagent there was added during a period of about 0.5 hr. while stirring 11.6 Gm. (0.2 mole) of dry acetone in 50 ml. of ether. After 2 additional hr. of stirring and refluxing, the reaction mixture was hydrolyzed by the addition of about 100 Gm. of cracked ice and 75 ml. of hydrochloric acid (10%). The organic layer was separated from the aqueous portion, and the aqueous layer extracted 3 times with 30-ml. portions of ether. The ether extracts were then combined and washed successively with several 25-ml. portions of sodium carbonate (10%) and 25 ml. of water. The organic layer was dried with fused sodium sulfate, filtered, and fractionated. There was obtained 14.5 Gm. (46.5%) of a colorless oil distilling at 97–100° (16 mm.).

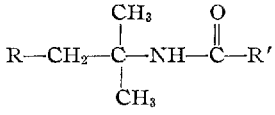
A second run twice as large gave 32.0 Gm. (51.2%) of product which distilled at 67–70° (2.2 mm.). The analytical sample distilled at 69° (2.2 mm.) and had a refractive index of 1.4650 at 20°.

Anal.—Calcd. for $C_{10}H_{20}O$: C, 76.92; H, 12.82. Found: C, 76.58; H, 12.60.

The alcohol gave a white, crystalline phenylurethan derivative which, when recrystallized 3 times from hexane, melted at 117–118°.

Anal.—Calcd. for $C_{17}H_{25}NO_2$: C, 74.18; H, 9.09. Found: C, 73.80; H, 9.09.

TABLE I. — *N*-(1,1-DIMETHYL-2-PHENYLETHYL)-AMIDES AND *N*-(1,1-DIMETHYL-2-CYCLOHEXYLETHYL)AMIDES



R	R′	M.p., C°.	Yield, %
C_6H_5	CH_3	89–91	80.5
C_6H_5	CH_3CH_2	95–97	84.5
C_6H_5	$CH_3CH_2CH_2$	62–64	60.7
C_6H_5	$(CH_3)_2CH$	111–113	79.5
C_6H_{11}	CH_3	80–82	81.3
C_6H_{11}	CH_3CH_2	59–61	85.4
C_6H_{11}	$CH_3CH_2CH_2$	79–81	87.7
C_6H_{11}	$(CH_3)_2CH$	105–106	89.0

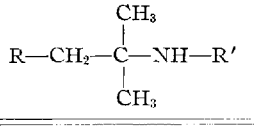
Preparation of 1,1-Dimethyl-2-cyclohexylethylamine (D-3-1)

N - (1,1 - Dimethyl - 2 - cyclohexylethyl)formamide.—This compound was prepared according to the method described by Ritter and Kalish (27) for the synthesis of *N-tert*-butylformamide.

Sodium cyanide, 19.6 Gm. (0.4 mole), and 50 ml. of glacial acetic acid was mixed with cooling and stirring. A solution of 100 Gm. of concentrated sulfuric acid in 50 ml. of glacial acetic acid was then added while continuing to stir the reaction mixture and maintaining the temperature at 20°. 1,1-Dimethyl-2-cyclohexylethanol, 62.4 Gm. (0.4 mole), was then added and the temperature permitted to rise spontaneously to 40–50°. The mixture was heated to 70° for 1 hr., stoppered, allowed to stand for 2 hr., diluted with 600 ml. of water, and neutralized with sodium carbonate. The formamide which separated as a viscous oil was extracted 3 times with 100-ml. portions of ether, dried over fused sodium sulfate, filtered, and the solvent removed under reduced pressure. The residue was fractionated to give 69.7 Gm. (95.5%) of product which distilled at 170–171° (15 mm.).

1,1 - Dimethyl - 2 - cyclohexylethylamine.—A mixture of 40.0 Gm. (1.0 mole) of sodium hydroxide in 60 ml. of water, 300 ml. of ethylene glycol, and

TABLE II.—*N*-SUBSTITUTED 1,1-DIMETHYLPHENETHYLAMINES AND *N*-SUBSTITUTED 1,1-DIMETHYL-2-CYCLOHEXYLETHYLAMINES



Code No.	R	R′	B.p., °C. (mm.)	Yield, %	Hydrochloride M.p., °C.	Formula	Cl, % Calcd.	Cl, % Found
D-3-2	C_6H_5	CH_3	114–120 (13)	95.0	176–177	$C_{11}H_{18}ClN$	17.78	17.80
D-3-4	C_6H_5	CH_3CH_2	107–109 (16)	61.8	214–215	$C_{12}H_{20}ClN$	16.61	16.60
D-3-6	C_6H_5	$CH_3CH_2CH_2$	119–121 (13)	91.2	205–206	$C_{13}H_{22}ClN$	15.59	15.68
D-3-8	C_6H_5	$CH_3(CH_2)_2CH_2$	131–132 (12)	58.7	160–161	$C_{14}H_{24}ClN$	14.70	14.68
D-3-10	C_6H_5	$(CH_3)_2CHCH_2$	126–128 (16)	51.7	209–210	$C_{14}H_{24}ClN$	14.70	14.88
D-3-3	C_6H_{11}	CH_3	96–99 (13)	71.0	155–156	$C_{11}H_{24}ClN$	17.20	17.14
D-3-5	C_6H_{11}	CH_3CH_2	103–106 (14)	60.0	220–221	$C_{12}H_{26}ClN$	16.12	16.15
D-3-7	C_6H_{11}	$CH_3CH_2CH_2$	115–116 (13)	54.3	189–190	$C_{13}H_{28}ClN$	15.18	15.10
D-3-9	C_6H_{11}	$CH_3(CH_2)_2CH_2$	133–136 (15)	72.0	173–174	$C_{14}H_{30}ClN$	14.32	14.32
D-3-11	C_6H_{11}	$(CH_3)_2CHCH_2$	130–139 (15)	48.8	174–175	$C_{14}H_{30}ClN$	14.32	14.37

TABLE III.—COMPARATIVE ACTIVITIES OF AMINES

Compd.	R	R′	Lethal Dose to Mice, mg./Kg.	Hexobarbital Anesthesia Mice, Ratio of Test/Control mg./Kg. 25	50	100	125	Blood Pressure Effect, % Change: Time After Drug, hr. 0	2	3	3.5	3.8	3.9	4	Atropine	Atropine and Vagotomy	Duration (min.) First Dose
D-3-2	C_6H_5	CH_3	200	0.81			0.57	−13/44[a]	−33	−32	−33	−28	−22	−21	−18	−22	1/60
D-3-4	C_6H_5	CH_3CH_2	400	1.0	0.8			−34/54	−25/62	−35/15	−38	−49	−50	—	−31	—	1/45
D-3-6	C_6H_5	$CH_3CH_2CH_2$	400	0.76	0.88			−22	−20	−22	−17	−19	−27	−35	−20	−23	1
D-3-8	C_6H_5	$CH_3(CH_2)_2CH_2$	200	1.08	0.83			−24	−26	−32	−34	−31	−35	−34	−35	−33	1
D-3-10	C_6H_5	$(CH_3)_2CHCH_2$	200	0.91	1.03			−21/6	−17/30	−13/28	−14/28	−19/11	−23/5	−26	−28	−26	1/15
D-3-1	C_6H_{11}	H	400	1.20	0.93			−29/51	−26/42	−17/37	−28/23	−31/2	−35/2	—	−44	−55	1/25
D-3-3	C_6H_{11}	CH_3	400		0.96	0.94		−40/39	−40/54	−33/23	−33/10	−37/5	−38	−37	−38	−42	2/35
D-3-5	C_6H_{11}	CH_3CH_2	400	0.73	0.58			−44/49	−40/34	−35/18	−44	−45	−43	−40	−42	−41	1/25
D-3-7	C_6H_{11}	$CH_3CH_2CH_2$	400	0.51	0.50			−51/7	−53/18	−40/4	−45	−38	−29	—	−46	−36	3/15
D-3-9	C_6H_{11}	$CH_3(CH_2)_2CH_2$	200	1.07	0.78			−15	−30	−32	−29	−31	−37	—	−41	−38	2
D-3-11	C_6H_{11}	$(CH_3)_2CHCH_2$	400	0.87	1.17			−69	−55	−51	−55	−43	−45	−45	−46	−44	2
Dextro-amphetamine			[b]					+60	−37	−35	−26	−30	−21	—	−19	−23	>45

[a] −13/44 = biphasic systemic blood pressure response; 13% decrease for 1 min., followed by a 44% increase for 60-min. duration. [b] LD_{50} in mice is 95 mg./Kg. [c] Ratios for 5 mg./Kg. were 1.0 and 0.93, while 10 mg./Kg. gave 1.21 and 0.61.

70.0 Gm. (0.382 mole) of *N*-(1,1-dimethyl-2-cyclohexylethyl)formamide was refluxed for 36 hr. It was then cooled and extracted 3 times with 100-ml. portions of ether. The combined ether extracts were washed with 50 ml. of water, dried over potassium carbonate, and distilled. The yield of product which boiled at 92–93° (17 mm.) was 48.2 Gm. (81.2%).

The base was dissolved in anhydrous ether and converted into the hydrochloride salt with gaseous hydrogen chloride in the usual way. The salt which was soluble in the ether–hydrogen chloride solution was obtained by evaporating off the ether. The crude product was recrystallized several times from ethyl acetate to give a pure product melting at 152–153°. [Reported (28) m.p. 147–148°.]

Anal.—Calcd. for $C_{10}H_{22}ClN$: Cl, 18.52. Found: Cl, 18.42, 18.30.

Preparation of *N*-Substituted 1,1-Dimethylphenethylamines and *N*-Substituted 1,1-Dimethyl-2-cyclohexylethylamines

N - (1,1 - Dimethyl - 2 - phenylethyl)amides and N-(1,1-Dimethyl-2-cyclohexylethyl)amides.—These amides were prepared by a slight modification of the method described by Ritter and Kalish (27). All amides listed in Table I were synthesized by the following procedure.

To a solution of 10.0 Gm. (0.1 mole) of concentrated sulfuric acid in 50 ml. of glacial acetic acid maintained at about 20° there was added portionwise 0.11 mole of the appropriate nitrile. One-tenth mole of 1,1-dimethyl-2-phenylethanol or 1,1-dimethyl-2-cyclohexylethanol, depending upon which of the amides was to be prepared, was added slowly. The mixture was stirred mechanically while raising the temperature to 50°, then stirred without heat for 0.5 hr. longer, during which time the temperature rose spontaneously to 60–80° leading to complete solution of the alcohol. The mixture was allowed to stand for 8 hr. and then diluted with 300 ml. of water to precipitate an oil which after several hours solidified to a crystalline mass. The solid was broken up, filtered, washed with water, and then air dried.

The yield and melting points for each *N*-substituted amide are listed in Table I. All products were recrystallized from dilute ethyl alcohol (50%).

N-Substituted 1,1-Dimethylphenethylamines and N - Substituted 1,1 - Dimethyl - 2 - cyclohexylethylamines.—For the preparation of these compounds a modified procedure of the method used by Moffett (29) for synthesizing 2,2-dimethylpyrrolidine was employed. All amines listed in Table II were prepared by the following procedure.

To a mixture of 3.8 Gm. (0.1 mole) of pulverized lithium aluminum hydride and 75 ml. of anhydrous ether, which had been refluxed gently for 1/4 hr. was added a solution of 0.05 mole of the amide in 150 ml. of anhydrous ether at such a rate that the solution refluxed gently without any further external heating. When the addition was complete and the initial reaction subsided, the mixture was stirred and refluxed for 15 hr. The reaction mixture was allowed to cool, and then 5 ml. of ethyl acetate was added slowly with vigorous stirring and, finally 50 ml. of 6 *N* hydrochloric acid was added in the same manner. The mixture was then steam distilled several minutes after the boiling point reached 100° and the

distillate discarded. The mixture in the flask was cooled and to it was added carefully with stirring 35 ml. of 12 *N* sodium hydroxide solution. The alkaline mixture was then steam distilled until the distillate was no longer basic (about 600 ml.). The amine was extracted from the distillate with three 100-ml. portions of ether, the combined ethereal extracts dried over potassium carbonate, filtered, and the solvent removed *in vacuo*. The residue was then fractionated under reduced pressure.

The base was dissolved in anhydrous ether and then precipitated as the hydrochloride salt by passing anhydrous hydrogen chloride into the solution in the usual way.

The yield and boiling points of each *N*-substituted amide are listed in Table II. The melting point for each *N*-substituted amine hydrochloride is listed in the same table. All amine salts were recrystallized from ethyl acetate–ethanol as solvent.

PHARMACOLOGY

Comparative pharmacological studies were conducted on all of the amine hydrochlorides prepared. Their effects were observed on the duration of hexobarbital anesthesia in mice and systemic blood pressure and bronchodilatory activity in dogs.

Groups of 10 male, albino Swiss-Webster mice were pretreated subcutaneously with the test compound 2 hr. before the intraperitoneal administration of a dose of 100 mg./Kg. of sodium hexobarbital. The end point was taken as that time when the mouse moved from a supine position. Similar groups of control mice were tested simultaneously, and the results presented as a ratio of the average duration of anesthesia of test mice to control mice. A ratio of 1.0 indicates the results were the same for both groups: a value less than 1.0 suggests antagonism of hexobarbital anesthesia. In addition, these compounds were administered orally in increasing doses to determine the degree and type of toxicity produced. The results are recorded in Table III. All test compounds were orally active as indicated by the fact that they were all lethal when administered orally to mice. Only compound D-3-5 and D-3-7 produced appreciable alteration in the hexobarbital anesthesia of mice. In both cases it was a decrease probably indicating central nervous system stimulation.

Adult mongrel dogs, unselected as to sex, were employed in the cardiovascular and bronchodilator experiments. Anesthesia was maintained by the judicious use of sodium pentobarbital. Solutions of test compounds were freshly prepared and injected intravenously. The ability of these compounds to alter systemic blood pressure at a constant dose of 4 mg./Kg. was investigated in 2 or 3 dogs for each compound and the results averaged. Furthermore, the same dose was repeated at various intervals to determine whether tolerance developed to the blood pressure effects. These results are also presented in Table III. For the phenyl derivatives the first dose of *N*-methyl (D-3-2) and *N*-ethyl (D-3-4) produced a pressor effect that became negligible after subsequent doses; with progressively longer chain length, including *N*-propyl (D-3-6), *N*-butyl (D-3-8), and *N*-isobutyl (D-3-10), no pressor activity was found. Similarly, for the cyclohexyl derivatives the magnitude of the pressor effect decreased with an increase in chain length of the *N*-substituent and completely disappeared with *N*-butyl (D-3-9). For some unexplained reason it was again present at *N*-isobutyl (D-3-11). As was expected tachyphylaxis developed readily to the pressor effect. The vasopressor effect following tachyphylaxis was not influenced by atropinization of the dog or by a combination of atropinization and bilateral midcervical vagotomy.

Changes in bronchiolar resistance of dogs were recorded by a modification of the method of Konzett and Rossler (30). In brief, under constant volume respiration variations in bronchiolar resistance were indicated by changes in the volume of air in excess of that required for adequate ventilation. Histamine, 10 mcg./Kg., or carbachol, 1 to 4 mcg./Kg., administered intravenously, were used as the bronchoconstricting agents. The ability of the test compound to block this bronchoconstriction was determined following intravenous administration of 2 and 4 mg./Kg. doses of the test compound. No consistent or appreciable bronchodilatory effect was observed.

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Stability and Flocculation of Oil Droplets in Dilute Emulsions

By HIROYUKI MIMA and NOBUYUKI KITAMORI

By using a Coulter counter, it was found that flocculation of oil droplets stabilized with acacia occurred even in dilute emulsions (8×10^{-3} to 10^{-4} vol. per cent oil). This phenomenon was reversible and the equilibrium in floc size distribution was attained rapidly, and the flocculation depended upon the concentration of oil droplets, the size distribution of primary droplets, and the amount of salts added. Since the rate of creaming depends upon the size of particles, the stability of emulsions is affected by the flocculation of emulsion droplets. The stability of emulsions in the presence of 0.1 per cent sodium chloride was measured quantitatively by measuring the fluorescence of anthracene dissolved in oil phase, and it is concluded from the experimental results that there is a distinct relationship between the floc-size and the stability of emulsions.

MANY STUDIES have been made on the stability of o/w emulsions. However, the previous methods to determine the stability, especially the rate of creaming, including measurement of the time elapsed to the beginning of creaming or the volume of the cream separated after a definite time, were not quantitative (1–5).

According to Stokes' law, it is known that the rate of creaming is affected by the size of particles, the difference between the gravities of oil phase and water phase, and the viscosity of water phase. The size of particles in emulsion depends

$$u = \frac{(P_1 - P_2)\, gd^2}{18\eta}$$

not only upon the size of primary droplets, but also upon the degree of flocculation of the primary droplets. As for suspensions, a study of the effect of suspensoid concentration on the degree of flocculation has been reported (6). However, no papers have yet dealt with the relationship between flocculation and concentration of oil in emulsions.

During the authors' studies on the applications of emulsions in the fields of pharmacy and foodstuff, it was observed that the creaming occurred rapidly in the original emulsions, which contained a high concentration of oil phase, while in the diluted emulsion, the rate of creaming was not so rapid. To clarify these phenomena, a Coulter counter was used to measure particle-size distribution of flocculated droplets (7, 8). From these experimental results, it was found that the more the emulsion was diluted, the more the modal diameter of the dispersed particles became smaller, and the ratio of the number of droplets per concentration of oil phase increased. These facts indicate that the deflocculation depends upon the concentration of oil in the emulsion, but all the emulsion droplets were deflocculated to primary ones in very diluted concentration, where there was no relation between flocculation degree and oil concentration.

In order to elucidate the relationship between flocculation of oil droplets and emulsion stability, the rate of change in concentration of oil at a fixed depth was also determined quantitatively as a function of time by measuring the fluorescence of anthracene which had been dissolved in the oil phase of emulsion.

From these results, it was shown that there was a distinct relationship between the floc-size measured with the Coulter counter and the stability of emulsions.

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EXPERIMENTAL

Material.—Liquid paraffin, olive oil, and orange oil were all reagent grade materials. Ethyl dihydroabietate was synthesized from abietic acid by esterification and hydrogenation; b.p. 160–195°/0.2 mm. Hg, d_{20}^{20} 1.0269, n_D^{20} 1.5230, acid value 3.0.

Gum arabic was used as an emulsion stabilizer. Sodium chloride and anthracene were of special grade. Ether used for extraction of anthracene was freed from fluorescent impurities by passage through an alumina column.

Preparation of Emulsions.—Anthracene was dissolved in oils to give 0.2% (w/v) solutions. Ten grams of gum arabic was dissolved in 1 L. of distilled water under stirring with a homo-mixer (Type Tokusyu Kikai Kogyo Co.). Twenty milliliters of oil (containing anthracene) was then added and pre-emulsified for 2 min. This relatively coarse emulsification was then followed by passing through a Monton Gaulin homogenizer under about 4500 p.s.i. Size distribution of primary droplets of emulsions depended upon the pressure under which the emulsion was forced through the valve of homogenizer. In addition, viscosity of oils and number of times of homogenization had effects on the droplet size distribution of the resultant emulsions. The emulsions containing 2.0% oil phase by volume were used as the original emulsions.

Measurement of Size Distributions.—The method for measurement of droplet-size distribution of emulsions with the Coulter counter has been reported previously in detail (9). Our experiment followed this method. The aperture of 30 or 50 μ was used in accordance with the size of droplets. The threshold scale of the instrument was calibrated with polystyrene latex,[1] 0.769 and 1.305 μ in diameters. One-tenth per cent of saline was used as suspending medium. Since the floc-size distribution varied slightly with stirring, the measurement was carried out without stirring in this experiment. As the reproducible results were obtained with the same sample in the repeated measurements, it was thought that creaming did not occur during the measurement. The size distribution of emulsion was measured at various concentrations by diluting with 0.1% saline solution. Deflocculation was facilitated by dilution of emulsion, and at last the further dilution of emulsion resulted no longer in any change of the characteristic shape of the distribution curve. The size distribution of primary droplets was obtained at a diluted concentration under which the modal diameter of the distribution curve did not become smaller. The droplet size was also determined photomicroscopically. The floc-size distributions of emulsions were corrected according to the table of coincident passages supplied by Coulter Electronics, Inc. The modal diameters obtained from the floc-size distribution curves were used as the criterion of the degree of flocculation of droplets in emulsions.

With olive oil emulsion, the size distribution was measured at constant emulsion concentration (2×10^{-3}% oil) by diluting with varying salt concentration. The threshold scale of instrument was calibrated on every occasion of measurement.

Stability Measurement of Dilute Emulsions.—In these experiments, the concentration of oil phase of emulsion was 1×10^{-3} to 4×10^{-3}% and for this low concentration, a special method was necessary to determine the concentration of the oil in a small amount of samples collected from these emulsions. For example, when 1 ml. of a sample was collected, the amount of oil in the sample was only about 10^{-5} ml., which was too small to determine quantitatively with gas chromatography. For this reason, fluorescent analysis was used in order to determine the small amount of oil. The method is as follows:

The previously prepared stock emulsion containing anthracene was diluted to 1×10^{-3} to 8×10^{-3}% oil phase by volume with 0.1% saline solution. The diluted emulsions were allowed to stand in large glass cylinders (A) equipped with a small side orifice (B) closed by a rubber stopper (C) at the lower part of the cylinder. The vessel was about 9 cm. in diameter and about 60 cm. in height. The small orifice was fitted at the height of about 2 cm. from the bottom of the vessel. A long needle (D) was put into the vessel through a rubber stopper, and the tip of the needle was located at the center of the cylinder and just 2 cm. in height from the bottom (Fig. 1). Samples were easily collected through the needle at certain time intervals at the constant depth. Two milliliters of a sample emulsion was taken for one test, and the oil composition and the anthracene, of course, were extracted from the sample with 5 ml. of refined ether, and the fluorescent strength of this ethereal solution was measured with Aminco's spectrophotofluorometer at 404 mμ by activating at 252 mμ. The volume of the emulsion in the cylinder was 2.1 L., and the total volume of the collected samples was 20–25 ml., which is only about 1% of the whole volume of emulsion, and is negligibly small. It was found that the fluorescence of the extract remained constant within an error with the same sample, in which the concentration of oil remained constant at a certain depth, and also there was a linear relationship between anthracene concentration (parallel to oil concentration) and strength of fluorescence. However, the strength of fluorescence of anthracene in orange oil decreased with time. Therefore, in the case of orange oil, it was necessary to use controls that were equal in concentration to that of test emulsion and were shaken at certain intervals in order to prevent creaming. The difference in fluorescence between the test emulsion and the control was considered as the decrease of anthracene due to creaming.

RESULTS AND DISCUSSION

Floc-Size Distribution.—The floc-size distribution of olive oil, liquid paraffin, and orange oil emulsions at various concentrations measured with a Coulter counter are given in Figs. 2 and 3 and Table I. The droplet-size distributions are included in these figures. These figures clearly show that the peak of the floc-size distribution curve migrates to larger droplet size with increase in emulsion concentration, and to the contrary, with decrease in emulsion concentration, they shift to smaller size. Table I shows the total number particles counted by the Coulter counter at various oil concentrations. According to these data, particle number is not only parallel to oil concentration, but less than the number calculated from the numbers of primary droplets. The measured number

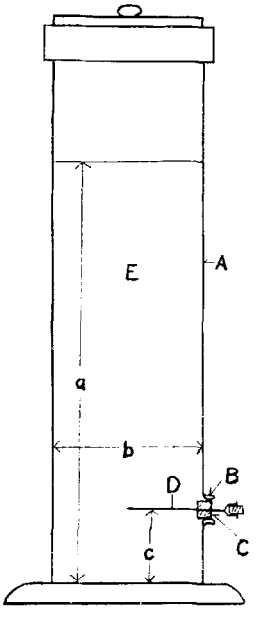

Fig. 1.—Apparatus for the determination of emulsion stability. Key: A, cylinder; B, side orifice; C, rubber stopper; D, needle; E, test emulsion; a, about 50 cm.; b, about 9 cm.; c, 2 cm.

[1] Supplied by J. W. Vanderhoff, Dow Chemical Co.

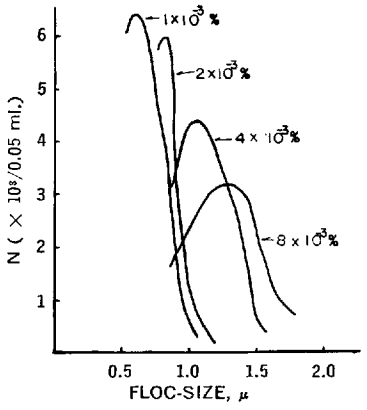

Fig. 2.—Change of floc-size distributions with concentration of emulsion (orange-oil emulsion).

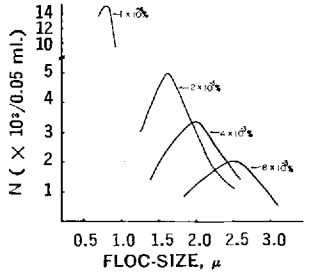

Fig. 3.—Change of floc-size distributions with concentration of emulsion (olive-oil emulsion).

TABLE I.—TOTAL NUMBERS OF PARTICLES IN ORANGE OIL EMULSION AT VARIOUS CONCENTRATIONS MEASURED WITH A COULTER COUNTER (IN 0.1% SODIUM CHLORIDE SOLUTION)

Emulsion Concn. ($\times 10^{-3}$%)	No. of Particles in 0.05 ml.	Relative No.	Relative No./ Emulsion Concn.
8.0	61,500	1.000	1.00
4.0	58,000	0.943	1.89
2.0	48,500	0.789	3.16
1.0	49,000	0.797	6.38

of particles approached to the calculated number with dilution. At a very dilute concentration, for example, in 1×10^{-3}% orange oil emulsion, the characteristic shape of the distribution curve did not change, and only the peak height of curve decreased according to the concentration after dilution. At this concentration, the size distribution curve was regarded as that of primary droplets in emulsions. These size distributions were in good agreement with the photomicroscopic observation. It may be reasonable to explain these phenomena as the deflocculation of flocculated emulsion by dilution. The scheme of these phenomena is illustrated in Fig. 4.; *i.e.*, in concentrated emulsion, which represents 4×10^{-3} to 8×10^{-3}% of orange oil emulsion in Fig. 2, the flocculation occurred highly; in medium concentrated emulsion, which represents 2×10^{-3}% in Fig. 2, the flocculation is not so high, and in diluted emulsion which has 1×10^{-3}% of orange oil, the particles were separated to primary ones. Thus, determination of size distribution with a Coulter counter confirmed the presence of flocs in emulsion. In the previous studies of flocculation and deflocculation of oil droplets in soap-stabilized emulsions (2, 7), the rate of creaming of emulsion or cream volume after a definite time period has been taken as the criterion of flocculation. In addition, microscopic observation and turbidity measurement have been made for studies of flocculation. In comparison with these methods, a Coulter counter method is excellent for the determination of floc-size, as it makes it possible to measure more exact floc-size distribution than that obtained by the previous methods. However, this method is not suitable for the investigation of the effect of surfactant only on flocculation, as an electrolyte solution must be employed as a medium in the measurement.

In the present work, the flocculation of emulsion droplets stabilized with acacia (which was one of the strong anticoalescent agents) in 0.1% saline was investigated. Here only the effect of oil phase concentration on flocculation of droplets in the same kind of emulsion and the same salt concentration was studied.

Because the rate of flocculation seems to be rapid and 20–30 min. were necessary for a determination of the distribution curve, the rate of flocculation could not be determined with this method. Therefore, the determination of the distribution curve was carried out after 5–10 min. of dilution of emulsion.

According to Smoluckowski's theory of coagulation kinetics (10, 11), the time required for the droplets to collide by Brownian motion is a function of the Boltzman constant, the temperature, the viscosity of the continuous phase, and the number of primary droplets. The time required for the flocculation of these emulsion droplets (about 0.6–0.7 μ, 8×10^{-3} to 10^{-4}%) was not more than 10 min. The numbers measured at various concentrations with the Coulter counter (N) were regarded as the total numbers of flocs in emulsion and the numbers

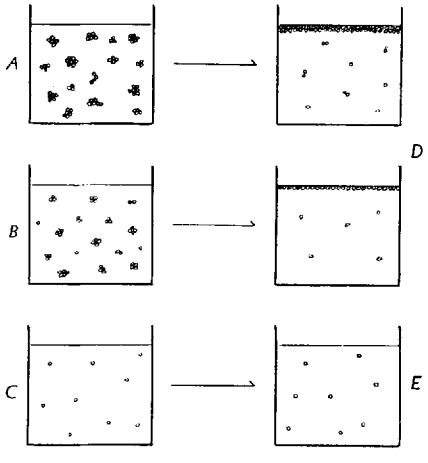

Fig. 4.—Schematic representation of the principal types of emulsion. Key: *A*, concentrated; *B*, medium; *C*, diluted; *D*, creaming occurs (unstable); *E*, no creaming occurs (stable).

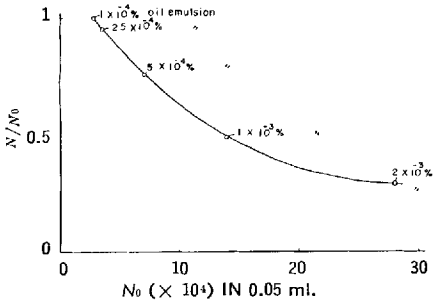

Fig. 5.—Relation between N/N_0 and N_0 obtained with ethyl dihydroabietate emulsion in 1% sodium chloride solution.

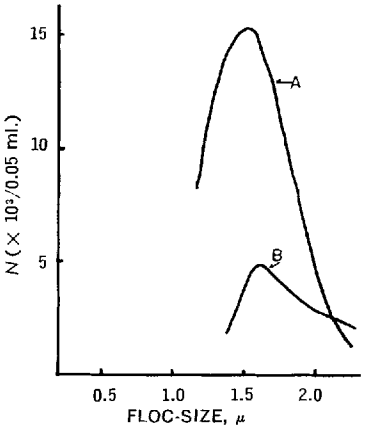

Fig. 6.—Floc-size distribution curves of 2×10^{-3}% olive oil emulsion in different salt concentrations. Key: A, in 1% NaCl solution; B, in 0.1% NaCl solution.

of primary droplets (N_0) which constituted the flocs were calculated from the total numbers of droplets measured in very diluted concentration, where the droplets did not form any flocs. The relation between N/N_0 and N_0 obtained with ethyl dihydroabietate emulsion in 1% saline is given in Fig. 5. The curve in Fig. 5 shows the relation similar to that of Smoluckowski's theory of coagulation. From these results, it is concluded that the flocculation is attributed to the collision of droplets, and the flocculation is reversible so that the equilibrium is attained rapidly after dilution.

In these cases, the flocculation of emulsion droplets was demonstrated in 0.1% saline solution. As pointed out earlier (12), it can be considered that the flocculation was affected by the concentration of salt. Actually, as shown in Fig. 6, the numbers of smaller particles in 1% NaCl solution were more than those in 0.1% NaCl solution at constant emulsion concentration.

Emulsion Stability.—Figures 7–10 illustrate the emulsion stabilities at various concentrations of olive oil, liquid paraffin, orange oil, and ethyl dihydroabietate emulsions. The changes in oil concentration of emulsion at fixed position upon standing indicate the floating or settling of oil droplets in emulsion at a certain rate.

The floating or settling rate depends mainly upon the size and the density difference between oils and media, and upon Brownian motion in small droplet size range. Actually, with ethyl dihydroabietate emulsions, oil concentration at the lower position of the test cylinder inclined to increase, because its density is slightly larger than that of water (Fig. 10), while with other emulsions oil concentration inclined to decrease. As shown in

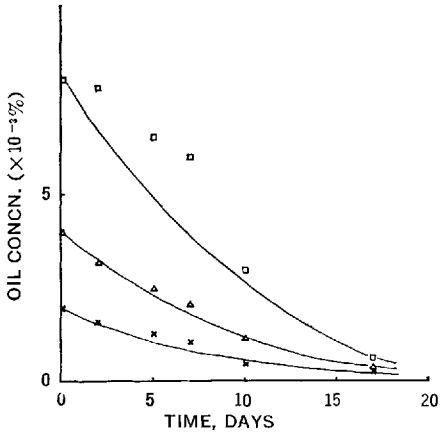

Fig. 7.—Stability of olive oil emulsion as reflected in changes with time of the concentration of the oil phase near the bottom of the test vessel. The 3 plots correspond to 3 different initial concentrations.

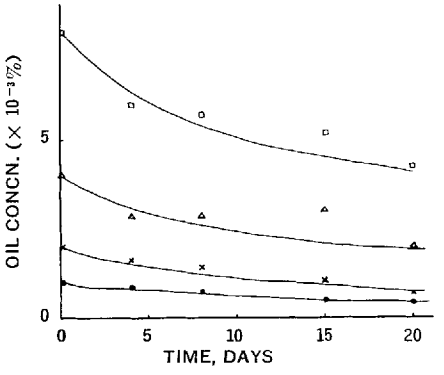

Fig. 8.—Stability of liquid paraffin emulsion.

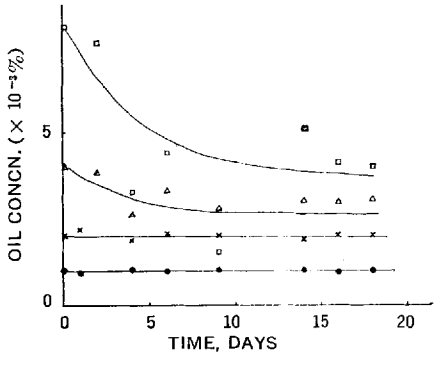

Fig. 9.—Stability of orange oil emulsion.

Figs. 8 and 9, the rates of decrease in oil concentration were initially rapid, gradually became slower, and attained to equilibria. The rate of decrease of concentrated emulsion is much more than that of diluted one, but the stability curve of the olive oil emulsion illustrated in Fig. 7 is slightly different from that of Figs. 8 and 9. In the very diluted emulsion of any oil, floating or settling of droplets does not occur, and therefore oil concentration remains constant. Even if droplets in emulsion float equally at any concentration, it follows that the rates of decrease in oil concentration of more concentrated emulsion are larger than those of more dilute emulsions. Therefore, the rate of creaming must be compared by using the specific rate of decrease in oil concentration, $1/c\ (dc/dt)$, where c is oil concentration at time t at a constant depth.

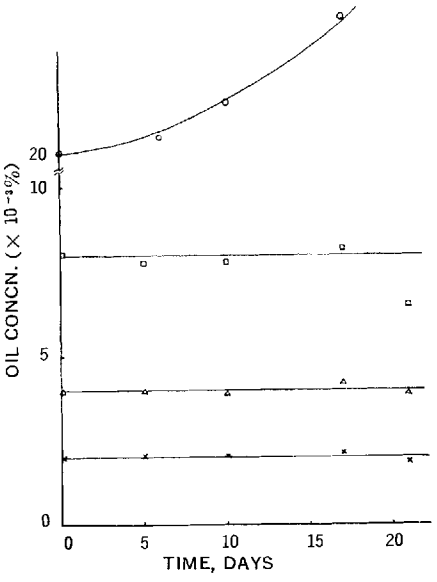

Fig. 10.—Stability of ethyl dihydroabietate emulsion.

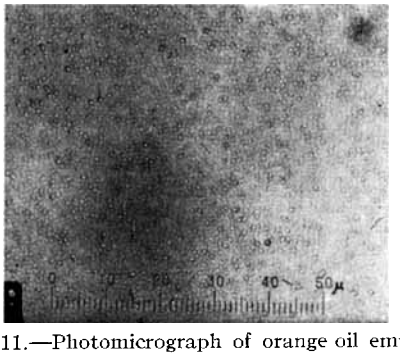

Fig. 11.—Photomicrograph of orange oil emulsion.

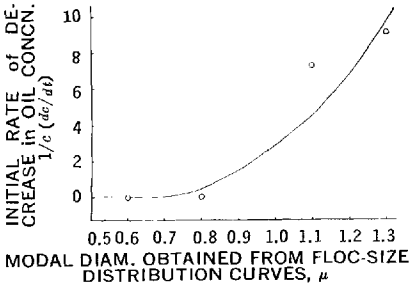

Fig. 12.—Relation between stability and floc-size in orange oil emulsion.

Relation between Flocculation and Stability of Emulsions.—Creaming of emulsions is the phenomenon that occurs in consequence of separation of oil droplets due to the density difference between the dispersed and continuous phases. Hence, the rate of creaming depends upon the density difference, the size of particles, and others according to Stokes' law. If the flocculation occurs in emulsion, a flocculated particle behaves as a larger droplet, although primary droplets were small. As shown in Figs. 2 and 3, the droplet size of the primary particles of emulsions prepared in this laboratory was about 0.6–0.7 μ and this was confirmed with photomicrograph (Fig. 11), in which none of the larger droplets were contained. From the data of the emulsion stability and the floc-size distribution measured with the Coulter counter, the relation between them may be concluded as follows.

For smaller droplets, a continuous random zig-zag motion called Brownian motion was expected to account for the stability against creaming; while, in the concentrated emulsion, Brownian motion of droplets affects the growth of flocs, and the larger flocs float according to Stokes' law. The relation between flocculation and emulsion stability of orange oil emulsion is given in Fig. 12. The modal diameter of floc-size measured with the Coulter counter was used as the size of flocs, and the specific rate of decrease in oil concentration was used as the measure of emulsion stability. The stability decreased rapidly with the increase of floc-size above a certain size. Below this size, creaming did not occur to any extent and emulsion was stable.

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Specific Activity of Trypsin

By ROBERT C. PETERSON

Trypsin has been fractionated electrophoretically on cellulose acetate into four zones. The lead zone, which contains the tryptic activity, was treated with specific substrate for enzyme activity and with the Lowry test for protein content. Specific activity of pure trypsin appears to be slightly greater than 8000 N.F. u./mg. Unexpected interference from commercial cellulose acetate was found with the protein test; this was reduced to a practical level. The phenomenon of tailing of protein in electrophoresis was studied.

THE ELECTROPHORETIC heterogeneity of commonly available trypsin is well established (1–13). Investigations in this laboratory on the purity of trypsin suggested the desirability of a simplified method for determining specific activity which could be applied to micro amounts of this enzyme and with slight modification to other enzymes. Electrophoresis on cellulose acetate combined with colorimetry offers such a method.

Earlier work included attempts at quantitative elution from cellulose acetate. It was found that the tryptic activity remaining on the cellulose acetate could be followed with the assay of Schwert and Takenaka (14) using the specific substrate benzoyl arginine ethyl ester (BAEE). From this it was decided that a method for specific activity might be derived from a combined direct determination of protein and tryptic activity in the electrophoretically fractionated zones while on the cellulose acetate. This article reports (*a*) the fractionation of trypsin by cellulose acetate electrophoresis, (*b*) determination of the amount of protein and tryptic activity in the isolated enzyme zone, and (*c*) a calculation of specific activity for the enzyme isolated under these conditions.

Previous similar work includes the extraction or elution of chromatographically or electrophoretically produced zones followed by reaction and measurement (9, 13, 15, 16), the production of color on the strip followed by extraction and measurement (15, 17–21), and direct reaction of the substance in the separated zone without a preceding or following elution (22–27).

Considering the difficulty of total elution, the time needed, and the viability of enzymes, direct test-tube reactions of the excised zones seemed more practical. While fluorigenic substrates (25) or the ninhydrin reaction for amino acids (22–24, 26, 27) have been used in this fashion, the author is not aware of a combination of similar tests being used to determine the specific activity of an enzyme.

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METHODS AND MATERIALS

The standard for all determinations was N.F. trypsin, lot 6040, 3226 N.F. u./mg. (28).

Electrophoresis was carried out in a greatly modified, horizontal cell.[1] Rigidity of components was provided, and evaporation was controlled by supplying inner and outer seals, a slotted inner plate, and by filling air spaces with Lucite blocks and sponges.

Cellulose acetate[2] was selected as the supporting medium. Several brands were tried, and in no case was sufficient precision of mobility found among several strips on a single run for quantitative purposes. Excellent precision was acquired on a single strip, however, by spotting 2 zones at the origin, 0.5 in. from either edge on a 5-cm. wide strip and using one half (a longitudinal cut) as a guide. (See Fig. 1.) The guide strip was dyed for 1 min. when tryptic activity was to be determined; a longer time was permissible when protein was determined. The dye solution was 0.1% Ponceau S in 5% trichloroacetic acid.

Some investigators found that the electrophoretic heterogeneity of trypsin depended to some extent upon the presence of calcium or other ions (2, 4–7, 9) while others did not (11, 13). The greater stability of trypsin in the presence of calcium ions and at an acid pH is well known (29–32). With this in mind, it was decided to use calcium ions in the assay but not during electrophoresis. The buffer was formic acid–pyridine–water/15 of 90%: 2.5: 982.5 by vol., pH 2.65. The strips were prerun 15 min. with reversed polarity before application of sample. The runs were for 3 hr. at room temperature and about 7.8 v./cm. For determination of tryptic activity, the wet strips were used within 1 min. after removal from the chamber; for protein determination, the strips were air dried.

Protein was determined with the Lowry (33) modification of the Folin-Ciocalteu (34) reagent using phenol reagent prepared in this laboratory according to Folin-Ciocalteu (34). The phenol reagent was tested for optimal dilution according to Oyama and Eagle (35), and their Lowry reagent modifications were used. In practice, the protein zone on the cellulose acetate was cut out and placed in a test tube. To this was added 0.5 ml. of water and 2.5 ml. of Lowry's reagent C, and the preparation was allowed to stand exactly 10 min. with frequent agita-

[1] Gordon-Misco, Microchemical Specialties Co.
[2] Oxoid, Colab Laboratories, Inc.

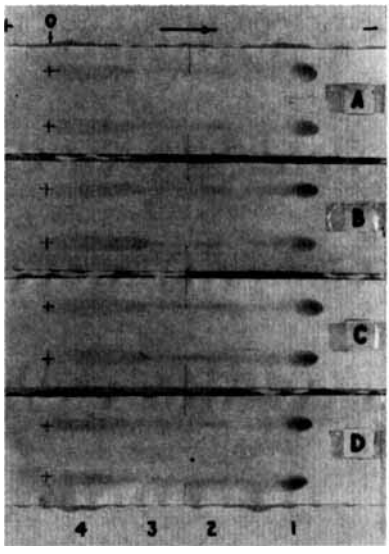

Fig. 1.—Photograph showing resolution and precision of patterns with duplicate spotting on same strip of cellulose acetate. Trypsin, 40 mcg., in aqueous formic acid–pyridine, pH 2.65; application 2.5 in. from anode end. Stained with naphthol blue black, 1% in 7.5% acetic acid. The leading zone contains the tryptic activity. Key: A, Eli Lilly; B, Mann Research Laboratories; C, Nutritional Biochemical Corp.; D, National Formulary.

tion. Folin reagent at the proper dilution, 0.25 ml., was jetted in by syringe and immediately mixed. The tubes were agitated frequently, and the absorption was determined at 1 hr. at 750 mμ with a spectrophotometer.[3] The reference solution was prepared similarly from a blank piece of cellulose acetate cut from the same strip immediately next to the test zone. Early inability to gain precision of results with the Lowry test when trypsin was spotted or electrophoresed on cellulose acetate was found to be due, in great part, to the presence of material in the cellulose acetate, as received, which gave a Lowry-positive test. From experience, it was found that an overnight soak in 0.01 *N* HCl followed by 2 rinses in fresh 0.01 *N* HCl and 2 rinses in deionized water would reduce this Lowry-positive material to usable values.

The Lowry test as described in this report was applied to 5 different lots (strips received at different times were considered different lots) of the cellulose acetate. One strip from each lot was used. Each strip was divided in half; equal sections from each half, with and without the special wash, were submitted to the Lowry test. The results are in Table I. All strips used for the Lowry test in this report were washed as above before being placed in the proper buffer. The contaminant has not been identified.

With each series of protein determinations, freshly dissolved trypsin in water and L-tyrosine[4] [stored in 0.2 *N* HCl, 0.5% by vol. in 37% formaldehyde (36)] were run as standards to assure stability of the Folin reagent.

Benzoyl-DL-arginine-*p*-nitroanilide (BAPA)[4] was

[3] Beckman model DU.
[4] Mann Research Laboratories, Inc.

Table I.—Lowry Protein Test of Cellulose Acetate without Application of Protein and with and without Special Wash to Minimize This Contaminant

	Absorption at 750 mμ			
	Unwashed Strips Sections		Washed Strips Sections	
Lot	1	2	1	2
A	0.095	0.091	0.032	0.025
B	0.196	0.162	0.030	0.030
D	0.089	0.087	0.020	0.017
E	0.079	0.103	0.029	0.034
H	0.087	0.090	0.021	0.022

used as the chromogenic substrate for tryptic activity (37–39). The substrate solution consisted of 100 mg. of BAPA dissolved in 20 ml. of redistilled dimethylsulfoxide and diluted to 100 ml. with pH 8, 0.1 *M* Tris buffer, 0.005 *M* in $CaCl_2 \cdot 2H_2O$. Five milliliters was used for each test; the reaction was carried out for 15 min. at 37° and stopped with 1 ml. of 30% acetic acid. Absorption was measured at 410 mμ *versus* a reference solution containing acetic acid as inhibitor.

RESULTS AND DISCUSSION

Determination of Protein.—The Lowry test was applied to the determination of protein (trypsin) which had been spotted on cellulose acetate under conditions appropriate for, but without, electrophoresis. The linear relationship between absorption values and amounts of protein is shown in Fig. 2. The span of values indicated at each point on the curve is ±1 standard error. Trypsin (30 mcg.) and L-tyrosine (15 mcg.), in solution, were brought through the Lowry test as standards simultaneously with the above series. The daily integrity of the Folin reagent and the test is indicated by the 7 absorbance averages and their standard deviations (trypsin, $\bar{x} = 0.361$, % coefficient of variation = ±1.4; L-tyrosine, $\bar{x} = 0.467$, % coefficient of variation = ±0.8). The plot in Fig. 2 provides a standard for determination of protein in

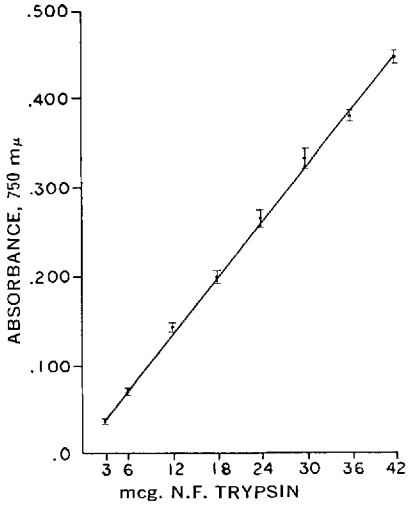

Fig. 2.—Lowry test for protein (trypsin) spotted on cellulose acetate.

the trypsin zone resolved by electrophoresis. Since the Lowry test varies with different proteins, such a curve would have to be constructed for each protein for which such a quantitative procedure would be established.

By incubating the various electrophoresed zones with gelatin, the main part of the tryptic activity was found in the leading zone. Very small amounts of tryptic activity were found all along the strip from the leading zone to the origin; this tailing will be clarified later in this report.

The Lowry test was applied to the lead zone after electrophoresis of varying amounts of N.F. trypsin. For determination of specific activity, only one level is needed, but it was of interest to determine its applicability over the range generally used in cellulose acetate electrophoresis. With the electrophoresis procedure as described, three 5 × 25.4-cm. strips were electrophoresed at one time and used for a single assay. The same amount of enzyme was spotted in duplicate on each strip. A longitudinally cut half served as guide—either after brief dyeing or after partial drying during which the protein zones became opaque and visible. By the latter method a fourth zone can be seen which Ponceau S is too insensitive to stain. Three electrophoretic runs (3 assays) were averaged to give the value at one level of application. Table II gives these values. At the 3-mcg. level there is insufficient protein in the lead zone for application of the test, and, in addition, it can be seen that not until about 18 mcg. is applied is a constant percentage of the applied protein found in the lead zone. This results from the tailing of trypsin activity with a certain saturation of the attracting sites on the cellulose acetate. A plot of applied trypsin against Lowry test absorption from the lead zone is given in Fig. 3. Linearity is found in the range of 18–42 mcg. applied trypsin.

TABLE II.—LOWRY PROTEIN DETERMINATION OF LEAD ZONE AFTER ELECTROPHORESIS OF N.F. TRYPSIN

Applied Trypsin, mcg.	Av. A, 750 mμ	±% Coeff. Var.	Protein[a] in Lead Zone, mcg.	% of Applied Protein in Lead Zone
3	0.002	85		
6	0.019	9	1	16.7
12	0.063	4	5	42
18	0.110	3.5	9.5	53
24	0.144	4.6	12.8	53
30	0.181	4.1	16.3	54
36	0.207	3.3	19	53
42	0.249	3.3	23	55

[a] From standard curve of N.F. trypsin spotted on cellulose acetate.

As with the Lowry tests on trypsin spotted on cellulose acetate, the tests on each level of electrophoresed trypsin were accompanied by standards of trypsin and L-tyrosine. The average absorption values were 0.360 for trypsin (compared to 0.361 before) and 0.465 for L-tyrosine (compared to 0.467 before). The Folin reagent and method have been stable during the course of this work.

Determination of Tryptic Activity.—The chromogenic (BAPA) assay was applied to the determination of enzyme or tryptic activity in zones when various amounts were spotted on cellulose acetate.[2]

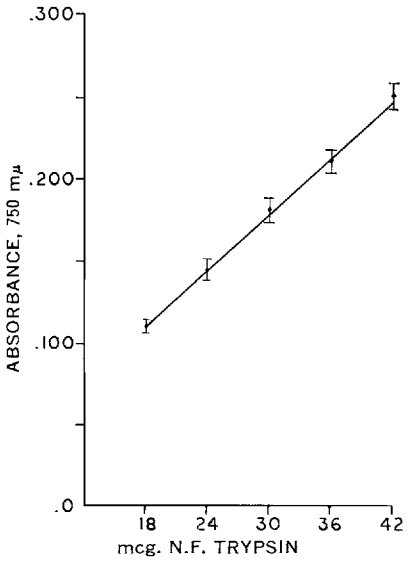

Fig. 3.—Lowry test for protein (trypsin) applied to lead zone (tryptic activity) after electrophoresis. Data are from Table II.

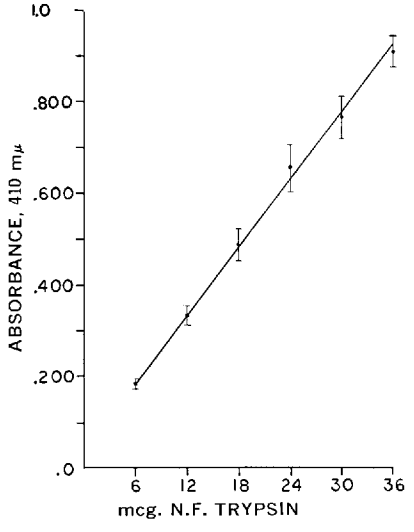

Fig. 4.—BAPA assay of tryptic activity in zones when N.F. trypsin is spotted in various amounts on cellulose acetate.

The cellulose acetate was prepared as for electrophoresis before application. As soon as the applied zone had soaked in, it was cut out and dropped into the reaction solution. Equal-size zones were cut according to a pattern (1 in. wide and 1.5 in. long with length of strip; these corresponded to the enzyme-zone size for the Lowry test), and the time of cutting was staggered every 1 min. A straight line can be drawn to relate the amount of enzyme applied and the average absorption value (Fig. 4).

The BAPA assay was applied to a quantitative study of tailing after electrophoresis of 30 mcg. of N.F. trypsin. The entire pattern of the wet strip was sectioned into 10 equal parts using a dyed guide strip and cardboard template to insure uniformity of zone size. Three electrophoretic runs gave the

average values listed in Table III. The range of absorption values for the lead zone was 0.707–0.720. The average total absorption of all sections was 0.812 with a range of 0.809–0.817. Reasonable agreement of results from application of the BAPA assay to spotted enzyme and to electrophoresed enzyme is indicated by the total absorption values at the 30 mcg. level, 0.763 and 0.812, respectively. N.F. trypsin was used as an indicator of the integrity of BAPA substrate. Ten micrograms in 1 μl. of buffer solution was treated simultaneously with the trypsin zones on cellulose acetate. Indicator values ranged from 0.448 to 0.510 with an average of 0.477; the substrate was satisfactory during the tests. The use of an indicator is necessary since BAPA is not stable indefinitely, even under refrigeration.

TABLE III.—STUDY OF TAILING OF N.F. TRYPSIN DURING ELECTROPHORESIS ON CELLULOSE ACETATE BY MEANS OF BAPA ASSAY

Acetate Section	A, 410 mμ (Tryptic Activity)							
	1,2[a]	3	4	5	6	7	8	9	10
	.712	.031	.018	.012	.009	.009	.008	.007	.006

[a] The absorption values for two equal sections were combined to give the lead zone value.

Since the enzyme activity does not coincide with the dyed protein zones (other than the lead zone), but decreases gradually toward the origin, this is tailing, and zones other than the lead zone do not have trypsin activity. This type of study could be applied to many supporting media in chromatography and electrophoresis in order to determine the influence of the medium on pattern formation.

Determination of Specific Activity.—Following electrophoresis of 30 mcg. of enzyme, the protein in the lead zone was determined with the Lowry test, and the percentage of applied enzyme found in this zone was determined with BAPA. From an assay of N.F. u./mg. of enzyme before electrophoresis the total amount of applied enzyme in N.F. units was determined. The assay values in N.F. u./mg. for these samples before electrophoresis are: *A*, 3226; *B*, 3615; *C*, 3095; *D*, 3565. (See Table IV for coding.) An assumption is made at this point, that since linear plots of concentration *versus* absorbance are obtained with both enzyme assays, a constant ratio exists between the two assays. It is realized that some variation might exist because of a difference in reaction of the N.F. trypsin and the more pure trypsin in the electrophoretic zone, but this is considered to be insignificant. Since in all cases the BAPA assay showed that 87–88% of the applied activity was found in the lead zone, a multiplication of the initial assay in N.F. u./mcg. by the 30 mcg. applied and the per cent recovered, gives the total amount of enzyme found in the lead zone as N.F. units. These totals are found in the second column of Table IV. The specific activity in the lead zone was calculated from these totals and from the totals of protein found in this zone. It appears from these data that the absolute specific activity of pure trypsin is above 8000 N.F. u./mg. The variation in specific activity may be due to the presence of varying amounts of denatured trypsin. All samples were stored as solids under refrigeration, but it has been our experience that occasional degradation is found even under these conditions. It should be pointed out that these samples had been refrigerated for varied amounts of time.

TABLE IV.—SPECIFIC ACTIVITY OF ELECTROPHORETICALLY FRACTIONATED TRYPSIN FROM VARIOUS SOURCES

Source[a]	N.F. u.	Protein, mcg.	Specific Activity, N.F. u./mg.
A	84.2	16.3	5166
B	95.4	11.5	8296
C	81.7	13.8	5920
D	94.1	13.7	6869

[a] A, N.F. lot 6040; B, Eli Lilly, H-1671-B; C, Nutritional Biochem. Corp., lot 2214; D, Mann Research Laboratories lot DA 3566.

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Substrate Specificity with Palladium-on-Silica Gel Catalysts

By ROBERT L. BEAMER and W. WENDELL LAWSON

Hydrogenation studies using palladium deposited upon silica gels prepared in the presence of various alkaloids indicate substrate selectivity. The selectivity appears analogous to that observed with enzymes (biocatalysts).

PADGETT and Beamer (1) have demonstrated the stereospecific hydrogenations of α-methylcinnamic acid catalyzed by palladium deposited upon silica gels precipitated in the presence of various cinchona alkaloids. That similarly prepared silica gels possess adsorption specificity has been demonstrated by Dickey (2) and Haldeman and Emmett (3). Beckett and co-workers (4) have shown stereoselective absorption on gels prepared in the presence of various alkaloids.

The present work is concerned with extending the hydrogenation studies of Padgett and Beamer (1) to include substrates in addition to α-methylcinnamic acid and silica gel carriers prepared in the presence, not only of cinchona alkaloids, but a modification of the cinchona alkaloidal structure (quinene). Studies using gels prepared in the presence of strychnine and brucine also were made.

EXPERIMENTAL

Reagents.—α-Methylcinnamic acid (K and K Laboratories), quinine sulfate N.F., quinidine sulfate U.S.P., cinchonine sulfate U.S.P. IX, cinchonidine sulfate U.S.P. X, strychnine sulfate N.F., brucine (Nutritional Biochemicals Corp.), propiophenone (Eastman Organic Chemicals), and ethylphenyl carbinol C.P. (Columbia Organic Chemicals).

Quinene.—Confusion exists in the literature concerning the melting point of quinene which reportedly melts indefinitely between 67 and 90° (5).

Comstock and Koenigs (6) first prepared quinene in 1884 and reported a melting point of 81–82° for the dihydrate. Giemsa and Halberkann (7) also prepared quinene but reported a melting point of 67°. The compound then resolidified and remelted from 90–91°. Both papers describe the preparation of quinene by dehydrohalogenations of 9-chloro-9-deoxyquinene using alcoholic potassium hydroxide, but differ in their methods of isolation.

The isolation described by Comstock and Koenigs proceeded through the zinc chloride–hydrochloric acid double salt (6). Giemsa and Halberkann crystallized quinene from a 50% acetone–water solution without prior conversion to the zinc chloride–hydrochloric acid double salt (7).

The discrepancy in melting points possibly could arise from the different isolation procedures. Therefore, we chose to isolate quinene by both procedures and to compare the products as to melting point, infrared spectra, and optical rotation.

Quinene was prepared by dehydrohalogenation of 9-chloro-9-deoxyquinine as described by both Koenigs and Giemsa. The product was isolated by use of each of the described procedures (6, 7) and compared as to yield, melting point, infrared spectra, and optical rotation. The infrared spectra were obtained in chloroform solution using a Perkin-Elmer Infracord model 137 spectrophotometer and were identical for the products of each separation. The specific rotation values were obtained using a Rhudolph spectropolarimeter model 200. The data for the yields, melting points, and specific rotations are summarized in Table I. A mixed melting point of the two products produced no depression.

The authors also prepared quinene by starting with 9-chloro-9-deoxyquinidine. The product of this reaction was identical with that prepared from 9-chloro-9-deoxyquinine. These results are in accord with those reported by Koenigs (8).

9-Chloro-9-deoxyquinine.—Preparation of this compound followed the procedure of Pouwels and Veldstra (9) which involves the replacement of the hydroxy group of quinine by chlorine through thionyl chloride.

9-Chloro-9-deoxyquinidine.—This compound was prepared according to the thionyl chloride procedure of Pouwels and Veldstra (9).

Propiophenone Oxime.—Thirty grams of hydroxylamine hydrochloride were added to 33.5 Gm. of propiophenone in 150 ml. of pyridine and 150 ml. of absolute alcohol. The mixture was refluxed 24 hr. Following the reaction period, the mixture was concentrated *in vacuo* with a rotary evaporator and left stoppered in the hood for 3 days, during which time white crystals formed.

The crystals were collected by filtration and

TABLE I.—COMPARISON OF QUINENE BY DIFFERENT ISOLATION METHODS

Method	Yield, Gm.	% Yield[a]	M.p.[b]	Specific Rotation[c]
Comstock-Koenigs	3.67[d]	39.7	76–80°	+48.93[e]
Giemsa-Halberkann	3.40[d]	36.8	75–78°	+45.98[e]

[a] Based on 9-chloro-9-deoxyquinine and on quinene dihydrate (5). [b] Uncorrected. [c] In 95% alcohol using the dihydrate (C = 1.776 Gm./ml.), temperature = 20°, wavelength = 589 mμ, length of tube was 200 mm. [d] Starting with 9.4 Gm. of 9-chloro-9-deoxyquinine. [e] The literature value for the dihydrate was unavailable.

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washed with a small amount of water. The filtrate was concentrated further on the rotary evaporator and cooled in a refrigerator. The additional crystals were collected by filtration, washed with water, and recrystallized from ethanol and water. The weight of the product after recrystallization was 23.0 Gm., representing a yield of 62% of theory. The crystals melted from 52–55°. [Reported (10) m.p. 53°.]

Preparation of Carriers.—The silica gel carriers were prepared by the procedure of Padgett and Beamer (1). The silica gels were precipitated from an aqueous sodium silicate solution using a solution of the alkaloid in 5.7 *N* hydrochloric acid. The gel was dried under a current of air in a fume hood, and the alkaloid was removed by washing with methanol in a Soxhlet extractor.

Hydrogenation Studies.—The catalysts were prepared by the method of Hartung (11) using 100 mg. of palladous chloride per gram of gel and sodium acetate as a buffer. Hydrogenations were performed using 0.05-mole substrate in absolute ethanol on a low-pressure Parr hydrogenation apparatus at an initial pressure of 4.2 Kg./sq. cm. and using initially 1.0 Gm. of catalyst. Heating was accomplished with an infrared heat lamp. After 24 hr., an additional 1.0 Gm. of catalyst was introduced, and the hydrogenation continued for another 8 hr. for a total reduction time of 32 hr. The reaction period of 32 hr. was used in all experiments and was based on the time required for reduction of 0.05 mole of α-methylcinnamic acid (1). No kinetic studies were attempted because of the abnormally long reduction period and the lack of an adequate temperature control.

Following hydrogenation, the catalyst was removed by filtration, and the alcohol evaporated at room temperature.

Infrared Studies.—Infrared spectra of α-methylcinnamic acid, propiophenone, and quinene were determined in chloroform using a Perkin-Elmer model 137 Infracord spectrophotometer. The concentrations of the solutions were 10%. The spectra of α-methylcinnamic acid and dihydro-α-methylcinnamic acid were also determined as 5% solutions.

Infrared spectra of mixtures of varying proportions of α-methylcinnamic acid and dihydro-α-methylcinnamic acid were determined and their absorptions *versus* concentrations were plotted at 910 and 1625 cm.$^{-1}$.

The same degree of absorption at 1710 cm.$^{-1}$ (carboxyl group) was obtained with the spectra of each of the mixtures. Following evaporation of solvent, the infrared absorptions of the hydrogenation mixtures were determined at 910, 1625, and 1710 cm.$^{-1}$, and estimations of composition were made.

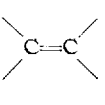
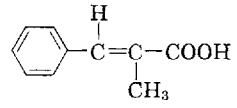
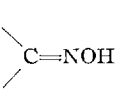
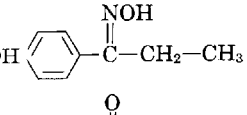
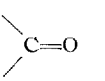
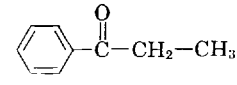
Gas Chromatographic Studies.—These determinations were made using a Beckman G.C.-2A gas chromatograph. The column was composed of silicone 550, 50% on C-22 firebrick (42/60 mesh). The column length was 6 ft. The instrument was equipped with a Bausch & Lomb Vom 7 recorder and a thermal conductivity detector.

The studies were made at a column temperature of 160° and with a flow rate of 60 ml./min. Helium was used as the carrier gas. The sample size was 0.005 ml. and was introduced using a Beckman 22400 liquid sampler.

RESULTS

When substrates other than α-methylcinnamic acid are employed with palladium-on-quinine silica gel catalysts, a lowered reactivity or a lack of reactivity was noted. These results are summarized in Table II.

TABLE II.—HYDROGENATIONS WITH PALLADIUM-ON-QUININE SILICA GEL

Group	Compd. Employed	% Yield
		81.1
		0.0 (With alc. HCl) 15.6 (No. alc. HCl)
		0.0

It should be noted that of the substrates employed, only α-methylcinnamic acid and propiophenone oxime in the absence of alcoholic hydrogen chloride gave a detectable yield. A lack of reaction was determined by no significant fall in hydrogen pressure and isolation within experimental error of all the starting material.

The absence of hydrogen chloride from the propiophenone oxime reduction introduced the possibility of secondary amine formation. So far, no secondary amine has been detected by infrared spectroscopy.

Vapor phase chromatography produced only two peaks having retention times of 52 sec. and 18 min., respectively. The peak at 52 sec. was identified as the solvent, ethanol. The peak at 18 min. was identified as 1-phenyl-1-propyl amine. Treatment with benzenesulfonyl chloride followed by sodium hydroxide indicated only primary amine.

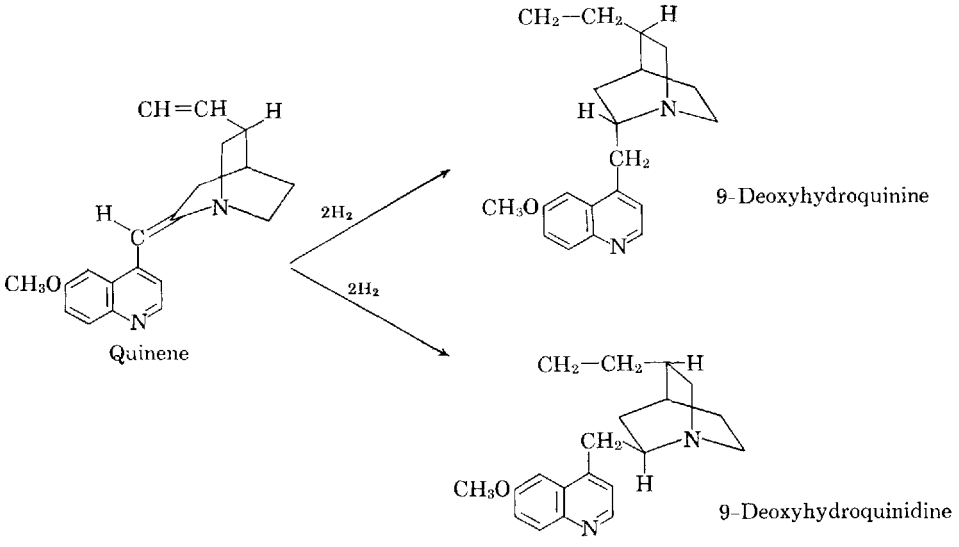
That only starting material was isolated from the attempted reduction of propiophenone was shown by infrared spectroscopy and gas chromatography and a comparison with known propiophenone. The possible hydrogenation products, ethylphenyl carbinol or *n*-propylbenzene (12), could not be detected by infrared spectroscopy or vapor phase chromatography.

The choice of quinene as a substrate for hydrogenation with palladized quinine silica gel as the catalyst was made because of its structural similarities to quinine.

The complete reduction of quinene is shown in Scheme I and leads to either dihydro-9-deoxyquinine or dihydro-9-deoxyquinidine. It was hoped that a stereoselective carrier would direct the reduction to one of the possible diastereoisomers.

Thus far, only quinene has been isolated from the reaction mixture. However, partial reduction could also lead to 9-deoxyquinine, 9-deoxyquinidine, or dihydroquinene. The authors are currently using other isolation techniques to attempt isolation of any of the possible products.

In view of the substrate selectivity with the



Hydrogenation of Quinene

Scheme I

palladium-on-quinine silica gel catalyst, varying the catalysts while employing the same substrate should give further information concerning the substrate selectivity of these catalysts. The results of these experiments are summarized in Table III.

TABLE III.—HYDROGENATIONS OF α-METHYLCINNAMIC ACID USING PALLADIUM-ON-SILICA GELS

Carriers	% Yield (After 32 hr.)
QN 0.5 SG[a]	81.1
QDN 0.5 SG	68.0
CN 0.5 SG	77.1
CDN 0.5 SG	87.9
Strych. SG	00.0
Brucine SG	00.0
Quinene SG	00.0
Plain SG	44.3

[a] Quinine silica gel which was prepared using 0.5 Gm. of quinine sulfate in the gel preparation. QDN = quinidine sulfate; CN = cinchonine sulfate; CDN = cinchonidine sulfate.

Only the catalysts prepared with the plain gel and the cinchona gels, with the exception of quinene, afforded reduction with α-methylcinnamic acid. Attempted hydrogenations employing catalysts prepared in the presence of strychnine, brucine, or quinene showed no indication of reduction after 32 hr. of hydrogenation and all of the unreacted α-methylcinnamic acid could be accounted for following the hydrogenation period.

However, the presence of large amounts of the starting material, α-methylcinnamic acid, can interfere with the isolation of dihydro-α-methylcinnamic acid by vacuum distillation (1). Therefore, we were never certain that where we have indicated no reduction that such was entirely the case. In other words, reduction too slight to be noted by a fall in the hydrogen gauge pressure might have occurred. Infrared spectra were determined with both α-methylcinnamic acid and D,L-dihydro-α-methylcinnamic acid. These spectra are shown in Figs. 1 and 2, respectively. The following absorption peaks are applicable to this work.

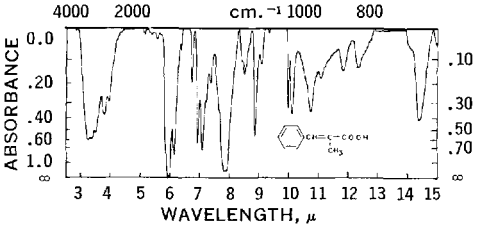


Fig. 1.—Infrared spectrum of α-methylcinnamic acid.

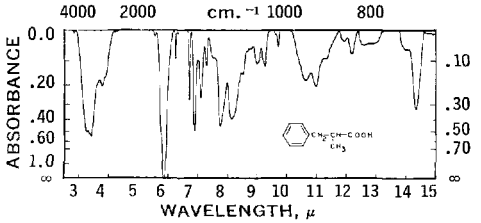


Fig. 2.—Infrared spectrum of D,L-dihydro-α-methylcinnamic acid.

α-methylcinnamic acid (Fig. 1):

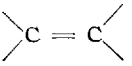
(a) —COOH absorption at 1710 cm.$^{-1}$

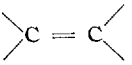
(b) $>C=C<$ absorption at 1625 cm.$^{-1}$

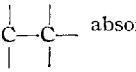
(c) $>C=C<$ absorption at 930 cm.$^{-1}$

D,L-dihydro-α-methylcinnamic acid (Fig. 2):

(a) COOH absorption at 1710 cm.$^{-1}$

(b) disappearance of  absorption at 1625 cm.$^{-1}$

(c) disappearance of  absorption at 930 cm.$^{-1}$

(d) appearance of  absorption at 910 cm.$^{-1}$

The absorption of mixtures of varying proportions of α-methylcinnamic acid and D,L-dihydro-α-methylcinnamic acid were plotted at 1625, 930, and 910 cm.$^{-1}$, as described earlier in this report. In each case a straight line was obtained. However, the plots at 910 and 1625 cm.$^{-1}$ were best applied to the determination of α-methylcinnamic acid and D,L-dihydro-α-methylcinnamic acid, and these are shown in Fig. 3. Use of these plots and the absorption at 1710 cm.$^{-1}$ (COOH) indicates the reaction mixture from the strychnine gel catalyst was 93% α-methylcinnamic acid and 7% dihydro-α-methylcinnamic acid.

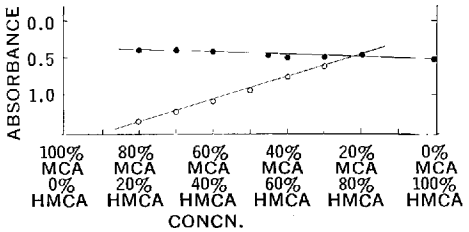


Fig. 3.—Plots of mixtures containing varying proportions of α-methylcinnamic acid and D,L-dihydro-α-methylcinnamic acid (HMCA). Key: ●, plot at 910 cm.$^{-1}$; ○, plot at 1625 cm.$^{-1}$.

DISCUSSION

The data indicate that the substrate selectivity observed with palladium-on-silica gel catalysts is similar to that observed with enzymes.

Beckett and co-workers have attributed the stereospecific adsorption of alkaloids by cinchona and other alkaloidal silica gels to imprints left on the gel surface by the alkaloid present during the precipitation of the gel (4). Likewise, the substrate selectivity of palladium-on-silica gel catalysts must arise from imprints left on the catalytic surface. The nature of these imprints is not known but may arise from some charge distribution on the surface of the adsorbent.

Such an explanation necessitates the presence of catalytic centers or sites similar to those proposed to explain enzyme specificity (1, 13–15).

According to Beckett's hypothesis, quinene should have been hydrogenated to a greater extent than α-methylcinnamic acid. That such is not the case could arise from steric hindrance (16, 17).

Another explanation for the substrate selectivity of α-methylcinnamic acid over quinene involves the nature of the silica gel surface as influenced by the cinchona alkaloids. This hypothesis supposes the gel surface to be of basic character, thus attracting the carboxylic acid. The incorrectness of this explanation is indicated by the results with the brucine and strychnine, the reduction of propiophenone oxime in the absence of alcoholic hydrogen chloride, and by Beckett's observations (4).

Therefore, the most probable explanation appears to involve active sites of proper size, shape, and charge distribution to accommodate the substrate molecules. These sites are imprinted on the carrier surface by the alkaloid. That α-methylcinnamic acid is not hydrogenated by the catalysts prepared from the brucine and strychnine gels can be explained by sites of improper size, shape, and charge distribution to accommodate the substrate molecules. This does not preclude the possibility of other substrates being hydrogenated by palladized strychnine or brucine gels.

Before definite answers concerning the nature and extent of the substrate selectivity of these catalysts can be obtained, studies must be made employing other substrates and carriers.

SUMMARY

Hydrogenations using palladium-on-quinine silica gel and various substrates indicate a degree of substrate selectivity by this catalyst. Substrate specificity also was shown in studies using α-methylcinnamic acid as a substrate and palladium deposited on various alkaloidal gels as catalysts. The selectivity may be explained by imprints left on the carrier surface by the alkaloid.

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Biological-Chemical Indicator for Ethylene Oxide Sterilization

By JOHN H. BREWER and RUSSELL J. ARNSBERGER

The use of a biological-chemical indicator for ethylene oxide sterilization of disposable medical and pharmaceutical materials is reported. The indicator system affords an immediate visible indication of gas penetration into the materials during the sterilization cycle and also serves as a biological (spore) control. Preparation of the indicator is discussed in some detail, including the organisms and chemicals employed and the basic chemical reactions. The usefulness of the indicator for determination of gas penetration is discussed in terms of diffusion of the gas and permeability of the materials, including latex and plastic films.

A NUMBER of indicators have been suggested for use with ethylene oxide. For the most part these depend on a pH change or other type of chemical reaction, and some attempt has been made to have these indicate time as well as the presence of ethylene oxide. Since moisture content and other factors have a great deal to do with the efficiency of gas sterilization, the most satisfactory indicator of sterilization is the use of positive spore controls. These have the disadvantage that one must wait for the required incubation before reading the results.

In the sterilization of many of the disposable medical and pharmaceutical materials such as gloves, syringes, transfusion, and infusion sets, it is of the utmost importance to determine whether the gas is reaching the intricate spaces within these items and sterilizing them. As pointed out by Rubbo and Gardner (1), this is particularly true of such items as syringes with rubber plungers which might have two or more ribs in contact with the glass or plastic barrel.

If one depended on pressure alone to get the gas into these spaces, then this pressure would be too great to be reached by most gas-sterilization processes. One must also take into consideration the diffusion characteristics of the materials employed, and this makes a visual indicator quite valuable. Most of the chemical indicators are so sensitive that a very small amount of gas might cause a color change and yet not be sufficient to kill any bacterial spores present. For this reason the authors have sought an indicator system that combined both a chemical indicator, which did not depend on a pH change alone, and bacterial spores.

CHEMICAL INDICATOR

The compound 4-(4-nitrobenzyl) pyridine[1] has been described as a sensitive reagent for the determination of alkylating agents (2, 3). This reagent reacts with ethylene oxide to form a methine dye which is blue in an alkaline medium. (Scheme I.) The mechanism in the reaction of the reagent with an alkylating agent has been reported (4).

4-(4-Nitrobenzyl) pyridine has been used in this laboratory as an ethylene oxide indicator in solution, dried on filter paper, and adsorbed on silica gel. It has been found useful to indicate ethylene oxide permeability of latex and plastic films, ethylene oxide retention of plastics and elastomers, and as an exposure indicator for ethylene oxide sterilization.

The indicator paper is prepared by first dipping Whatman No. 1 filter paper in a 2% aqueous solution of sodium carbonate. After drying, the paper is dipped in a 2% solution of the indicator reagent in acetone. The indicator paper thus formed can then be cut into convenient-sized strips and inoculated with bacterial spores.

Indicator strips prepared as described will turn blue within a few seconds when exposed to the vapors of pure ethylene oxide. The same strips have been exposed to sterilization gas mixtures of ethylene oxide and carbon dioxide in ratios of 10:90 and 20:80, and it was observed that as long as the strip remained in the mixed-gas atmosphere the color did not change. Furthermore, if the strip was sealed in a permeable plastic container which would retain some residual gas mixture after removal from the sterilizer, no color change would occur. However, when the paper was removed to the air after exposure, the blue color would develop. This effect was explained by the fact that carbon dioxide prevented the reaction product from assuming the alkaline form until the CO_2 was diffused from the paper.

EXPERIMENTAL

In order to determine ethylene oxide penetration to the area between the plunger-stopper rings of both glass and plastic syringes, indicator strips were cut to size and inserted in this area. (Fig. 1.) The assembled syringes were placed in a glass desiccator; the desiccator was evacuated to approximately 25 in. of mercury and ethylene oxide admitted to a concentration of approximately 1000 mg./L. No color change of the strips in the syringes was noted until after about 1.5 hr. of exposure. After this time, a faint blue color was noted, which continued to darken. After 3 hr. of exposure, the syringes were removed from the desiccator. For the next 24 hr. the indicator strips continued to darken to a deep blue.

It appeared from the above experiment that dif-

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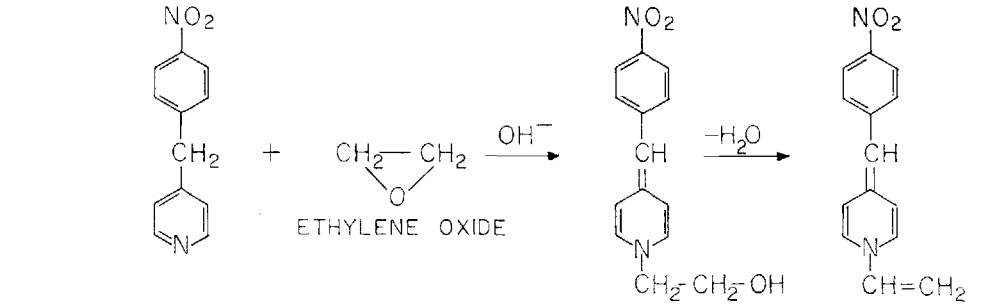
[1] The authors acknowledge the suggestion of Dr. David N. Kramer, Army Chemical Center, Md., that we investigate this compound as an indicator.

The cost of the preparation of the color picture which appears in this article has been borne by the authors.



Scheme I

fusion of ethylene oxide gas through to the area between the syringe stopper rings is slow; however, the gas will eventually penetrate to this area and in sufficient concentration to kill the control organisms. There was also an indication that ethylene oxide continues to diffuse from the stopper for a period of time after the syringes are removed from a sterilizer. To investigate these observations further, syringes were assembled with 5 stoppers and indicator strips in each barrel and exposed as in the previous experiment. When the syringes were removed from the desiccator after 3 hr. of exposure, the strips in the 2 outer stoppers were blue and the 3 inner stoppers were white. Twenty-four hours after the syringes were removed, the strips in the stoppers next to the outer stoppers had turned blue and the center strip remained white; 36 hr. after exposure, the center strip had turned blue. These are exaggerated laboratory tests, since no syringe has over 1 rubber plunger.

This might indicate that the gas remains in the rubber or within the syringe, tubing sets, or other items, and might possibly contaminate drugs being administered to the patient. To prove this is not the case, gas chromatographs are run to show that all the gas has diffused out within the 10-day quarantine period and is further demonstrated by the toxicity tests which are run on each lot.

BIOLOGICAL INDICATOR

As spore controls we have employed both *Bacillus stearothermophilus* and *Bacillus globigii*. For our

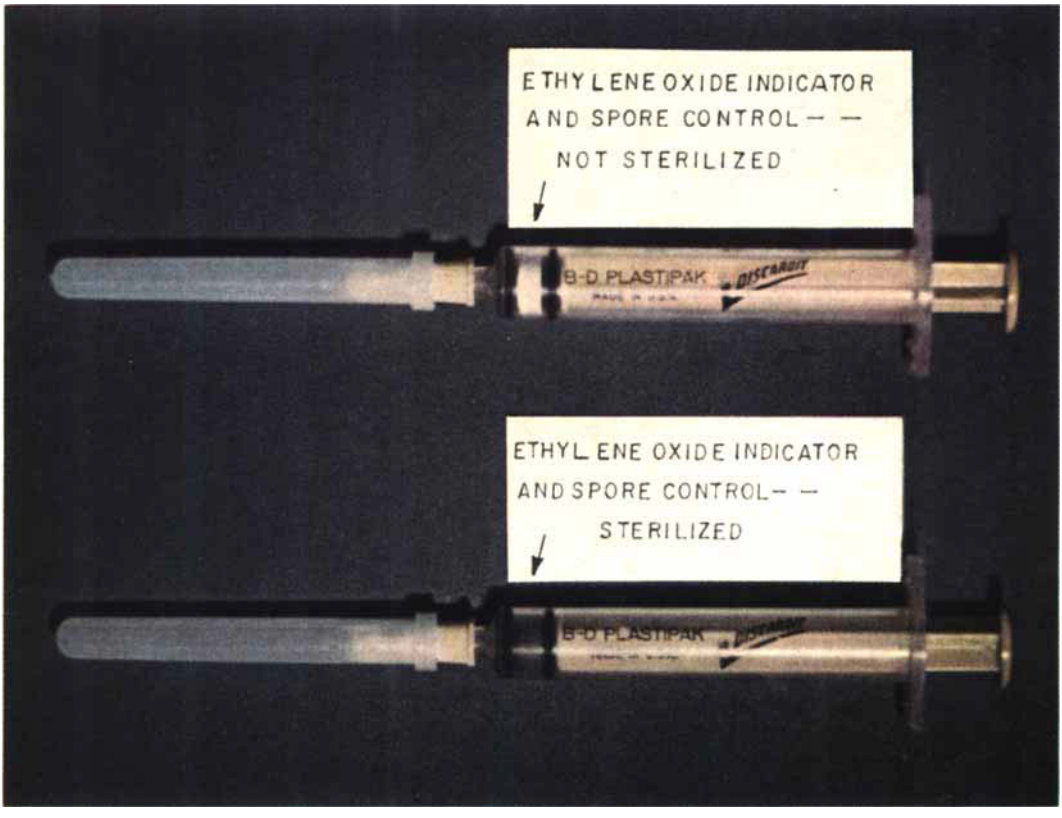


Fig. 1.—Sterilized and unsterilized ethylene oxide indicators and controls.

purposes we have found the thermophilic organism much more satisfactory. One is not likely to encounter this type of organism as a laboratory contaminant in the sterility test, thereby causing a false positive reaction. This would necessitate the retesting of the load and delay production.

A very important consideration in the selection of the chemical indicator was to make sure that it did not inhibit the growth of the control organisms. Bacteriostatic-type tests were made using diluted spore suspensions with the paper strips, both before and after exposure to the sterilizing gas. No inhibition was noted and controls from small inocula grew quite luxuriantly in the test media.

The spores used to inoculate the indicator strips were prepared by suspending washed spores of *B. stearothermophilus* in distilled water containing 1% sorbitol. The suspension was standardized to approximately 5 million spores per ml. Spore counts were determined by appropriate dilution of the suspension and plating on trypticase soy agar. Plates were incubated at 60° for 24 hr. Paper strips were inoculated with 0.02 ml. of the suspension or approximately 100,000 spores. After sterilization, strips were transferred to fluid thioglycollate medium and incubated at 60° for 7 days to determine survival of spores. Control tubes containing indicator strips, both before and after exposure to ethylene oxide and inoculated with diluted spore suspension, showed no inhibition of the control organism.

APPLICATION

In commercial lots, dye-spore strips with 100,000 spores are prepared in a size to fit the item being sterilized. They have been prepared in sizes similar to the commercially available spore strips[2] for use in the index finger and in the cuff of rubber gloves, since it is felt that these are the most difficult places for the gas to reach in this item. These controls in the index finger change color, and the organisms are killed even if a knot is tied in the wrist part of the glove, indicating adequate penetration. In the sterilization of commercial lots of gloves,[3] the use of these indicators throughout the sterilizer load give assurance that the entire lot has been sterilized.

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[2] Kilit spore strips, Baltimore Biological Laboratory, Baltimore, Md.
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Pharmacokinetic Model for Nalidixic Acid in Man I

Kinetic Pathways for Hydroxynalidixic Acid

By G. A. PORTMANN, E. W. MCCHESNEY, H. STANDER, and W. E. MOORE

A model which includes the parameters of absorption, metabolism, and excretion of hydroxynalidixic acid (HNA) is presented. Individual rate constants for the absorption and disappearance of HNA in 5 subjects were determined. Theoretical plasma-level curves based upon these constants were calculated, and good agreement with the experimental data was obtained. Rate constants for glucuronide formation, oxidation, and excretion of HNA were calculated.

HYDROXYNALIDIXIC acid (1-ethyl-1, 4-dihydro-7-hydroxymethyl-4-oxo-1, 8-naphthyridine-3-carboxylic acid) is 1 of 4 metabolites of nalidixic acid[1] formed in man. It has been isolated and shown to have an *in vitro* antibacterial spectrum similar to that of nalidixic acid (1). The other 3 metabolites, all of which have not shown biological activity, are nalidixic acid glucuronide, hydroxynalidixic acid glucuronide, and the 3,7-dicarboxylic acid.

A previous article by the authors (2) described a simplified working model which permitted calculation of the apparent kinetics of the biologically active and inactive forms of nalidixic acid as separate groups.

Because nalidixic acid has been found to be clinically effective in the treatment of Gram-negative infections (3, 4), more definitive studies of its pharmacokinetics have been continued. These experiments would also illustrate the mechanisms which enabled the simplified model to describe absorption and elimination parameters.

In order to describe the complete pharmacokinetic profile of nalidixic acid, it is necessary to quantitate all the rate processes, including those occurring after its oxidation to the 7-hydroxy derivative. This article deals with a study of the absorption, metabolism, and excretion of hy-

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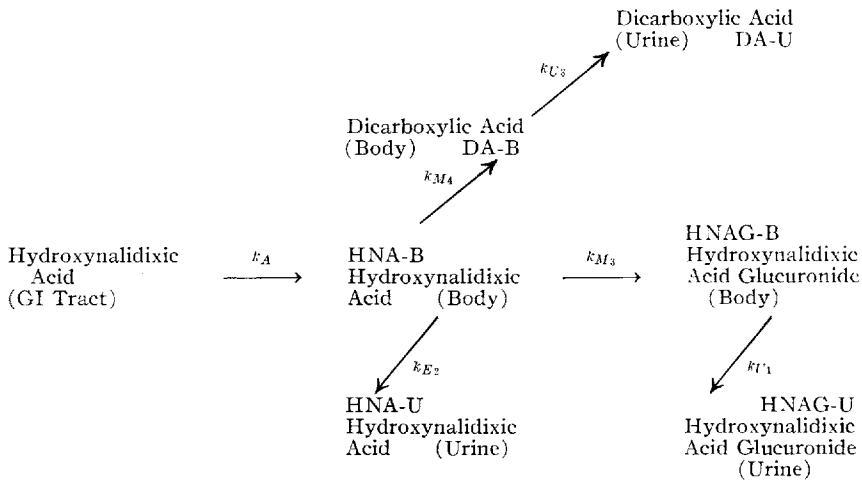

Kinetic pathways of hydroxynalidixic acid in man; k_A = availability rate constant; k_{M_i} = metabolic rate constants; k_{E_2} and k_{U_i} = excretion rate constants. Designations for the rate constants subnumber are those used in the larger model for nalidixic acid (7).

Scheme I

droxynalidixic acid, gives basic information about the compound, and [as will be illustrated (7)] permits a detailed description of the pharmacokinetics of nalidixic acid.

THEORETICAL

The work of McChesney and co-workers (1) leads to the model depicted in Scheme I. This represents the present knowledge of the kinetic pathways for hydroxynalidixic acid in man.

Equations describing the change of each component in Scheme I with respect to time were derived assuming total absorption and first-order rate processes. The total urine recovery was found to be 98.3 ± 2.9% (±S.E.), indicating complete absorption of the drug in every case. Those equations that were used in the present study are listed below. Lag time is defined as the time interval between ingestion of the dosage form and the appearance of hydroxynalidixic acid in the plasma. A_0 is equal to the administered dose since complete absorption occurs. Time (t) is measured after the lag time interval. The disappearance rate constant (k_{d_2}) for hydroxynalidixic acid is taken as the sum of $k_{E_2} + k_{M_3} + k_{M_4}$. The availability rate constant (k_A) from the gastrointestinal tract encompasses many factors, including motility, dissolution rate, and the intrinsic absorption rate.

$$[\text{HNA-B}] = \frac{k_A A_0}{(k_A - k_{d_2})}\left(e^{-k_{d_2}t} - e^{-k_A t}\right) \quad \text{(Eq. 1)}$$

$$k_{E_2} = \frac{k_{d_2}\,[\text{HNA-U}]_\infty}{A_0} \quad \text{(Eq. 2)}$$

$$k_{M_4} = \frac{k_{d_2}\,[\text{HNAG-U}]_\infty}{A_0} \quad \text{(Eq. 3)}$$

$$k_{M_4} = \frac{k_{d_2}\,[\text{DA-U}]_\infty}{A_0} \quad \text{(Eq. 4)}$$

EXPERIMENTAL

Medication and Sampling Protocol.—The sodium salt of HNA was given in hard gelatin capsules

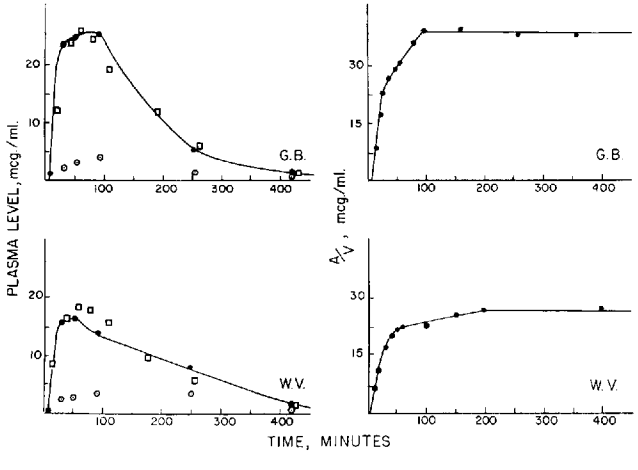

Fig. 1.—Plasma levels and A/V as a function of time. Key: ●, hydroxynalidixic acid; ⊙, hydroxynalidixic acid glucuronide; □, calculated values for hydroxynalidixic acid. Initials identify subjects.

(equivalent to 500 mg. of the acid per capsule) to 5 healthy subjects in a 1-Gm. dose (2 capsules) after overnight fasting. Food, but not water, was withheld until 3 hr. postmedication. Blood samples were taken after 0, 10, 30, 50, 90, 250, and 420 min. after drug administration. Urine collections were made at 0, 8, 24, and 48 hr. after administration of the drug. Plasma and urine concentrations of HNA and HNAG were determined in each sample using a previously published method (1). DA also was determined in the urine samples as previously described (1).

METHODS OF CALCULATION

The rates of availability of HNA to the plasma were determined by the method of Wagner and Nelson (5). Typical plots of A/V values are shown in Fig. 1 and represent the cumulative amount absorbed/apparent volume of distribution as a function of time. Individual disappearance rate constant values (k_{d_2}) were obtained from semilogarithmic plots of plasma hydroxynalidixic acid *versus* time. These rate constants multiplied by the cumulative area under experimental plasma level curves (Fig. 1) at different times, plus the plasma level at each time, gives A/V as defined by

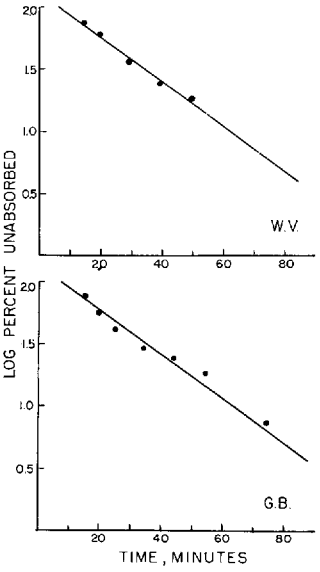

Fig. 2.—Log per cent hydroxynalidixic acid unabsorbed as a function of time. Initials identify subjects.

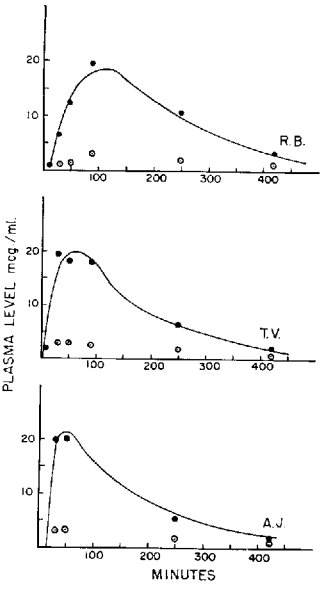

Fig. 3.—Plasma levels as a function of time. Key: ●, experimental hydroxynalidixic acid; ⊙, experimental hydroxynalidixic acid glucuronide; —, calculated curves for hydroxynalidixic acid. Initials identify subjects.

Wagner and Nelson. With these values, the per cent of the drug unabsorbed at different times was calculated. Availability rate and lag time were determined from graphs of log per cent unabsorbed as a function of time as shown in Fig. 2 and as listed in Table I.

Individual values for the excretion rate constant (k_{E_2}) and metabolic rate constants (k_{M_3} and k_{M_4}) were calculated using Eqs. 2, 3, and 4. It was found, as expected, that the 0–48 hr. urinary excretion amounts would represent infinity values for HNA-U, HNAG-U, and DA-U.

From $[A/V]_\infty$ values, the apparent volumes of distribution were calculated and are expressed in Table I as a percentage of body weight.

RESULTS AND DISCUSSION

Theoretical curves of HNA plasma levels *versus* time were calculated using Eq. 1 and the individual parameters. In order to obtain concentration values, HNA-B was divided by the individual's apparent volume of distribution. Excellent agreement was obtained between the theoretical plasma level values based on the model and the experimental values as shown in Figs. 1 and 3. This indicates that small deviations from the first-order rate process (*e.g.*, Fig. 2, G.B.) as measured from the experimental curve are not of great significance.

TABLE I.—KINETIC PARAMETERS FOR SODIUM HYDROXYNALIDIXATE

Subject	% Urinary Recovery	Lag Time, min.	k_A (min.$^{-1}$) × 10^3	k_{d_2} (min.$^{-1}$) × 10^3	k_{E_2} (min.$^{-1}$) × 10^3	k_{M_3} (min.$^{-1}$) × 10^3	k_{M_4} (min.$^{-1}$) × 10^3	Apparent Vol. of Distribution as % of Body Wt.
A.J.	105	13	70	7.37	1.44	4.30	1.99	45.9
G.B.	91	8	41	7.88	2.94	3.50	0.74	40.8
T.V.	97	5	39	7.70	2.39	3.78	1.31	55.5
W.V.	100	6	40	7.00	0.96	4.16	1.44	52.3
R.B.	78[a]	10	17	7.37	...	...	...	41.5
Mean values	98	8	42	7.46	1.93	4.05	1.37	49.0

[a] Incomplete urine collections.

The short lag time of 8 ± 1.5 min. (±S.E.) shows the rapid appearance of the drug in the plasma. That the drug is also rapidly absorbed is apparent from the high values for k_A. Sodium salts of acidic drugs usually have fast dissolution rates (6), and in the case of nalidixic acid (NA), the sodium salt had a shorter lag time and greater availability rate than the acid in capsule form (2). The mean lag time of 8 min. for the sodium salt of HNA (Table I) is very similar to the previously reported mean lag time of 3 min. for the sodium salt of NA (2). Also, the mean availability rate constant for HNA (sodium salt) of 0.042 $min.^{-1}$ is identical to this rate constant for NA (sodium salt) (2). This similarity in lag time and availability rate constant between the sodium salts of NA and HNA is related to their similar pKa, dissolution rate, and chemical structure.

All 5 subjects showed remarkably similar disappearance rate constants (k_{d_2}, range from 7.00–7.88) with a threefold difference in excretion rate constants (k_{E_2}, range from 0.96–2.94). A high excretion rate constant was compensated by a low metabolic rate constant (k_{M_3} and k_{M_4}) and vice versa. This fact could be predicted from the model because of the small variation in disappearance rates.

Glucuronide formation (k_{M_3}) is 2 to 5 times faster than the oxidative formation of the dicarboxylic acid (k_{M_4}). Hydroxynalidixic acid excretion (k_{E_2}) is slower in all cases than the formation of glucuronide (k_{M_3}), and in 2 cases (subjects A. J. and W. V.) slower than the formation of the dicarboxylic acid (k_{M_4}). This high rate of glucuronide formation plus the observed low glucuronide plasma levels (Figs. 1 and 3) indicates that HNAG has a large volume of distribution and/or a rapid excretion rate.

A relatively high apparent volume of distribution is evident for HNA [49.0 ± 3.0% (±S.E.)], indicating that it is distributed in extravascular compartments. The data of McChesney and coworkers (1) of tissue levels in dogs and monkeys showed that NA and HNA have a relatively uniform tissue distribution. This is particularly significant since nalidixic acid is used to treat infections which are localized in tissues.

CONCLUSION

A pharmacokinetic model for hydroxynalidixic acid has been presented and has been shown to be applicable in 5 subjects after oral administration of the sodium salt in capsule form. The good fits of theoretical plasma level curves with the actual data support the definitions of such parameters as lag time, k_A, k_{d_2}, and V_D. Calculated rate constants for excretion and metabolism will be used in a subsequent article in which an extensive model for nalidixic acid will be assumed.

APPENDIX

Expressions relating the amounts of DA-U, HNAG-U, and HNA-U as a function of time are presented. By letting time (t) approach infinity, equations are obtained which enable the calculation of metabolic constants (k_{M_3} and k_{M_4}) and the excretion constant (k_{E_2}) without making any assumptions about the relative values of consecutive rate constants.

These equations were derived from Scheme I where the rate constants are assumed to be first order and the compartments are defined as the (a) GI tract, (b) body or apparent volume of distribution, and (c) urine. Definitions of terms are stated under *Theoretical*.

$$[\text{HNA-U}] = \frac{k_{E_2}k_A A_0}{(k_A - k_{d_2})}\left(\frac{e^{-k_A t}}{k_A} - \frac{e^{-k_{d_2}t}}{k_{d_2}}\right) + \frac{k_{E_2}A_0}{k_{d_2}} \quad \text{(Eq. 1}a\text{)}$$

$$[\text{HNAG-U}] = \frac{k_{U_1}k_{M_3}k_A A_0}{(k_A - k_{d_2})(k_{d_2} - k_{U_1})}\left(\frac{e^{-k_{d_2}t}}{k_{d_2}} - \frac{e^{-k_{U_1}t}}{k_{U_1}}\right) - \frac{k_{U_1}k_{M_3}k_A A_0}{(k_A - k_{d_2})(k_A - k_{U_1})}\left(\frac{e^{-k_A t}}{k_A} - \frac{e^{-k_{U_1}t}}{k_{U_1}}\right) + \frac{k_{M_3}A_0}{k_{d_2}} \quad \text{(Eq. 2}a\text{)}$$

$$[\text{DA-U}] = \frac{k_{U_3}k_{M_4}k_A A_0}{(k_A - k_{d_2})(k_{d_2} - k_{U_3})}\left(\frac{e^{-k_{d_2}t}}{k_{d_2}} - \frac{e^{-k_{U_3}t}}{k_{U_3}}\right) - \frac{k_{U_3}k_{M_4}k_A A_0}{(k_A - k_{d_2})(k_A - k_{U_3})}\left(\frac{e^{-k_A t}}{k_A} - \frac{e^{-k_{U_3}t}}{k_{U_3}}\right) + \frac{k_{M_4}A_0}{k_{d_2}} \quad \text{(Eq. 3}a\text{)}$$

It is apparent that when time (t) is permitted to approach infinity, the above equations reduce to their last terms and give on rearrangement Eqs. 2, 3, and 4 of the text.

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Effect of Inert Tablet Ingredients on Drug Absorption I

Effect of Polyethylene Glycol 4000 on the Intestinal Absorption of Four Barbiturates

By PARVINDER SINGH*, J. KEITH GUILLORY†, THEODORE D. SOKOLOSKI‡, LESLIE Z. BENET, and V. N. BHATIA

The effect of polyethylene glycol 4000 on dissolution and absorption rates of 4 barbiturates has been studied. It has been shown that in the case of phenobarbital, which formed a complex of reduced solubility with polyethylene glycol 4000, the dissolution and intestinal absorption rates of the drug were reduced. The other 3 barbiturates did not interact with the polyethylene glycol 4000, and its presence did not affect their dissolution and absorption rates. The reduction in the absorption rate of the phenobarbital was closely related to the reduced dissolution rate of the complex, although only the phenobarbital was shown to be absorbed through the gut. It was also shown that the absorption rate of the phenobarbital from the complex was independent of the degree of dissociation of the latter in the mucosal fluid. An hypothesis is proposed to explain that phenomenon.

IN THE PREPARATION of medicinal tablets, a number of so-called inert ingredients are used as adjuvants. These adjuvants are used as binding agents, disintegrators, lubricants, diluents, or coating materials. Since the adjuvants often constitute a considerable portion of a tablet (in some cases as much as 30% or more), there is good reason to believe that they might influence drug availability and absorption in those instances where drug–adjuvant interactions occur. This point of view has been expressed in numerous reports (1, 2). More recently, studies have been concerned specifically with quantifying the effects of selected complexations on drug absorption rates (3, 4).

The objectives of this study are to determine the influence of 8 selected adjuvants on a group of selected drugs with respect to (*a*) the occurrence of interactions between the adjuvants and the drugs, (*b*) the effect of interactions on the dissolution rates of the drugs, and (*c*) the effect of the interactions on the absorption rate of the drugs.

The adjuvants selected for this study have been picked because they are among the ones frequently used in the manufacture of tablets. They are: polyethylene glycols, sodium carboxymethylcellulose, sodium alginate, cation-exchange resins, starch, acacia, cellulose acetate phthalate, and polyvinylpyrrolidone.

Twelve drugs have been chosen for this study. These drugs have been selected because either they or closely related chemicals are frequently used in tablet form, and because, as a group, they provide a wide range of physical properties including solubility, partition coefficient, and pK useful for this study. The 12 drugs are: barbital, phenobarbital, pentobarbital, barbituric acid, salicylamide, salicylic acid, benzyl penicillin, caffeine, acetanilide, phenacetin, sulfadiazine, and sulfathiazole.

This first report deals with the descriptions of the methods and procedures as well as the study of the effect of the adjuvant, polyethylene glycol 4000 (hereafter referred to as PEG 4000), on the dissolution and absorption of 4 barbiturates: barbital, phenobarbital, pentobarbital, and barbituric acid. In general, the procedure consisted of the following steps. (*a*) Determination of any complex formation between the drugs and the adjuvant by the phase solubility method. (*b*) Determination of the dissolution rates of pure drug tablets in pH 5.3 buffer, both in the presence and in the absence of the adjuvant. (With phenobarbital where an insoluble complex could be isolated, the dissolution rate of the pure complex tablets was studied.) (*c*) Determination of the absorption rate of the drug by the "everted intestinal sac" technique, both in the presence and in the absence of the adjuvant. (In the case of phenobarbital where an insoluble complex could be isolated, the absorption rate of the complex was studied.)

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‡ Present address: College of Pharmacy, Ohio State University, Columbus.

EXPERIMENTAL

Reagents.—Recrystallized phenobarbital U.S.P., m.p. 175–176°; recrystallized pentobarbital U.S.P. m.p., 128–129°, (prepared from sodium pentobarbital); recrystallized barbital N.F., m.p. 188–190°; recrystallized barbituric acid (Eastman Kodak); PEG 4000 (Union Carbide Chemicals Co.); isotonic phosphate buffer,[1] pH 5.3; isotonic phosphate buffer (5), pH 7.4; sodium borate buffer (0.05 *M*), pH 9.1.

Complexation.—All interaction studies between PEG 4000 and the barbiturates under consideration were carried out in a manner similar to that used by Higuchi and Lach (6). An excess of the barbiturate was placed in each of a series of 125-ml. glass-stoppered flasks and varying amounts of PEG 4000 added. Specifically, 250 mg. each of phenobarbital and pentobarbital, 500 mg. of barbital, and 1.9 Gm. of barbituric acid were used in each flask in these studies. Fifty milliliters of isotonic phosphate buffer solution (pH 5.3) was added to each flask, and the contents were equilibrated in a mechanical shaker bath at 37° for 24 hr.

Measured aliquot portions were removed and analyzed for their barbiturate content in a Beckman DB recording spectrophotometer. (The absorption maxima for phenobarbital, barbital, and pentobarbital were taken to be 240 mμ and for barbituric acid 258 mμ.) All spectrophotometric analyses were conducted in a sodium borate buffer having a pH of 9.1.

Manufacture of Tablets.—Cylindrical tablets of the pure drug were compressed in a single punch Colton machine using $^{9}/_{32}$ in. flatfaced punches. Tablet hardness ranged between 5 and 7 on the Strong-Cobb scale.

Phenobarbital tablets weighed 190 mg., pentobarbital 155 mg., barbital 180 mg., and barbituric acid 200 mg.

Dissolution.—The barbiturate tablets were firmly fixed into cylindrical Plexiglas containers with the aid of paraffin wax in such a way that only one surface of the tablet was exposed to the solution. [This is a variation of the technique first introduced by Nelson (7).] The Plexiglas disks were weighted down with a metal plate which prevented them from overturning or sliding during an experimental run (Fig. 1). The tablets were so fixed that their upper surfaces were level with those of the disks. Two such affixed tablets were placed in each of a set of 250-ml. glass-stoppered flasks. One hundred milliliters of isotonic phosphate buffer (pH 5.3) was added to each flask, and the flasks were placed in a constant-temperature (37°) shaker bath and mechanically stirred at 120 strokes/min.

Aliquot portions of the solution were removed at fixed time intervals and analyzed spectrophotometrically for their barbiturate content.

Dissolution in the Presence of PEG 4000.—The experimental procedure was similar to that used for pentobarbital, barbital, and barbituric acid (the 3 barbiturates that did not complex with PEG 4000) as described above, except that a known amount of PEG 4000 was added to the solution (0.880 Gm./100 ml. of PEG 4000 in each flask).

[1] One liter contains 0.568 Gm. of anhydrous disodium hydrogen phosphate, 13.248 Gm. of sodium phosphate, monobasic, and 3.40 Gm. of sodium chloride.

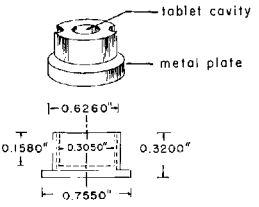

Fig. 1.—A Plexiglas disk with tablet cavity used to hold tablets for dissolution study.

With phenobarbital, which formed an insoluble complex in the presence of PEG 4000, a different procedure was adopted. To a saturated solution of phenobarbital in pH 5.3 buffer, sufficient PEG 4000 was added so that the ratio of adjuvant to drug was identical to the ratio found in the complexation studies (*vide infra*). Specifically, 77.38 mg. of PEG 4000 was added to each 100 ml. of a pH 5.3 buffered solution of phenobarbital having a concentration of 170 mg. %. The phenobarbital–*polyethylene glycol 4000 complex* (hereafter referred to as PB–PEG complex) was allowed to form over a period of 24 hr., filtered, and dried, m.p. 145–147°. The dry powder was then compressed into cylindrical tablets having the same specifications as the drug tablets and weighing 175 mg. The tablets were fixed into the Plexiglas disks, and the dissolution study was conducted. Spectrophotometric determinations were carried out by measuring the phenobarbital equivalent of the complex in solution. It should be noted that the presence of PEG 4000 had no effect on the absorption maxima of the barbitals, and that the pH of all solutions, containing either pure drug or the drug in the presence of PEG 4000, remained constant at pH 5.3.

Absorption.—The "everted intestinal sac" technique of Wilson and Wiseman (8) was adopted in all absorption studies conducted in the present series. Female Sprague-Dawley rats weighing about 250 Gm. were starved for 24 hr. prior to the experiments. The small intestine was freed of all adhering tissue, removed, and cut into 6-cm. segments. No more than 5–6 segments were used from any rat, and these segments were continually bathed in pH 5.3 buffered medium which was aerated with oxygen. Preliminary experiments showed that under our experimental conditions, there was no difference in absorption rates obtained from segments taken from different parts of the small intestine. This is corroborated by Schanker *et al.* (9) in the case of perfused rat intestines. Once cut, the segments were everted and sacs containing 0.4 ml. of isotonic 7.4 buffer were prepared by established techniques. The sacs were kept in a aerated pH 5.3 buffered medium.

One segment was introduced into each of a series of 250-ml. glass-stoppered flasks containing 100 ml. of a solution of known strength of a barbiturate drug. The flasks were placed in a shaker bath at 37°. Before starting the experiment, the air in the flasks was replaced by oxygen.

Corresponding to each sample flask containing the barbiturate solution, a control flask was also run simultaneously. The contents of the control flask were similar to the sample flask except that it did not contain the drug.

The flasks were mechanically stirred at 120 strokes/min., and 2 flasks (a sample and a control)

were removed at regular time intervals. The 2 intestinal sacs were removed from solution, wiped dry with tissue paper, punctured, and 0.16 ml. of the solution contained in each was removed and diluted to an appropriate extent with pH 9.1 buffer, so that the barbiturate content of each sample could be analyzed spectrophotometrically.

Absorption in the Presence of PEG 4000.—With pentobarbital, barbital, and barbituric acid the experiments described above were repeated except that a known weight of PEG 4000 was added to both the sample and control flasks (0.880 Gm./100 ml. of PEG 4000 in each flask).

With phenobarbital, however, the solution in the flask was prepared by placing a weighed quantity of the powdered complex described above into 100 ml. of phosphate buffer (pH 5.3). Initially an attempt was made to have the amount of phenobarbital available (170 mg. %) correspond to the amount used in the absorption studies run without PEG; therefore, 247.38 mg. of the complex (each 247.38 mg. of complex contained 170 mg. of phenobarbital and 77.38 mg. of PEG 4000) was added to 100 ml. of the 5.3 buffer. Since the complex only has a limited solubility (61.7 mg. % measured as the phenobarbital content[2]) an excess of the complex was always present as a solid in all of the sample flasks. Absorption studies were also run at lower complex concentrations (48.0 mg. % and 25.8 mg. % measured as the phenobarbital content), where, in these cases, all of the complex present was in solution.

RESULTS

Complexation.—Phenobarbital was the only one of the 4 barbiturates which complexes with PEG 4000. The complex formed in this case was an insoluble one, the ratio being 1:2.4 (*i.e.*, 1 Gm. molecular weight of phenobarbital and 2.4 Gm. equivalents of the ether linkages of PEG 4000). The data for the complexation are illustrated in Fig. 2. It should be noted that Higuchi and Lach (6) obtained a ratio of 1:2 for this complexation; however, this value was obtained at a different temperature (30°) and in a nonbuffered system at a different pH.

Dissolution.—Figure 3 shows that the dissolution rate of the PB–PEG complex differs from that of phenobarbital by a factor of about 3. (Rate for phenobarbital was 0.208 mg. %/min., and rate for PB–PEG complex was 0.07 ± 0.002 mg. %/min.) The dissolution rates of pentobarbital, barbital, and barbituric acid were not affected by the presence of PEG 4000. An example of this (barbital) is shown in Fig. 4. The dissolution rate values for all 4 drugs, as determined under the authors' experimental conditions, are given in Table I. (In each case, the method of least squares was used to calculate the rates. Each rate listed is a statistically treated average of 4–6 experiments.)

Absorption.—Figure 5 shows the absorption rates of the free phenobarbital and the PB–PEG complex (when excess complex was present). The absorption rate of the complex is about one-third that of pure phenobarbital, but these values must be

[2] All spectrophotometric measurements were made with reference to phenobarbital in solution. Therefore, the abscissas of Figs. 2 and 5 and the ordinate of Fig. 7 should be read as concentration of phenobarbital.

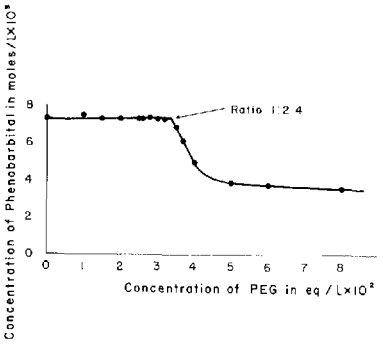

Fig. 2.—Phase diagram showing the effect of varying concentrations of PEG 4000 on the solubility of phenobarbital at 37° and at a pH of 5.3.

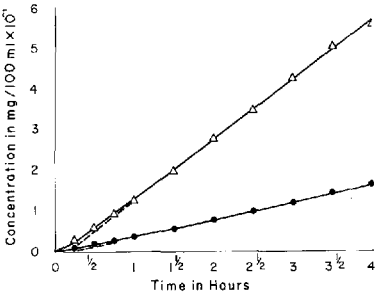

Fig. 3.—Dissolution rate of phenobarbital (△) and PB–PEG complex tablets (●). Average rate: △, 0.208 mg.%/min.; ●, 0.07 ± 0.002 mg. %/min.

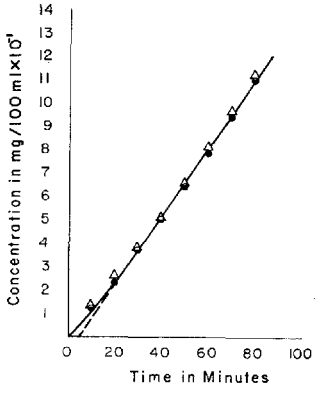

Fig. 4.—Dissolution rate of barbital tablets. Key: △, without PEG; ●, with PEG. Average rate: 1.33 mg. %/min. Range of error: △, ± 0.015 mg. %/min.; ●, ± 0.03 mg. %/min.

TABLE I.—DISSOLUTION RATES OF THE FOUR BARBITURATES

Drug	*Dissolution* Rate, mg. %/min.	Dissolution Rate in Presence of PEG 4000, mg. %/min.
Pentobarbital	0.140 ± 0.004	0.140 ± 0.005
Barbital	1.330 ± 0.015	1.330 ± 0.030
Barbituric acid	3.960 ± 0.400	3.960 ± 0.210
Phenobarbital	0.208	...
PB–PEG complex	...	0.070 ±0.002

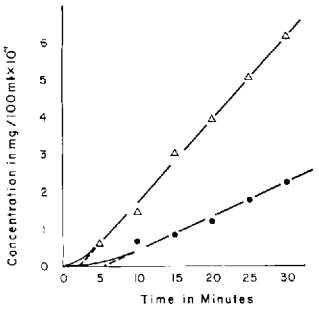

Fig. 5.—Absorption rate of phenobarbital (△) and of the PB–PEG complex (●) (when excess complex present). Average rate: △, 2.36 ± 0.17 mg. %/min.; ●, 0.81 ± 0.14 mg. %/min.

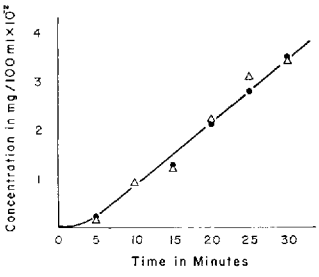

Fig. 6.—Absorption rate of barbital. Key: △, without PEG; ●, with PEG. Rate: 11.7 mg. %/min.

TABLE II.—ABSORPTION RATES OF THE FOUR BARBITURATES

Drug	Absorption Rate, mg. %/min.	Absorption Rate in Presence of PEG 4000, mg. %/min.
Pentobarbital (initial concn. = 115 mg./100 ml.)	1.91	1.90
Barbital (initial concn. = 807 mg./100 ml.)	11.7	11.7
Barbituric acid (initial concn. = 1,580 mg./100 ml.)	29.87	31.48
Phenobarbital (initial concn. = 168 mg./100 ml.) (av. of 5 expt.)	2.36 ± 0.17	...
PB–PEG complex[a] (initial concn. = 61.7 mg./100 ml.) (av. of 5 expt.)	...	0.81 ± 0.14
(initial concn. = 48.0 mg./100 ml.) (av. of 3 expt.)	...	0.52 ± 0.09
(initial concn. = 25.8 mg./100 ml.) (av. of 6 expt.)	...	0.31 ± 0.04

[a] Measured as phenobarbital equivalents.

related to the concentration of phenobarbital equivalents in the outside solution. For free phenobarbital the outside concentration was 170 mg. %, while the concentration of phenobarbital equivalents was only 61.7 mg. % when 247.38 mg. of the PB–PEG complex was added to 100 ml. of buffer. The absorption rates of the 3 other barbiturates were not affected by the presence of PEG 4000. An example of this (barbital) is shown in Fig. 6. The absorption rates of all 4 barbiturates, in addition to the absorption rates of the complex at 3 different outside concentrations, are given in Table II. In each case, the method of least squares was used to calculate the rates.

DISCUSSION

Schanker *et al.* (9) have stated that most drugs are absorbed by passive diffusion. This hypothesis applied to the drugs in this study. Figure 7 shows that in absorption studies carried out by using phenobarbital solutions of different concentrations (closed circles), the absorption rate bears a linear relationship to the initial concentration of the solutions. The open circles on this plot correspond to average absorption rates found for the 3 different concentrations of PB–PEG complex listed in Table II.

A modification of Fick's first law of diffusion has been employed by Higuchi (10) to explain the phenomenon of percutaneous absorption. The equation can also be applied to absorption through the small intestine. The rate of absorption is then given by

$$\frac{dq}{dt} = \frac{DA}{L}(\text{P.C.})\,C \qquad \text{(Eq. 1)}$$

where dq/dt is the absorption rate, D is the diffusion coefficient, A is the cross-sectional area of the gut wall exposed to the drug, L is the thickness of the gut wall, C is the concentration of the drug solution outside the everted sac, and P.C. is the partition coefficient of the barbiturate between the wall and the drug solution.

Similar equations can be written to describe both the phenobarbital absorption rate and the PB–PEG complex. The ratio of the absorption rates is given by

$$\frac{\text{absorption rate of phenobarbital}}{\text{absorption rate of the PB–PEG complex}} = \frac{D_{PB}\,A/L\,(\text{P.C.}_{PB})C_{PB}}{D_{PB-PEG}A/L\,(\text{P.C.}_{PB-PEG})\,C_{PB-PEG}} \qquad \text{(Eq. 2)}$$

Equation 2 is only applicable if the PB–PEG complex can be shown to diffuse through the gut membrane as an intact complex. This seems unlikely in light of recent work which shows that PEG 4000 does not pass through the biological membrane (11). In addition, experiments were run with uneverted whole (40 cm.) rat guts. These guts were first flushed with distilled water, and then filled with a solution of the PB–PEG complex in distilled water (60 mg. % measured as phenobarbital equivalents). The guts were continually aerated as previously described and were placed in flasks containing 25 ml. of distilled water. The flasks were shaken at 37° for 1 hr., after which the gut was discarded, and the outside solution was centrifuged and filtered so as to remove insoluble gut particles. The solution was concentrated by evaporation, and the precipitate was dried overnight at 60°. The melting point of the solid was then observed on a microscope equipped with a hot stage. Although the concentrate was not purified,

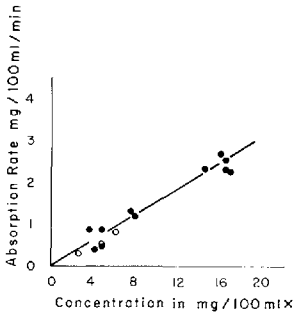

Fig. 7.—Plot showing the linear dependence of the absorption rate on the initial concentration of phenobarbital in the solution surrounding the everted sac. Key: ●, absorption runs with pure phenobarbital; O, average values obtained for absorption runs with excess complex (61.7 mg. % phenobarbital equivalent) and for a series of runs with all complex in solution (48.0 mg. % and 25.8 mg. %).

particles were seen to melt beginning at 173°.[3] Similar results were obtained when pure phenobarbital was placed in the large uneverted gut segments. Although the above experiments were not run under the same conditions as the absorption studies, it seems reasonable to conclude that only phenobarbital passes through the gut wall.

Under this assumption Eq. 2 will reduce to Eq. 3, since D, A, L, and (P.C.) are the same for both the solutions of pure phenobarbital and PB–PEG complex.

$$\frac{\text{absorption rate of phenobarbital}}{\text{absorption rate of phenobarbital from complex}} = \frac{C_{PB}}{C_{PB-PEG}\ \text{(measured as PB equiv.)}} \quad \text{(Eq. 3)}$$

If Eq. 3 is valid, the rate of absorption of phenobarbital from the complex will be a function of the concentration of phenobarbital in solution as the complex, and, therefore, the rate of absorption of the complex should follow the same linear relation with concentration as does the rate of absorption of pure phenobarbital. This relation is shown in Fig. 7, and seems to be true within the experimental error expected from biological work.

It should be pointed out that Fig. 7 implies that the rate of absorption of phenobarbital from the complex is a function of the *total* phenobarbital in solution, not just the concentration of the free phenobarbital resulting from dissociation of the complex. This was checked by running everted gut absorption studies with solutions containing PB–PEG complex (at the 3 concentrations studied) and various concentrations of excess PEG 4000 (up to 1:1 weight–weight, complex to PEG). If absorption was a function of the concentration of free phenobarbital resulting from complex dissociation, the rate of absorption should decrease as excess PEG 4000 is added. In all cases the rates of absorption of phenobarbital from the complex were similar to the rates reported in Table II when no excess PEG was present, indicating that the rate of absorption of phenobarbital from the complex is, indeed, a function of the total phenobarbital in solution, and is not dissociation related.

In light of the evidence presented it would seem that the gut is acting as a "dissociating membrane," causing the complex to dissociate and allowing the diffusible phenobarbital to be absorbed. This hypothesis has previously been proposed by Levy and Matsuzawa (4) to explain the fact that eosin-B was absorbed through the cannulated everted intestine at the same rate from solutions containing the free dye as from solutions containing the dye (at an identical concentration) complexed with atropine and pheniramine. In the previous work (4) the complexes dissociated into 2 components, both of which passively diffused through the intestine, while in the present study the complex dissociates to give 1 passively diffusible component, and 1 component which cannot pass through the intestinal membrane.

Although the above reasoning is hypothetical, it appears that complex dissociation does take place either at the gut surface or in an outer layer of the gut, since the absorption rates are related to total phenobarbital concentration. Additional tests of the above hypothesis are being carried out in this laboratory on a PEG-sulfathiazole complex which increases the total solubility of the drug.

CONCLUSIONS

This portion of the study has demonstrated that the absorption of phenobarbital in the presence of PEG is markedly decreased and that the decrease is probably a function of the decreased solubility of the PB–PEG complex. It has also been demonstrated that the dissolution rate of the complex is only about one-third the rate for pure phenobarbital. As has been pointed out in the literature (12, 13), dissolution is usually the rate-limiting step in attaining therapeutic blood levels. Thus, a PB-PEG complex would markedly decrease the therapeutic efficacy of phenobarbital at both the level of availability rate (dissolution) and at the level of maximum availability attainable (solubility). It was also observed that the dissolution and absorption rates of 3 other closely related drugs were not affected when the same adjuvant showed no interaction with them. Thus, the work so far supports the validity of the hypothesis that tablet adjuvants may not be as inert in their effect as has been traditionally assumed.

SUMMARY

1. The effect of a tablet adjuvant (polyethylene glycol 4000) on the dissolution and absorption rates of 4 barbiturates has been studied.

2. It has been shown that in the case of pentobarbital, barbital, and barbituric acid, all of which did not interact with polyethylene glycol 4000, the dissolution and absorption rates were not affected.

3. Phenobarbital formed a complex of reduced solubility with polyethylene glycol 4000. The effect of this interaction was to reduce the dissolution and absorption rates of the drug.

4. The reduction in the absorption rate of phenobarbital was found to be closely related to the reduced solubility of the complex, and although it was

[3] Phenobarbital, m.p. 174–178°; PEG 4000 congeals at 53–56°; PB–PEG complex, m.p. 145–147°.

demonstrated that only phenobarbital was absorbed through the gut, it was also shown that the rate of absorption was independent of the degree of dissociation of the complex in the mucosal fluid. It has been proposed [after Levy and Matsuzawa (4)] that the intestinal membrane has a dissociating effect on the complex, allowing the phenobarbital to be absorbed, but preventing absorption of PEG.

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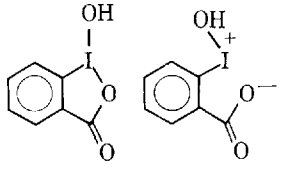
Chemistry and Biochemistry of Polyvalent Iodine Compounds V

Ionization of Heterocyclic Polyvalent Iodine Compounds

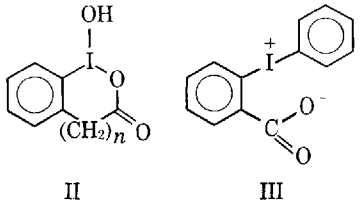
By WALTER WOLF, JAMES C. J. CHEN, and LAUREEN L. J. HSU

The ionization constants of several polyvalent compounds were determined and found to be consistent with the ionization of an hydroxyl group of polyvalent iodine. The pKa of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole is 7.35 ± 0.13, and those of its two immediate higher homologs are 7.54 ± 0.29 and 7.37 ± 0.17, respectively. The behavior of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole was studied in varying concentrations of sulfuric acid. Two protonation steps seem to occur, with apparent pKa values of −0.58 and −5.75, on the H_0 scale. The structural implications of these findings on the heterocyclic nature of the benziodoxole ring are discussed.

RECENT WORK on the structure of certain polyvalent iodine compounds (1, 2) confirms their heterocyclic nature. The structure of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole (I) has been unequivocally determined by X-ray crystallography (3). This study revealed a significant difference between the two I—O bonds; the intra-annular bond between iodine and oxygen is 2.30 Å. long, while the bond between iodine and the hydroxylic oxygen is 2.00 Å. This difference can be ascribed either to the steric strain of a 5-membered ring or to a strong ionic contribution to the iodine-oxygen (ring) bond. An alternative possibility, that a betaine type of ring (iodonium-carboxylate, I*a*) makes a significant contribution to the structure of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole had to be evaluated. The behavior of 2-carboxy-diphenyl iodonium (III), containing such a ring system, had been studied recently (4). The ionization constants of I and its homologs (II, $n = 1$ or 2) were investigated in order to provide further information on the structure of these iodine-containing heterocyclic rings, and to evaluate the possible contribution of betainic structures such as (I*a*). This study was conducted both in dilute aqueous



I Ia



II III

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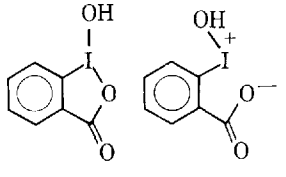

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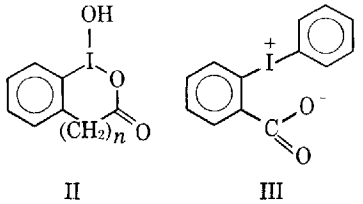

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solutions and in media of very high acidity (H_0 to -10).

EXPERIMENTAL

Products.—1,3 - Dihydro - 1 - hydroxy - 3 - oxo-1,2-benziodoxole was prepared by the method of Meyer and Askenasy (5) as modified by Wolf and Hsu (1). 3,4-Dihydro-1-hydroxy-3-oxo-1*H*-1,2-benziodoxin and 1,3,4,5-tetra-hydro-1-hydroxy-3-oxo-1,2-benziodoxepin were prepared as described previously (1, 2). Baker and Adams reagent grade sulfuric acid (95%) was used.

Methods.—The method used for the determination of the pKa in dilute aqueous solutions was that of Albert and Sarjeant (6), using a Cary 14 recording spectrophotometer, a Beckman DB recording spectrophotometer, and a Beckman-Gilford direct-reading spectrophotometer. The pH measurements were performed on a Photovolt 115 pH meter, which was standardized against Beckman buffers before each measurement. All measurements were conducted at 23 ± 3°.

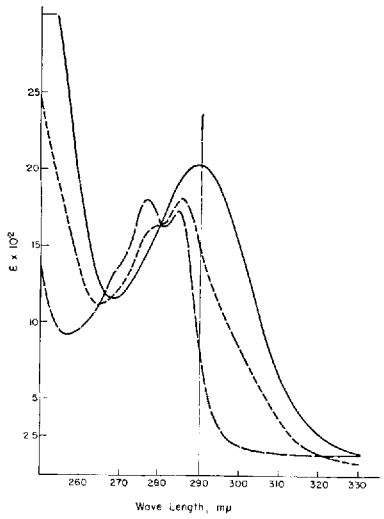


Fig. 1.—Absorption spectra of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole in $^1/_{15}$ *M* phosphate buffer. Key: ——, pH 11.19; - - -, pH 7.5; – – –, pH 1.85.

TABLE I.—MOLAR ABSORPTIVITIES OF POLYVALENT IODINE COMPOUNDS AND THEIR ANIONS[a]

Compd.		Anion[b]	Un-ionized Molecule[c]
1,3-Dihydro-1-hydroxy-3-oxo-1,2-benziodoxole	2900	2044	849
	2845	1915	1739
	2765	1267	1811
3,4-Dihydro-1-hydroxy-3-oxo-1,2-benziodoxin	3200	337	184
	2900	491	521
	2500	1717	1687
1,3,4,5-Tetrahydro-1-hydroxy-3-oxo-1,2-benziodoxepin	3200	326	24
	2900	496	809
	2500	1547	2619

[a] In phosphate buffers, 1/15 *M*. [b] pH 10–12. [c] pH 1–2.

The spectrophotometric method as modified by Davis and Geissman (7) was used to determine the ionization constants in the concentrated sulfuric acid solutions. Stock solutions of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole in 96% H_2SO_4 were prepared and their stability checked periodically. No apparent decomposition could be observed during the course of this work. The stock solutions were diluted, under refrigeration, to the desired final concentrations in 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole and sulfuric acid. The H_0 scale used was that of Paul and Long (8) for the 40–95% sulfuric acid region and that of Bascombe and Bell (9) for the 0–40% sulfuric acid concentration.

RESULTS AND DISCUSSION

The changes in the absorption spectra of a solution of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole in $^1/_{15}$ *M* phosphate buffers at pH 1.85, 7.5, and 11.19 are illustrated in Fig. 1. By using 290 mμ ($\lambda_{max.}$ for the anion, Table I) as the analytical wavelength, the pKa values listed in Table II were obtained.

Similar determinations for 3,4-dihydro-1-hydroxy-3-oxo-1*H*-1,2-benziodoxin and 1,3,4,5-tetrahydro-1-hydroxy-3-oxo-1,2-benziodoxepin are illustrated in Tables III and IV.

The pKa and ionization constants of the 3 heterocyclic polyvalent iodine compounds are listed in Table V.

For comparison, the pKa values, as reported in the literature for some related iodo compounds, are listed in Table VI.

The influence of the iodo group on the ionization of the benzoic and phenylacetic acids is seen to be strongest when the halogen is in the *ortho* position. The decrease in pKa has been ascribed to both inductive and resonance effects (10, 13), which could be enhanced in *o*-iodobenzoic acid by the participation of a betaine type of structure.

The introduction of a methylene group shields the carboxylate function from such effects. Thus, for *o*-iodophenylacetic acid, the ΔpKa is 0.27, compared with a ΔpKa of 1.34 for *o*-iodobenzoic acid. The ΔpKa values are 0.15 and 0.34 for the *m*-analogs, respectively. While no data are available for the corresponding iodophenylpropionic acids, it can be assumed that the introduction of the second methylene group would further shield the carboxylic acid group. [*Cf.* pKa of propionic acid, 4.88 (6).]

In contrast, the authors' results on the ionization constants of the related polyvalent iodine deriva-

TABLE II.—IONIZATION CONSTANTS DETERMINATION FOR 1,3-DIHYDRO-1-HYDROXY-3-OXO-1,2-BENZIODOXOLE AT 23 ± 3°C.

pH	d	$d_I - d$	$\frac{d_I - d}{d - d_M}$	$\log \frac{d_I - d}{d - d_M}$	pKa
6.80	0.355	0.295	3.470	0.5403	7.34
7.00	0.390	0.260	2.160	0.3345	7.33
7.15	0.445	0.205	1.170	0.0682	7.22
7.50	0.480	0.170	0.807	−0.0931	7.40
7.60	0.500	0.150	0.650	−0.1870	7.41

TABLE III.—IONIZATION CONSTANTS DETERMINATION FOR 3,4-DIHYDRO-1-HYDROXY-3-OXO-1*H*-1,2-BENZIODOXIN AT 23 ± 3°C.

pH	d	$d_I - d$	$\frac{d_I - d}{d - d_M}$	$\log \frac{d_I - d}{d - d_M}$	pKa
6.80	0.058	0.052	6.500	0.813	7.61
7.20	0.070	0.040	2.000	0.300	7.50
7.50	0.075	0.035	1.400	0.146	7.65
7.60	0.088	0.022	0.785	−0.105	7.59
7.95	0.100	0.010	0.200	−0.700	7.25

TABLE IV.—IONIZATION CONSTANTS DETERMINATION FOR 1,3,4,5-TETRAHYDRO-1-HYDROXY-3-OXO-1,2-BENZIODOXEPIN AT 23 ± 3°C.

pH	d	$d - d_I$	$\frac{d - d_I}{d_M - d}$	$\log \frac{d - d_I}{d_M - d}$	pKa
6.80	0.680	0.225	3.200	0.5050	7.31
7.20	0.560	0.215	1.950	0.2900	7.49
7.50	0.600	0.145	0.850	−0.0700	7.42
7.70	0.530	0.075	0.314	−0.5031	7.20

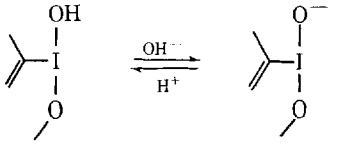
TABLE V.—pKa AND IONIZATION CONSTANTS OF THREE HETEROCYCLIC POLYVALENT IODINE COMPOUNDS

	pKa	Ionization Constant
1,3-Dihydro-1-hydroxy-3-oxo-1,2-benziodoxole	7.35 ± 0.13	$(2.2 \pm 0.5) \times 10^{-7}$
3,4-Dihydro-1-hydroxy-3-oxo-1*H*-1,2-benziodoxin	7.54 ± 0.29	$(3.4 \pm 1.6) \times 10^{-7}$
1,3,4,5-Tetrahydro-1-hydroxy-3-oxo-1,2-benziodoxepin	7.37 ± 0.17	$(2.3 \pm 0.8) \times 10^{-7}$

TABLE VI.—pKa VALUES OF SOME *o*-IODOPHENYL CARBOXYLIC ACIDS

Compd.	H	*o*-Iodo	*m*-Iodo	*p*-Iodo	Ref.
Benzoic acid	4.20	2.86	3.86	3.93	(10, 11)
Phenylacetic acid	4.31	4.04	4.16	...	(10, 12)
β-Phenyl propionic acid	4.66	...	...	...	(10)

tives fail to detect any such variations in their pKa values, and this suggests that the ionizable function is not located on a carboxylic acid. Instead, the authors suggest that a common ionizable function is present in the three heterocyclic polyvalent iodine systems, namely, the iodine-bonded hydroxyl group.



This function is independent, except for steric factors, of the number of methylene groups between the carbonyl group and the benzene ring.

Further arguments supporting this assignment can be made if we consider the pKa values of the proven betaine structures reported by Beringer (4), who studied the acidities of carboxydiphenyliodonium compounds in acetonitrile–water solutions at 24°. Under their conditions the apparent pKa values are 6.92 for benzoic acid, and 6.24, 6.45, and 6.64, respectively, for the *o*-, *m*-, and *p*-iodobenzoic acids, and 3.5 ± 1, 5.55, and 4.6 for the *o*-, *m*-, and *p*-phenyliodoniumbenzoic acids (2,3 and 4-carboxydiphenyliodonium). The iodonium substituted benzoic acids appear to be stronger acids than the corresponding iodo-benzoic acids under the conditions studied. Thus, while in all the above examples, the *o*-iodo or the *o*-iodonium derivatives were stronger acids than unsubstituted benzoic acid, the contrary is the case for 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole, which is a weaker acid than either benzoic or *o*-io-

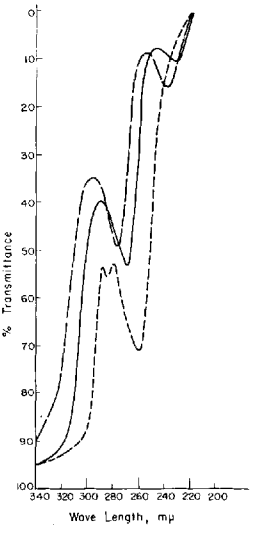


Fig. 2.—Absorption spectra of 1, 3-dihydro - 1 - hydroxy- 3-oxo - 1,2 - benziodoxole in sulfuric acid solution. Key: - - -, in 9.6% H_2SO_4; ——, in 48% H_2SO_4; – – –, in 86.3% H_2SO_4.

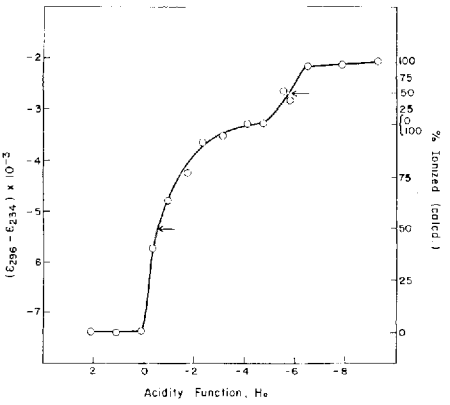


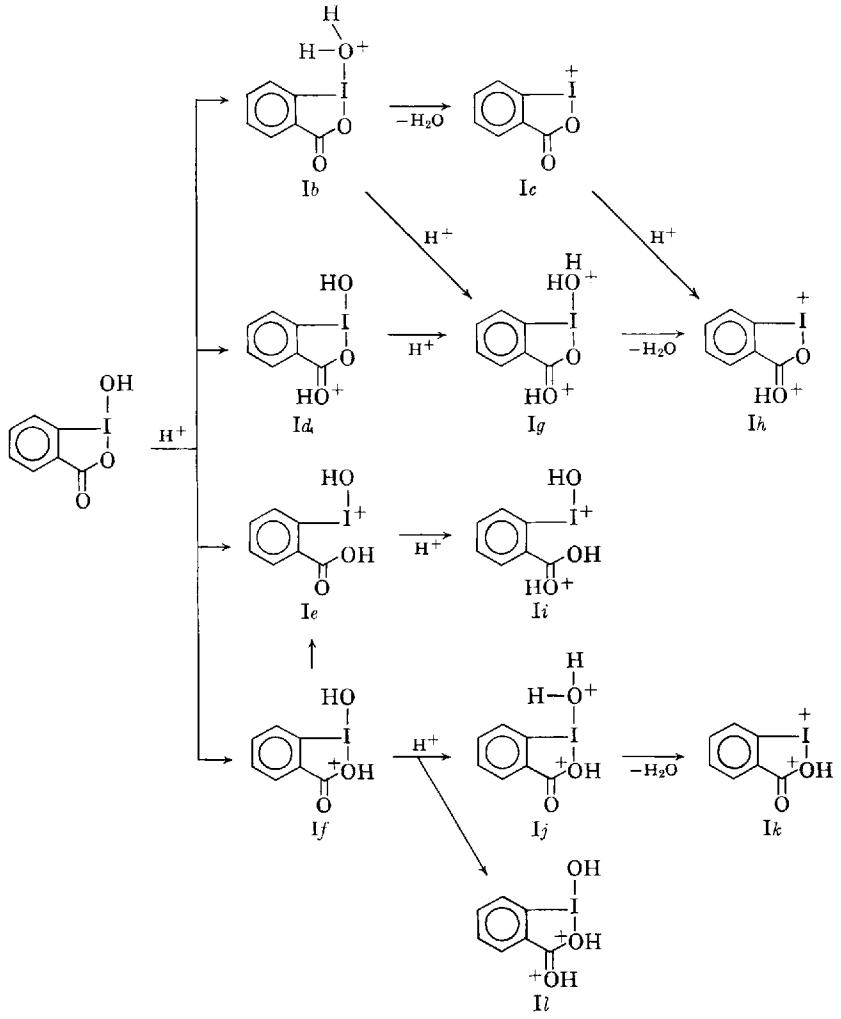
Fig. 3.—1,3-Dihydro-1-hydroxy-3-oxo-1,2-benziodoxole: acidity function *vs.* (ϵ 296–ϵ 234); points from spectral data, pKa's calculated from curve, −0.58 and −5.75.

dobenzoic acid. We can, therefore, safely assume that structures such as I*a* make only an extremely small, if any, contribution to 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole.

Carboxylic acids exhibit a second protonation in strongly acidic media (14), and the pKa's determined in the acidity function (H_0) region are −7.38, −7.78, −7.64, and −7.50 for benzoic acid, and its *o*-, *m*-, and *p*-iodo analogs, respectively. A single protonation step is observed, and the suggestion has been made that protonation occurs on the carboxylic oxygen atom rather than on the hydroxyl group (15).

The behavior of the benziodoxole ring in strongly acidic media is of interest, since this system provides a number of potential protonation sites. Particularly, it might be possible to observe if ring opening occurs or if a protonation of the carbonyl function, similar to that of other benzoic acids, is detectable.

The change in absorption of a solution of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole in various concentrations of sulfuric acid is illustrated in Fig. 2. A plot of $\Delta\epsilon$ (ϵ 296 − ϵ 234) *versus* H_0 is given in Fig. 3, and it shows a complex curve, which appears to have two inflection points at H_0 = −0.58 and at H_0 = −5.75. These results indicate



Scheme I

a two-step protonation. As a check of the method, the pKa of *o*-iodobenzoic acid was determined concurrently, yielding the value of −7.4 which is in good agreement with previously published data (12).

A first protonation of compound I could lead to structures I*b*, I*d*, I*c*, or I*f*; loss of water from I*b* will yield I*c*. (Scheme I.)

Of these structures for a single protonation, I*b* and I*f* are considered least likely. Protonation of a conjugated carbonyl function, such as that of benzophenone, occurs at pKa = −6.41 (16), and protonation of a lactone occurs at the carbonyl rather than the ether oxygen; an electronic shift would convert "structure" I*f* into I*e*. Examination of Fig. 2 reveals that protonation of compound I gives rise to a new peak at 256 mμ, while the doublet at 276–284 coalesces to a single peak at 288 mμ. By comparison, *o*-iodobenzoic acid shows a peak at 285 mμ which, in strong acid, shifts to 328 mμ. Thus, the 288 mμ peak of mono-protonated 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole could be indicative of the presence of a carboxylic acid function (I*e*). However, not enough is known about the spectral characteristics of iodonium compounds to rule out the possible contribution of structure I*d*.

The possibility of a sulfonation reaction appears unlikely, as dilution of the above concentrated sulfuric acid solutions resulted in an absorption spectra identical to that obtained by mild acidification of an aqueous solution.

The second protonation (H_0 = −5.75) may be associated with that of a carbonyl group, leading to structures such as I*g*, I*h*, I*i*, I*j*, or I*l*; loss of water could lead to I*k*, or I*h*. The shift of the absorption band from 288 mμ to 296 mμ is quite small. Structures I*j* and I*l* would contain a protonated lactone oxygen, and are therefore considered far less likely than structure I*i*, which represents a classically protonated carboxylic acid and is resonance stabilized. Although the pKa observed is significantly less than those recorded for other *o*-substituted benzoic acids (−6.78 to −7.78), the effect of an *o*-iodonium function must be taken into consideration. Thus, no conclusion can be made at present on the possible significance of these data on deciding between structures I*i* and I*h* for the doubly protonated species, or between I*e* and I*d* for the singly protonated molecule.

The ionization properties of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole and homologs thus support the heterocyclic nature of these compounds.

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Parameters for Absorption, Metabolism, and Elimination

By G. A. PORTMANN, E. W. McCHESNEY, H. STANDER, and W. E. MOORE

The absorption, metabolism, and excretion of nalidixic acid in man is illustrated by a model, and appropriate equations are derived. A total of 7 rate constants are calculated: 4 metabolic constants, 2 excretory constants, and 1 availability constant. All 5 components of the urine compartment and 2 components of the plasma compartment are measured. Comparisons between experimental and calculated values are good.

THE CONSTRUCTION of pharmacokinetic models, their utility in dosage form design, and the mathematical description of the fate of a drug in the body has been adequately treated by Wagner (1, 2), Nelson (3), Levy (4), and others (5).

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model for hydroxynalidixic acid (9) which is an active metabolite.

The present article gives further experimental results which permit the complete pharmacokinetic description of nalidixic acid in man. This model has been applied to blood levels and urinary excretion data obtained from two different tablet formulations.

THEORETICAL

Equations describing the change of each component in Scheme I with respect to time were derived, assuming total absorption and all rate processes to be first order. The recovery of the drug from the urine in terms of all forms detected analytically was found to be 95 ± 2% (± S. E.) indicating essentially total absorption in every case.

The equations used in the present study are listed below. Lag time is defined as the time interval between ingestion of the dosage form and the appearance of nalidixic acid in the plasma. A_0 is equal to the administered dose since complete absorption occurs. Time (t) is measured after the lag time interval. The nalidixic acid (NA) disappearance rate constant (k_d) is equal to the sum of $k_{E_1} + k_{M_1} + k_{M_2}$. The disappearance rate constant (k_{d_2}) for hydroxynalidixic acid (HNA) is the sum of $k_{E_2} + k_{M_3} + k_{M_4}$. The availability rate from the intestinal tract (k_A) is a resultant of such factors as gastrointestinal motility, dissolution rate, etc., and the absorption rate itself.

$$A = A_0 e^{-k_A t} \qquad \text{(Eq. 1)}$$

$$\text{NA-B} = \frac{k_A A_0}{(k_A - k_d)} (e^{-k_d t} - e^{-k_A t}) \qquad \text{(Eq. 2)}$$

$$\text{HNA-B} = \frac{k_{M_1} k_A A_0}{(k_A - k_d)(k_A - k_{d_2})} (e^{-k_{d_2} t} - e^{-k_d t}) - \frac{k_{M_1} k_A A_0}{(k_A - k_d)(k_A - k_{d_2})} (e^{-k_{d_2} t} - e^{-k_A t}) \qquad \text{(Eq. 3)}$$

$$\text{NA-U} = \frac{k_{E_1} A_0}{(k_d - k_A)} (1 - e^{-k_A t}) + \frac{k_{E_1} k_A A_0}{(k_d - k_A) k_d} (e^{-k_d t} - 1) \qquad \text{(Eq. 4)}$$

$$k_{E_1} = \frac{k_d [\text{NA-U}]_\infty}{A_0} \qquad \text{(Eq. 5)}$$

$$\text{HNA-U} = \frac{k_{E_2} k_{M_1} k_A A_0}{(k_A - k_d)(k_d - k_{d_2})} \left(\frac{e^{-k_d t}}{k_d} - \frac{e^{-k_{d_2} t}}{k_{d_2}} \right) - \frac{k_{E_2} k_{M_1} k_A A_0}{(k_A - k_d)(k_A - k_{d_2})} \left(\frac{e^{-k_A t}}{k_A} - \frac{e^{-k_{d_2} t}}{k_{d_2}} \right) + \frac{k_{E_2} k_{M_1} A_0}{k_{d_2} k_d} \qquad \text{(Eq. 6)}$$

$$[\text{HNA-U}]_\infty = \frac{k_{E_2} k_{M_1} A_0}{k_d k_{d_2}} \qquad \text{(Eq. 7)}$$

$$k_{M_2} = \frac{k_d [\text{NAG-U}]_\infty}{A_0} \qquad \text{(Eq. 8)}$$

$$[\text{HNAG-U}]_\infty = \frac{k_{M_3} k_{M_1} A_0}{k_d k_{d_2}} \qquad \text{(Eq. 9)}$$

$$[\text{DA-U}]_\infty = \frac{k_{M_4} k_{M_1} A_0}{k_d k_{d_2}} \qquad \text{(Eq. 10)}$$

$$k_{M_1} = \frac{k_d [\text{HNA} - \text{U} + \text{HNAG} - \text{U} + \text{DA} - \text{U}]^\infty}{A_0} \qquad \text{(Eq. 11)}$$

EXPERIMENTAL

Protocol.—Two tablet formulations,[1] both containing micropulverized nalidixic acid crystals, but with different tablet excipients, were given orally as a 1-Gm. dose (2 Caplets) to 8 subjects according to a crossover design, with 1 week between experiments. The drug was administered with water after overnight fasting. To maintain uniformity of absorption, food, but not water, was withheld until 3 hr. postmedication.

Blood samples were taken at 0, 20, 50, 80, 120, 270, and 420 min. postmedication. A 0- and 24-hr. urine sample was also collected. Previous experiments have shown essentially complete recovery in 24 hr.

Analytical Methods.—Nalidixic and hydroxynalidixic acids may be extracted from biological materials by means of toluene at pH 1–2, as previously described (7). Prior hydrolysis for 1 hr. at this pH releases both compounds from their glucuronide conjugates. It is possible to distinguish between NA and HNA on the basis of their differing extractabilities as a function of pH, as described below.

(*a*) Free (or total) naphthyridine is determined by extracting with toluene at pH 1.2 (0.1 *N* HCl), transfer from toluene to 0.5 *M* borate pH 9, and reading the fluorescence at 325/375 mμ after acidification as described previously (7). The results are calculated on the basis of NA standards carried through the same procedures; the recovery of NA, therefore, is 100%, while that of HNA is 134%. The latter figure is a result of 2 factors: the extractability of HNA under the conditions used in 77%, but, since its fluorescence on a weight basis is 172% of that of NA, a net value of 134% will be obtained for HNA read against NA standards.

(*b*) A second aliquot of the material to be analyzed (1–2 ml.) is diluted as necessary with an aqueous buffer of pH 5.63. (Composition of buffer per liter: *sodium chloride*, 10 Gm.; *potassium acid phosphate*, 12.25 Gm.; basic potassium phosphate, 1.75 Gm.) The solution is extracted with 30 ml. of toluene, of which 25 ml. is extracted with the 0.5 *M* borate, which is assayed in the usual way. Under these conditions the extractability of NA is 5.8 times that of HNA, but the relative fluorescence factor of 1.72 reduces the advantage in favor of NA to 3.4. When read against NA standards carried through the same steps, this method will recover 100% of the NA present, and 33% of the HNA. The following relationship, therefore, exists:

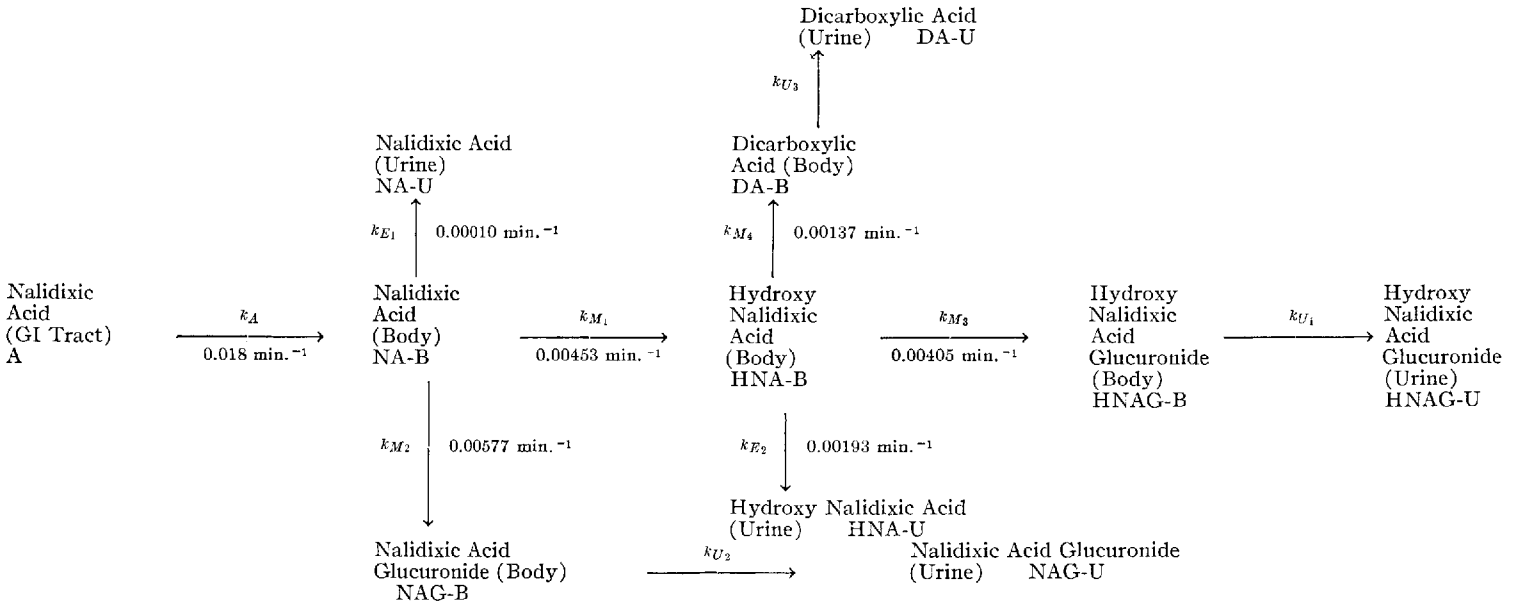
step *1* = 100% NA + 134% HNA

step *2* = 100% NA + 33% HNA

step *1* − step *2* = 101% HNA

For practical purposes, then, step *1* − step *2* is taken simply as equal to the amount of HNA present, and the amount of NA is readily calculated from either *1* or *2*. When applied to the analysis of mixtures of the 2 compounds ranging from 5–40 mcg. of NA and 60–5 mcg. of HNA, the recovery

[1] Negram Caplets, 500 mg./Caplet.



Kinetic pathways of nalidixic acid in man; k_A = availability rate constant; k_{M1} = metabolic rate constants; k_{E1} and k_{U1} = excretion rate constants.

Scheme I

TABLE I.—PARAMETERS FOR NALIDIXIC ACID IN FORMULATIONS A AND B

Subject	% Urinary Recovery A	% Urinary Recovery B	Lag Time, min. A	Lag Time, min. B	k_A (min.$^{-1}$) × 10^3 A	k_A (min.$^{-1}$) × 10^3 B	kd (min.$^{-1}$) × 10^3 A	kd (min.$^{-1}$) × 10^3 B	k_{M1} (min.$^{-1}$) × 10^3 A	k_{M1} (min.$^{-1}$) × 10^3 B	k_E (min.$^{-1}$) × 10^3 A	k_E (min.$^{-1}$) × 10^3 B	k_{M2} (min.$^{-1}$) × 10^3 A	k_{M2} (min.$^{-1}$) × 10^3 B	V_D as % of Body Wt. A	V_D as % of Body Wt. B
A. J.	96	97	35	12	40.5	15.1	12.8	12.8	5.64	4.16	0.35	0.13	6.25	8.15	29.7	45.1
G. B.	[a]	93	[a]	18	[a]	18.2	[a]	11.6	[a]	6.98	[a]	0.04	[a]	3.74	[a]	25.6
D. F.	103	94	47	28	18.3	15.3	13.6	12.8	5.40	4.59	0.15	0.33	8.60	6.81	38.2	40.0
J. R.	96	97	8	10	8.50	9.77	10.5	10.0	2.33	3.42	0.02	0.05	7.70	6.21	26.6	30.8
W. H.	90	97	9	15	25.6	15.3	7.7	6.1	2.20	1.72	0.02	0.03	4.71	4.18	39.2	43.5
R. B.	[b]	[b]	0	36	15.6	0.99%/min.[c]	11.6	12.8	...	...	...	...	...	...	...	...
J. D.	95	83	6	14	0.70%/min.[c]	0.59%/min.[c]	10.0	12.8	5.53	7.53	[d]	[d]	3.98	3.14	29.8	25.8

[a] Incomplete crossover. [b] Incomplete urine collection. [c] Zero order. [d] No detectable NA.

of HNA was 96.5 ± 5% of the amount added, and the recovery of NA was 103 ± 9%. If total NA + HNA is determined following acid hydrolysis in the same way, and the values for free NA and HNA are subtracted, the amounts present as conjugated NA and conjugated HNA may be estimated, NA and HNA being stable to this hydrolysis.

In addition, urine levels of NAG, HNAG, and DA were determined with previously described methods (7).

RESULTS AND DISCUSSION

The Wagner and Nelson method (8) of calculating availability rates of drug to plasma was used, and results (k_A) are shown in Table I. Typical plasma level curves and A/V values are shown in Figs. 1 and 2 for 2 individuals and 2 dosage forms. For these same subjects, the logarithm of the per cent unabsorbed at various times was calculated and is shown in Fig. 3. Lag time is read as the time when 100% is unabsorbed by extrapolating the line to log 2.

Three of 13 observations for availability rates are apparently of the zero-order type. The mean of the 10 first-order availability rates is 0.018 min.$^{-1}$ which is very similar to the mean rate of 0.020 min.$^{-1}$ reported for nalidixic acid in caplet form for 8 subjects (6). The use of total plasma naphthyridine for determining absorption rates (6) results in a small error because practically all of the naphthyridine is in the form of nalidixic acid.

With the exception of W. H., subjects showed little variation in disappearance rates, either among themselves or between dosage forms. Using Eq. 2, with division by V_D to obtain concentration, the theoretical values of NA were calculated for the 2 subjects shown in Figs. 1 and 2. Excellent agreement was obtained between the calculated values and those from the actual plasma level curve.

Because of low HNA plasma levels (Figs. 1 and 2), it is apparent that very little difference would be seen between the disappearance rates of NA and NA + HNA. In fact, in our previous study (6), where active naphthyridine (NA + HNA) was measured in 8 subjects, their apparent average disappearance rate was 0.011 min.$^{-1}$, which is identical to the average rate for NA (Table I).

Individual values for excretion (k_{E_1}) and metabolic (k_{M_1}, k_{M_2}) rate constants were calculated using Eqs. 5, 8, and 11 with the 0–24-hr. urinary data as the infinity values (Table I). Data previously obtained (6) indicated that a 24-hr. urine analysis would represent complete excretion. Rate constants for the formation of NAG (k_{M_2}) are the

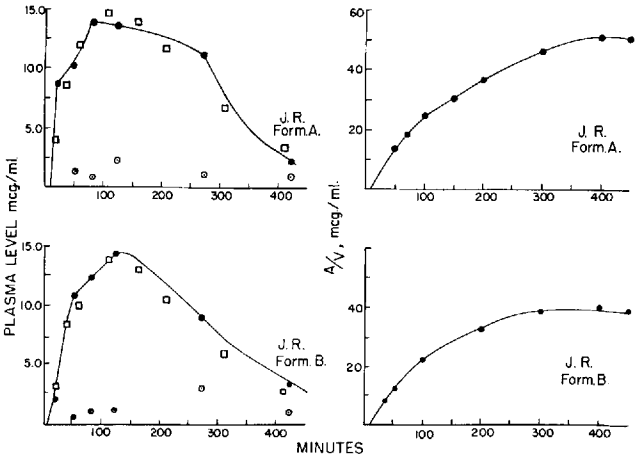


Fig. 1.—Plasma levels and A/V as a function of time. Key: ●, NA; ⊙, HNA; □, calculated values for NA. Initials identify subjects.

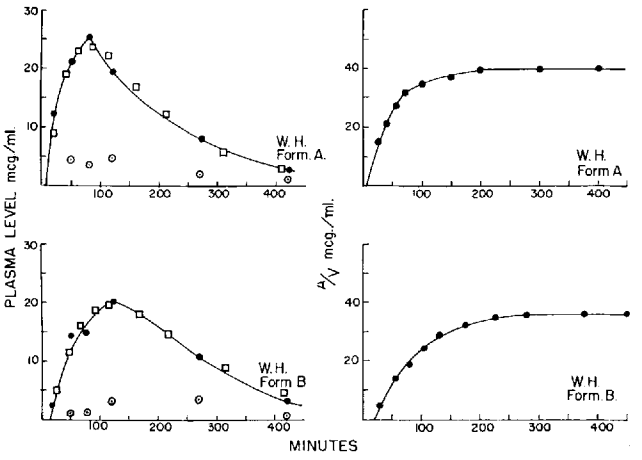


Fig. 2.—Plasma levels and A/V as a function of time. Key: ●, NA; ⊙, HNA; □, calculated values for NA. Initials identify subjects.

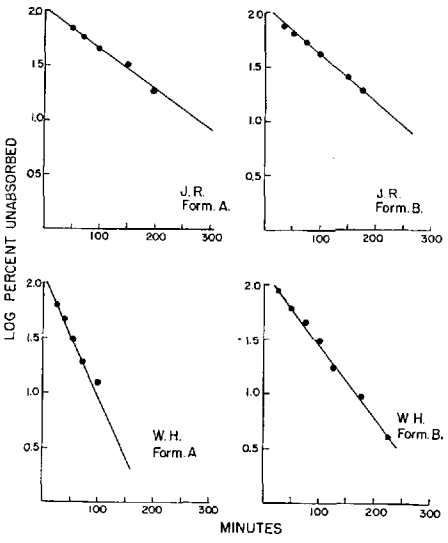


Fig. 3.—Log per cent nalidixic acid unabsorbed as a function of time. Initials identify subjects.

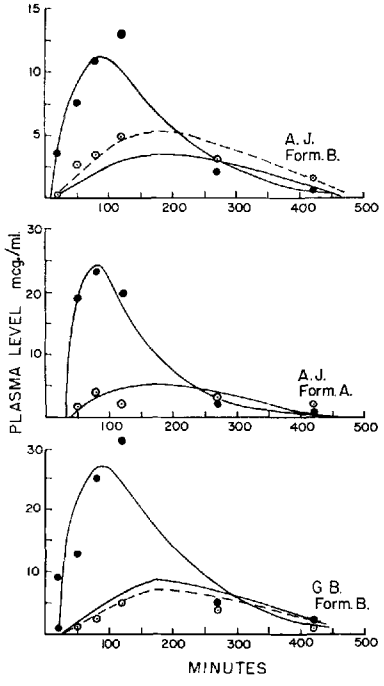


Fig. 4.—Plasma levels as a function of time. Key: experimental ●, NA; experimental ⊙, HNA; —, calculated curve for HNA and NA. (20 min. experimental value for A. J., form A was 0.)

highest of the various constants for disappearance in 4 of 6 subjects. The variation in metabolic rates with each individual between forms A and B is slight compared to the variation among individuals.

Because of the low excretion rate of nalidixic acid and its high metabolic rates, the over-all effect is an extremely small quantity of NA appearing in the urine. In fact, if the active drug is measured in the urine (NA + HNA), the observed apparent excretion rate constant should be essentially that of HNA. The apparent mean excretion rate constant of the active drug (NA + HNA) in the urine has been reported as 0.0015 min.$^{-1}$ ± 0.0003 (18 observations) (6) compared to an average excretion rate constant for HNA of 0.0019 min.$^{-1}$ ± 0.0004 (4 observations) (9).

The excretion of acidic and basic organic compound is sometimes modified greatly as a result of changes in urinary pH usually induced by large doses of sodium bicarbonate or ammonium chloride. McChesney and co-workers (7) have shown that the urinary excretion of active naphthyridine (NA + HNA) is increased with $NaHCO_3$ and decreased with NH_4Cl given simultaneously with nalidixic acid. No attempt was made to control urinary pH in the present study since normal variations in excretion rates were sought.

The apparent volumes of distribution for NA and HNA are inversely proportional to the per cent of plasma protein binding at pH 7.4 in therapeutic concentration ranges. These are 93% for NA and 63% for HNA using equilibrium dialysis techniques.

In order to evaluate the processes beyond the formation of HNA, the results of an experiment in which HNA is given orally (9) are needed. For 2 subjects (A. J. and G. B.) common to both these experiments, their individual disappearance rate constants (k_{d_2}) and apparent volumes of distribution for HNA (9) plus their k_A, k_{M_1}, k_d, and V_D values for NA (Table I) were used in Eqs. 2 and 3 to calculate plasma levels. These results which are shown in Fig. 4 indicate a reasonable agreement with the experimental data for NA.

For HNA, the agreement with experimental data for A. J. (form A) is reasonable, however, for G. B. and A. J. (form B) the general shape of the theoretical curve is good indicating that the rate constants are sufficiently good estimates, but the heights of the curves are too low or too high. The complexity of the HNA system is of such a magnitude that good fits should not be expected with the use of apparent distributive volumes and disappearance rate constants from separate experiments. Good fits can be obtained by only changing the volume of distribution. By increasing the V_D for G. B. from 40.8 to 50.0% of body weight, the height of the plasma level curve is lowered (dashed curve, Fig. 4), producing a good correlation with experimental values. For A. J. (form B), decreasing the V_D from 45.9 to 31% of body weight also gives a good fit with experimental values (dashed curve, Fig. 4).

Assuming average rate constants and using Eqs. 5, 7, 8, 9, and 10, the average urinary excretion values at infinity were calculated. A comparison between these values and the average experimental values is given in Table II. Again a reasonable agreement is apparent.

The plasma level comparison (NA and HNA) plus the good agreement with urinary excretion data indicate that these average rate constants (Table II) will give a good picture of the metabolism and excretion of nalidixic acid and its metabolite (HNA). Such a picture is presented in Figs. 5 and 6 where the changes occurring in plasma and urine

TABLE II.—COMPARISON OF CALCULATED AND EXPERIMENTAL VALUES FOR NALIDIXIC ACID AND METABOLITES

Compd.	Calcd.[a] Urinary Excretion, mg.	Exptl. Urinary Excretion (mg.) Av. ± S.E.
NA-U	9	8 ± 3
NAG-U	517	537 ± 49
HNA-U	105	129 ± 8
HNAG-U	221	229 ± 32
DA-U	74	43 ± 6

[a] Average rate constants are: k_d, 0.01116 min.$^{-1}$; k_{M_1}, 0.00453 min.$^{-1}$; k_{E_1}, 0.00010 min.$^{-1}$; k_{M_2}, 0.00577 min.$^{-1}$; k_{d_2}, 0.00746 min.$^{-1}$; k_{E_2}, 0.00193 min.$^{-1}$; k_{M_3}, 0.00405 min.$^{-1}$; k_{M_4}, 0.00137 min.$^{-1}$.

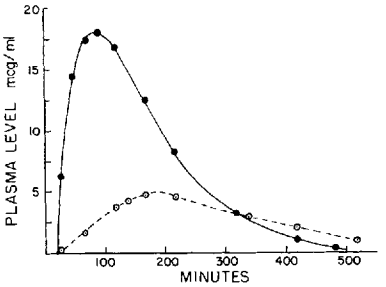


Fig. 5.—Calculated average plasma levels as a function of time. Key: ●, NA; ⊙, HNA.

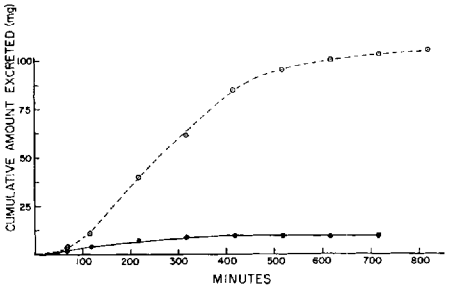


Fig. 6.—Calculated average cumulative urinary excretion as a function of time. Key: ●, NA; ⊙, HNA.

with respect to time for the active components of the model are presented. Average rate constants and V_D's were used in Eqs. 2, 3, 4, and 6 to generate these curves for a 1-Gm. dose of nalidixic acid.

SUMMARY

A differential extraction procedure has been developed which enabled the separate determination of NA and HNA in plasma and urine.

The solution of a pharmacokinetic model for nalidixic acid which involves 5 components in the body and urine compartments has been mathematically described in terms of rate equations. A total of 7 rate constants have been determined: 2 for glucuronide formation, 2 for oxidation, 2 for excretion of NA and HNA, and 1 for the availability of NA. The reasonable agreement between calculated and experimental curves has been illustrated.

APPENDIX

Equations relating the quantities of NAG-U, HNAG-U, and DA-U as a function of time are presented. By letting time (t) approach infinity, equations are obtained which enable the calculation of metabolic and excretion constants without making any assumptions about the relative values of consecutive rate constants.

These equations were derived from the model (Fig. 1) where the rate constants are assumed to be first order and the compartments are defined as the: (a) GI tract, (b) body or apparent volume of distribution, and (c) urine. Definitions of terms are stated under *Theoretical*.

NAG-U =

$$\frac{k_{U_2}k_{M_2}k_A A_0}{(k_A - k_d)(k_d - k_{U_2})}\left(\frac{e^{-k_d t}}{k_d} - \frac{e^{-k_{U_2}t}}{k_{U_2}}\right) - \frac{k_{U_2}k_{M_2}k_A A_0}{(k_A - k_d)(k_A - k_{U_2})}\left(\frac{e^{-k_A t}}{k_A} - \frac{e^{-k_{U_2}t}}{k_{U_2}}\right) + \frac{k_{M_2}A_0}{k_d} \quad \text{(Eq. 4a)}$$

HNAG-U =

$$\frac{k_{M_3}k_{M_1}k_A k_{U_1}A_0}{(k_A - k_d)(k_d - k_{d_2})(k_{U_1} - k_{d_2})}\left(\frac{e^{-k_{U_1}t}}{k_{U_1}} - \frac{e^{-k_{d_2}t}}{k_{d_2}}\right) - \frac{k_{M_3}k_{M_1}k_A k_{U_1}A_0}{(k_A - k_d)(k_d - k_{d_2})(k_{U_1} - k_d)}\left(\frac{e^{-k_{U_1}t}}{k_{U_1}} - \frac{e^{-k_d t}}{k_d}\right) - \frac{k_{M_3}k_{M_1}k_A k_{U_1}A_0}{(k_A - k_d)(k_A - k_{d_2})(k_{U_1} - k_{d_2})}\left(\frac{e^{-k_{U_1}t}}{k_{U_1}} - \frac{e^{-k_{d_2}t}}{k_{d_2}}\right) + \frac{k_{M_3}k_{M_1}k_A k_{U_1}A_0}{(k_A - k_d)(k_A - k_{d_2})(k_{U_1} - k_A)}\left(\frac{e^{-k_{U_1}t}}{k_{U_1}} - \frac{e^{-k_A t}}{k_A}\right) + \frac{k_{M_3}k_{M_1}A_0}{k_d k_{d_2}} \quad \text{(Eq. 5a)}$$

DA-U =

$$\frac{k_{M_4}k_{M_1}k_A k_{U_3}A_0}{(k_A - k_d)(k_d - k_{d_2})(k_{U_3} - k_{d_2})}\left(\frac{e^{-k_{U_3}t}}{k_{U_3}} - \frac{e^{-k_{d_2}t}}{k_{d_2}}\right) - \frac{k_{M_4}k_{M_1}k_A k_{U_3}A_0}{(k_A - k_d)(k_d - k_{d_2})(k_{U_3} - k_d)}\left(\frac{e^{-k_{U_3}t}}{k_{U_3}} - \frac{e^{-k_d t}}{k_d}\right) - \frac{k_{M_4}k_{M_1}k_A k_{U_3}A_0}{(k_A - k_d)(k_A - k_{d_2})(k_{U_3} - k_{d_2})}\left(\frac{e^{-k_{U_3}t}}{k_{U_3}} - \frac{e^{-k_{d_2}t}}{k_{d_2}}\right) + \frac{k_{M_4}k_{M_1}k_A k_{U_3}A_0}{(k_A - k_d)(k_A - k_{d_2})(k_{U_3} - k_A)}\left(\frac{e^{-k_{U_3}t}}{k_{U_3}} - \frac{e^{-k_A t}}{k_A}\right) + \frac{k_{M_4}k_{M_1}A_0}{k_d k_{d_2}} \quad \text{(Eq. 6a)}$$

It is apparent that when time (t) is permitted to approach infinity, the above equations reduce to their last term and give Eqs. 8, 9, and 10 of the text.

Equation 11 of the text is derived from the sum of the expressions for DA-U, HNAG-U, and HNA-U at $t = \infty$.

$$[\text{DA-U}]_\infty + [\text{HNAG-U}]_\infty + [\text{HNA-U}]_\infty = \frac{k_{M_4}k_{M_1}A_0}{k_d k_{d_2}} + \frac{k_{M_3}k_{M_1}A_0}{k_d k_{d_2}} + \frac{k_{E_2}k_{M_1}A_0}{k_d k_{d_2}} \quad \text{(Eq. 7a)}$$

which reduces to

$$[\text{DA-U}]_\infty + [\text{HNAG-U}]_\infty + [\text{HNA-U}]_\infty = \frac{k_{M_1}A_0}{k_d k_{d_2}}(k_{M_4} + k_{M_3} + k_{E_2}) \quad \text{(Eq. 8a)}$$

By definition $k_{d_2} = k_{M_4} + k_{M_3} + k_{E_2}$.

Therefore, Eq. 8a, upon substituting for k_{d_2}

and solving for k_{M_1} reduces to Eq. 11 of the text. This equation permits the calculation of the metabolic rate constant (k_{M_1}) for the oxidation of nalidixic acid to hydroxynalidixic acid.

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Drug Standards

Determination of Free Salicylic Acid in Aspirin and Aspirin Products

By JOHN D. WEBER and JOSEPH LEVINE

In a previously described method for the determination of free salicylic acid in aspirin, the salicylic acid is isolated on a partition chromatographic column as its purple ferric–phenol complex, using ferric chloride solution as immobile phase. With the addition of a high concentration of urea to the ferric chloride, the method is significantly improved. The separation is more sharply defined, making feasible the analysis of larger samples of aspirin and permitting the use of a more easily prepared chromatographic column.

A PROCEDURE has been described (1) for the isolation and determination of small amounts of salicylic acid which occur in aspirin and aspirin products. The salicylic acid is retained on a Celite:2% ferric chloride partition chromatographic column as its ferric complex while the nonphenolic aspirin is eluted with chloroform. The ferric complex is then dissociated with acetic acid and the free salicylic acid eluted with chloroform.

Several investigators have encountered difficulty with the published method (2). During the elution of the aspirin, the salicylic acid migrates slowly down the column (as evidenced by the position of the purple complex) and spreads out into a diffuse band, which sometimes becomes difficult to discern. Unless the chromatographic column is packed with great uniformity, channeling may occur during both the elution of aspirin and the recovery of salicylic acid.

A radical improvement in the chromatographic separation is achieved with a modified ferric chloride reagent, which contains a high concentration of urea. The band of the ferric–salicylate complex obtained with this reagent is much more deeply colored than that obtained with the simple ferric chloride reagent. The dense, sharply delineated band migrates only very slightly during the elution of aspirin. The use of shorter columns, which do not require extraordinary care in packing, is therefore feasible. Columns prepared with the modified reagent will also accommodate much larger samples of aspirin than those prepared with ferric chloride alone.

Addition of urea to the ferric chloride reagent was suggested from the report of a urea–salicylic acid complex by Bolton (3). It is apparent that the formation of this binary complex does not account for the trapping of salicylic acid by the ferric chloride–urea reagent, however, since urea solutions alone, at any concentration, are completely ineffective in removing salicylic acid from chloroform solution.

Optimum results are obtained with an immobile phase containing 5% ferric chloride and which is 10 *M* with respect to urea. It must be maintained at a pH between 3.1 and 3.3. At lower pH levels the salicylic acid band becomes diffuse and more loosely retained, while at higher levels recovery of salicylic acid from the column may be incomplete using the specified volume of eluant.

The concentration of urea in the reagent must

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and solving for k_{M_1} reduces to Eq. 11 of the text. This equation permits the calculation of the metabolic rate constant (k_{M_1}) for the oxidation of nalidixic acid to hydroxynalidixic acid.

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Determination of Free Salicylic Acid in Aspirin and Aspirin Products

By JOHN D. WEBER and JOSEPH LEVINE

In a previously described method for the determination of free salicylic acid in aspirin, the salicylic acid is isolated on a partition chromatographic column as its purple ferric–phenol complex, using ferric chloride solution as immobile phase. With the addition of a high concentration of urea to the ferric chloride, the method is significantly improved. The separation is more sharply defined, making feasible the analysis of larger samples of aspirin and permitting the use of a more easily prepared chromatographic column.

A PROCEDURE has been described (1) for the isolation and determination of small amounts of salicylic acid which occur in aspirin and aspirin products. The salicylic acid is retained on a Celite:2% ferric chloride partition chromatographic column as its ferric complex while the nonphenolic aspirin is eluted with chloroform. The ferric complex is then dissociated with acetic acid and the free salicylic acid eluted with chloroform.

Several investigators have encountered difficulty with the published method (2). During the elution of the aspirin, the salicylic acid migrates slowly down the column (as evidenced by the position of the purple complex) and spreads out into a diffuse band, which sometimes becomes difficult to discern. Unless the chromatographic column is packed with great uniformity, channeling may occur during both the elution of aspirin and the recovery of salicylic acid.

A radical improvement in the chromatographic separation is achieved with a modified ferric chloride reagent, which contains a high concentration of urea. The band of the ferric–salicylate complex obtained with this reagent is much more deeply colored than that obtained with the simple ferric chloride reagent. The dense, sharply delineated band migrates only very slightly during the elution of aspirin. The use of shorter columns, which do not require extraordinary care in packing, is therefore feasible. Columns prepared with the modified reagent will also accommodate much larger samples of aspirin than those prepared with ferric chloride alone.

Addition of urea to the ferric chloride reagent was suggested from the report of a urea–salicylic acid complex by Bolton (3). It is apparent that the formation of this binary complex does not account for the trapping of salicylic acid by the ferric chloride–urea reagent, however, since urea solutions alone, at any concentration, are completely ineffective in removing salicylic acid from chloroform solution.

Optimum results are obtained with an immobile phase containing 5% ferric chloride and which is 10 *M* with respect to urea. It must be maintained at a pH between 3.1 and 3.3. At lower pH levels the salicylic acid band becomes diffuse and more loosely retained, while at higher levels recovery of salicylic acid from the column may be incomplete using the specified volume of eluant.

The concentration of urea in the reagent must

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be 10 *M* to maintain dense salicylic acid bands during the elution of the aspirin. A quantity of salicylic acid which forms a zone 5 mm. deep with this reagent, for example, forms a zone spread throughout the column when the concentration of urea in the immobile phase is reduced to 7.5 *M*. In the absence of aspirin, the effect of the urea concentration is minor. Dense bands are obtained with urea concentrations over a range of 2.5 to 10 *M*.

It has been shown (1) that only dilute solutions of ferric chloride remove salicylic acid from chloroform solution; the U.S.P. ferric chloride test solution (9%) is virtually ineffective. In contrast, the effectiveness of the urea–ferric chloride reagent in maintaining a dense salicylic acid band increases with increase of ferric chloride concentration over a range of 1 to 15%. The color of the reagent at the higher concentrations deepens, however, lessening the visual contrast between its color and that of the salicylic acid complex. A concentration of 5% ferric chloride was selected as optimum for providing suitable color contrast while maintaining the compactness of the salicylic acid band on the column.

PROCEDURE

Reagent.—To 60 Gm. of reagent grade urea, add 8 ml. of 60% ferric chloride solution[1] and 42 ml. of 0.05 *N* HCl. Shake, without heating, to dissolve the urea. The pH of freshly prepared solution is about 3.2. It should be checked on a pH meter daily, and, as necessary, adjusted to between pH 3.1 and 3.3 by dropwise addition of 6 *N* HCl.

Salicylic Acid Standard.—Accurately weigh about 25 mg. of salicylic acid and dissolve in 100.0 ml. of chloroform. Transfer 5.0 ml. to a 100-ml. volumetric flask; add 2 ml. of glacial acetic acid, 20 ml. of ether, 20 ml. of methanol, and 4 drops of hydrochloric acid. Dilute to volume with chloroform.

Chromatographic Tube.—A 25- × 250-mm. test tube to which is attached a 50-mm. length of 6- or 8-mm. tubing. The tamping rod consists of a disk of stainless steel, aluminum, or glass, of a diameter 1 mm. less than that of the column, attached to a rod 12 to 18 in. long. Pack fine glass wool[2] in the base of the column as support.

Preparation of Trap Column.—To 3 Gm. of Celite 545[3] add 2 ml. of *Reagent*. Mix thoroughly, transfer to the column, and tamp, using gentle pressure, to a uniform mass. Cover with a pad of cotton about 20 mm. thick. (This insures uniform distribution of the solvent over the column cross section.)

Procedure.—Use water - saturated solvents throughout.

Aspirin and Aspirin Tablets.—Weigh a quantity of sample, equivalent to about 200 mg. of aspirin, into a beaker. Add 10 ml. of chloroform and stir for 3 min. to dissolve the sample. Transfer the solution to the column, rinsing the beaker with 10 ml. of chloroform in small portions. Wash the column successively with 10- and 50-ml. portions of chloroform to remove the aspirin. Rinse the tip of the column with a jet of chloroform. (If the purple zone reaches the bottom of the column, repeat with a smaller sample of aspirin.)

Place as receiver under the column a 50-ml. volumetric flask containing 10 ml. of methanol and 2 drops of hydrochloric acid. Elute the column with 10 ml. of ether containing 1 ml. of glacial acetic acid, followed by 30 ml. of chloroform, and dilute to volume with chloroform. Concomitantly determine the absorbance of this solution and of the standard solution at the maximum at about 306 mμ.

APC Tablets and Flavored Tablets.—Mount in series directly above the trap column a second column containing a pad of cotton about 20 mm. deep. Dissolve the sample as described above and transfer it to the upper column with the aid of 10 ml. of chloroform. Pass 20 ml. of chloroform over the columns; discard the upper column, and continue as described under *Aspirin and Aspirin Tablets*, beginning with "Wash the column successively with 10- and 50-ml. portions of chloroform."

DISCUSSION AND RESULTS

A moderate amount of urea is eluted together with the salicylic acid during the recovery of the latter from the column. Hydrochloric acid is added to insure acidity of the eluate, and methanol to achieve miscibility of the acid with the eluting solvent. The absorbance maximum of the resulting solution is at 306 mμ, as compared with the wavelength of 310 mμ in the absence of methanol. The standard reference solution is therefore prepared to have the same solvent composition as the eluate.

No measurable degree of hydrolysis of aspirin occurs during the analysis. The salicylic acid fraction obtained from 500-mg. samples of purified aspirin had an absorbance of 0.003, equivalent to about 0.001% of salicylic acid.

Measured amounts of salicylic acid were added to powdered aspirin U.S.P. and to powdered commercial tablets. Recoveries are presented in Tables I and II, respectively. The latter values show that the excipient materials present in these commercial formulations do not interfere with the procedure.

A number of samples of buffered aspirin tablets were assayed, employing the preliminary boric acid treatment described in the earlier procedure (1). Because of the precision of the analyses, it was thought that these represented the true content of free salicylic acid of the tablets. However, following a suggestion (4) that boric acid might not be fully effective in liberating salicylic acid from the buffering agents, it was indeed found that recovery of salicylic acid added to aluminum hydroxide gel, calcium carbonate, and magnesium trisilicate, which are commonly used in these formulations, was incomplete. A methanolic solution of oxalic acid effected complete recovery of salicylic acid from its calcium salt while producing only a negligible degree of hydrolysis of aspirin. However, neither this nor any other reagent thus

[1] Commercial reagent.
[2] Pyrex Filtering Fibre, Corning Glass catalog No. 3950.
[3] Johns-Manville Corp.

TABLE I.—STANDARD RECOVERIES OF SALICYLIC ACID ADDED TO ASPIRIN

Aspirin, mg.	Salicylic Acid Added, mg.	Total Wt. Salicylic Acid Found	Corrected Wt. Salicylic Acid Found, mg.	Recovery, %
218.5[a]	None	0.045 (0.020%)[c]	...	...
489.7[a]	None	0.093 (0.019%)	...	...
1005.4[a]	None	0.179 (0.018%)	...	...
1020.9[b]	None	0.225 (0.022%)	...	...
1005.6[b]	None	0.225 (0.022%)	...	...
1004.2[b]	None	0.225 (0.022%)	...	...
201.3	0.129	0.170	0.132	102.3
501.8	0.129	0.228	0.133	103.1
202.3	0.257	0.295	0.257	100.0
204.0	0.257	0.295	0.256	99.6
203.9	0.514	0.552	0.513	99.8
201.9	0.514	0.555	0.517	100.6
200.9	0.771	0.802	0.764	99.1
209.5	0.771	0.805	0.765	99.2
51.2	2.056	2.076	2.066	100.5
54.7	2.056	2.079	2.069	100.6

[a] Freshly opened container of powdered aspirin U.S.P. [b] Same sample 11 months after having been opened. [c] The accuracy at this level is limited by the reading of the spectrophotometer scale. The absorbance of the salicylic acid fraction in this analysis was 0.026; a deviation of ±0.001 would be equivalent to a deviation of ±4%. The accuracy at this level can be increased by increasing the size of the sample.

TABLE II.—STANDARD RECOVERIES OF SALICYLIC ACID ADDED TO COMMERCIAL TABLETS

Product	Sample Wt., mg.	Wt. Aspirin in Sample, mg.	Salicylic Acid Added, mg.	Total Wt. Salicylic Acid Found, mg.	Corrected Wt. Salicylic Acid Found, mg.	Recovery, %
5 gr.	234.0	196.9	...	0.096 (0.049%)	...	...
aspirin	253.8	213.6	...	0.105 (0.049%)	...	...
tablets	249.3	209.8	0.608	0.701	0.598	98.4
U.S.P.	251.8	211.9	0.608	0.701	0.597	98.2
	247.8	208.5	0.243	0.344	0.242	99.5
	250.0	210.4	0.243	0.341	0.238	97.9
1¼ gr.	548.7	195.5	...	0.111 (0.057%)	...	...
children's	555.0	197.8	...	0.113 (0.057%)	...	...
aspirin	538.3	191.8	0.489	0.596	0.487	99.6
	541.8	193.1	0.498	0.598	0.488	99.8
APC tablets	543.8	244.7	...	0.159 (0.114%)	...	...
N.F. (3.5	453.5	204.0	0.489	0.719	0.486	99.4
gr. aspirin)	420.3	189.1	0.489	0.693	0.477	97.6

far tested quantitatively releases salicylic acid from aluminum hydroxide gel without causing extensive hydrolysis of aspirin.

Analyses of buffered tablets using the oxalic acid reagent had a precision equal to that obtained with boric acid, and uniformly indicated a higher free salicylic acid content. Examination of the insoluble residue showed, however, that these analyses did not represent the entire nonaspirin salicylate content of the tablets. Despite this incomplete recovery, 4 of 7 samples analyzed gave values in excess of 1% salicylic acid.

TABLE III.—ANALYSIS OF COMMERCIAL SAMPLES

Sample	Salicylic Acid Found, %
Aspirin powder, U.S.P.	0.018, 0.019, 0.020
Aspirin tablets, 5 gr. U.S.P.	
Brand 1	0.045, 0.043
Brand 2	0.019, 0.020, 0.020
Brand 3	0.076, 0.078, 0.070
Brand 4	0.125, 0.122, 0.124
Brand 5	0.285, 0.280, 0.280
Aspirin tablets, flavored 1¼ gr.	
Brand 1*a*	0.199, 0.209, 0.196
Brand 1*b*	0.180, 0.168, 0.170
Brand 2*a*	0.094, 0.091, 0.091
Brand 2*b*	0.057, 0.057
Brand 3	0.534, 0.528, 0.531
APC tablets	
Brand 1	0.046, 0.044, 0.045
Brand 2	0.047, 0.045, 0.044
Brand 3	0.115, 0.120, 0.121
Brand 4	1.04, 1.06, 1.07
Brand 5	0.262, 0.264, 0.259

Aspirin products of various brands, both local and nationally advertised, were obtained from local retail pharmacies and food markets. The samples were purchased directly from the stock on the shelves; no information is available on their age. The analyses of these samples are presented in Table III.

Three of 5 samples of 5-gr. aspirin tablets were well within the U.S.P. XVII limits of 0.15% salicylic acid; 1 approached this value, and 1 exceeded it. Three of 5 samples of 1¼ gr. of children's flavored aspirin tablets exceeded this limit.

Four of the 5 samples of aspirin, phenacetin, and caffeine tablets were well within the N.F. XII limits of 0.75%, while a fifth, which had a loose bottle cap at the time of purchase, exceeded the limits.

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Film Coating Theory and Practice

By GILBERT S. BANKER

Recent theory and developments relating to the formation and modification of synthetic polymeric films are discussed in relation to the pharmaceutical uses of such films in dosage form development. Fundamental mechanical and physicochemical properties of films as affected by plasticization, solvent effects, polymer chemistry, film additives, and other factors are considered in relation to film dissolution, permeability, and diffusion properties.

POLYMERIC FILMS are finding an ever-increasing range of application in pharmaceutical research, development, and dosage form design. In the coating of tablets and other solid dosage forms there is presently no coating methodology that can match film coating in production capability or economy. Polymeric film coatings have been increasingly employed to coat drug particles and drug-containing pilules to produce products with a delayed or prolonged pharmaceutical action. Approximately 1000 pharmaceutical patents pertaining to polymeric materials as adjuvants, including polymeric coatings, have been issued in the last 15 to 20 years. In addition to application to all types of solid oral dosage forms, polymeric films are being employed for such diverse uses as the coating of suppositories, the encapsulation of liquids, and aerosol spray bandages. As film theory and technology continue to advance, both fundamentally and in selected pharmaceutical applications, increasing and more effective utility of polymeric films will be made by the pharmaceutical industry. The purpose of this paper is to relate some of the more recent theory and developments in film technology to pharmaceutical applications and practice.

THEORY OF FILM FORMATION

Forces in the Film.—In any pharmaceutical film coating operation in which a polymer film is being applied to a matrix, sets of forces operate between the film forming polymer molecules on the one hand (cohesion), and between the film and the substrate on the other hand (adhesion). Cohesion, also known as autohesion or self-adhesion, refers to the ability of contiguous surfaces of the same material, at a molecular or at a supermolecular level, to form a strong bond which prevents or resists separation at the point of contact. To obtain high levels of cohesion 2 phenomena are necessary: the cohesive (autoadhesive) strength of the material, molecule to molecule, must be relatively high, and the contiguous surfaces of the film material must coalesce on contact (1). Coalescence or the disappearance of boundary layers between adjacent polymer molecular layers or surfaces is explained by diffusion theory. According to theory, movement (diffusion) of individual macromolecules or segments of macromolecules between and within film layers may occur under a variety of conditions, including during gelation, when polymers are deposited in solution over a previous polymer layer, or at elevated temperatures corresponding to a semisolid state (2). The result, if there is adequate cohesive attraction between the molecules and sufficient diffusion and coalescence, will be a restoration of the polymer structure to a uniform nonlaminated matrix at the contact zone (Fig. 1) affected by the displacement (diffusion) of whole molecules or of individual segments of the macromolecular chains. Only high polymers, owing to their molecular structure, combine sufficient cohesive strength and capacity for coalescence to produce fiber and film structures, drawn or deposited from appropriate solvents.

The significance of the degree of cohesion in film structures is fundamental to film properties. An increase in cohesion in the structure of 1 polymer to another, or of 1 analog of a homologous series to another will increase film density and compactness, may decrease porosity and permeability, decrease flexibility, probably increase brittleness, as well as affecting other film properties either directly or indirectly (3).

Controllable Processing Factors Affecting Cohesion in Pharmaceutical Film Coatings.—The factors which may increase film cohesion, polymer surface to polymer surface, not all of which are readily controllable in the typical pharmaceutical film coating operation include: increased surface contact time (4), increased contact temperature (5), increased contact pressure (4), coat thickness, and control of coat solution or coat dispersion concentration, degree of polymer solvation, and viscosity. Increased coat-contact temperature,

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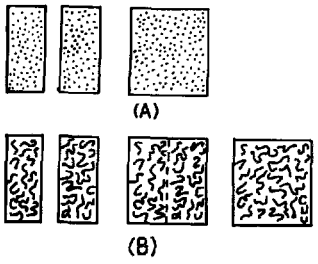


Fig. 1.—Schematic representation of the coalescence of (A) low molecular weight liquids, and (B) high polymers in a polymeric film structure.

time, or pressure all promote cohesion by promoting molecular diffusion at the contact boundaries.

Temperature.—Temperature is directly related to autohesion; indeed, the temperature dependence of cohesion is a proof of the diffusional basis of the process. As the contact temperature increases, the cohesive strength of the contiguous polymer surfaces increases over a definite temperature interval along an exponential curve, not unlike the exponential dependence of diffusion rate on temperature observed for low molecular weight materials (7). This exponential increase in diffusion rate with temperature is due to the increased thermal motion of complete polymer chain molecules or fractions of such molecules, and is probably secondarily related to decreased polymer, polymer gel, or polymer film density. That a more cohesive film generally results from the application of a warm coating solution to a warm substrate is well known to those skilled in coating technology. There are, of course, limits to the amount of heat which may be advantageously used, as excessive heat may cause premature spray drying of the coat, slipping and peeling of the coating as it approaches its melting or glass point, or the development of pinholes in the coat caused by solvent evaporation under high localized vapor pressure through a case-hardened film surface. It is also well known that an increased temperature generally greatly facilitates adhesion between polymer film and substrate, with the temperature effect probably eliciting the same phenomena as in cohesion (promoted diffusion).

Contact Pressure and Contact Time.—Contact pressure is not a readily controllable factor in the typical pharmaceutical film coating application. Contact time refers to the duration during which a newly deposited polymer film layer is "setting-up" and the polymer molecules, wholly or in part, are capable of diffusion and orientation. Reasonably rapid solvent evaporation rates are sought in most film coating applications to facilitate rapid coating. However, solvents which flash off prematurely not only may lead to spray drying of the atomized coat solution, but may produce noncohesive films due to a premature immobilization of the polymer molecules in the film structure prior to molecular orientation, as well as to poor diffusion of the polymer molecules between molecular layers in the film.

Film Coat Thickness.—Cohesive strength of films is commonly expressed as the peeling strength, represented as the work of ergs/cm.^{2}, required to separate bonded layers of film (5). The cohesive film strength (peeling strength) has been found to increase as a zero-order function of film thickness up to some fixed value, dependent on polymer film chemical class, after which the cohesive strength is constant with further increases in thickness.

Coat Solution Concentration, Solvation, and Viscosity.—Viscosity, hence polymer solvation and solution concentration, are of great importance to the self-adhesion of high polymers. At low viscosity or at high polymer solvation levels self-diffusion should be promoted. On the other hand, at low viscosities most coating solutions will be very dilute, coating times will be unduly long, and it will be comparatively easy for a selected deposited film component forming the bond to separate from the bulk of the previously homogeneous film substrate. Consequently, an intermediate viscosity will usually result in the highest cohesive strength (6). The viscosity at which the deposited coating solution gels will also affect cohesive strength, and this will be a function of the solvent or mixed solvent system used and the rate of desolvation and stereochemical displacement of the polymer from the solvent during evaporation.

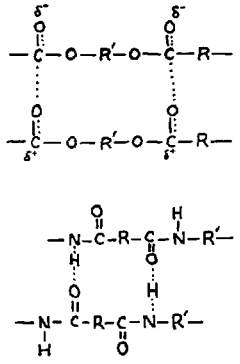
Formulation Factors Affecting Cohesion in Pharmaceutical Film Coatings.—The formulation factors primarily affecting cohesion in pharmaceutical films include polymer chemistry (stereochemistry and functionality) and polymer structural properties (molecular order and crystallinity in the film), solvent effects, the presence of added dispersed solids, and plasticization.

Polymer Chemistry.—The shape of polymeric molecules exerts a strong influence on cohesion in films, since molecular shape largely determines both the diffusibility of a macromolecule or its individual branches or segments, and the strength of its interlacing areas. Macromolecules with a regular structure, provided they are not in a strongly crystalline state, should be more diffusible than molecules with a highly irregular stereochemical structure (8, 9). Branched molecules in which the branching does not greatly hinder diffusion may have a greater cohesive strength than nonbranched equally noncrystalline polymers, based on a firmer anchoring of such macromolecules in the diffusion layer. In a homologous series, lower molecular weight fractions exhibit a greater cohesion, and show a greater change in cohesion strength with temperature changes (5). In strongly polar polymers, self-adhesion by diffusion is insignificant, due to the minimal flexibility and fixed order of the macromolecules caused by the intermolecular forces holding the polymer chains in a fixed form. Proteins and cellulosics are examples of such polymers exhibiting minimal molecular diffusion, proteins tending toward a helix, and cellulosics having a rigid ring structure chain backbone. In crystalline polymer structures, the cohesion between ordered polymer molecules may greatly exceed that possible by the diffusion process in amorphous polymers (10).

The theory of cohesion in polymer films is complex and may involve the concepts both of diffusion and ordered structure. The cohesive strength of the crystalline–amorphous polymers used pharmaceutically is related to the presence, concentration, location, and relative polarity of polar groups along the polymer chain, regularity of chain structure, branching, molecular weight, and molecular weight distribution.

Polymer Structural Properties (Molecular Order and Crystallinity).—A highly ordered, crystalline polymer represents a polymer system of maximum

cohesive structure (compactness) and cohesive strength (represented by crystal strength). The intermolecular forces which promote cohesion, particularly hydrogen bonding, also promote crystallinity (11). While polar groups diminish molecular self-diffusion, strongly polar groups or hydrogen bonding groups (such as —OH and —COOH groups of substituted cellulosics, —COOH groups of pendant carboxyl containing linear polymers such as acrylics, or —CO—NH— groups of polyamides or polypeptides), if they are regularly distributed along the chain, will produce a distinct tendency for the formation of laterally ordered chains (structure I). The closer the polar groups



(A)

(B)

I

The lateral order arrangement (crystalline centers) in films. A, Polyester chains laterally ordered by dipole interaction forces. B, Polyamide chains laterally ordered by hydrogen bonding forces.

are to one another along the chain and the better their lateral fit, the more pronounced will be their effect to promote crystallinity (12, 13). Regardless of the factors promoting molecular order, it is extremely doubtful that any polymer is 100% crystalline, due to the molecular weight distribution found in polymer systems and imperfect chemical and stereochemical repeating molecular structures (14).

Typical noncrystalline polymers include those in which an irregularity of structure occurs, as in copolymers of two or more dissimilar monomer constituents, and in polymers having atactic configurations (a random sequence in the branching of substituents about the polymer chain). There are a few exceptions of atactic polymers such as poly(vinyl alcohol) which do crystallize. This is because certain groups such as CH_2, CHOH, CF_2, and C=O, are small enough to fit into the crystal lattice of the polymer structure. However, most atactic polymers, such as poly(vinyl acetate), due to their stereoirregularity, are noncrystalline and have never been crystallized (15).

The nearly limitless gradations between substantially completely crystalline polymers and truly amorphous polymers are typical of the great majority of pharmaceutically significant polymers which are composed both of distinct crystalline and amorphous phases. Such polymers commonly possess some features leading to disorder, such as atactic configuration, irregular substitution, or bulky side groups which space the individual linear macromolecules apart from each other, plus features leading to an ordered structure, such as a rigid chain or polar substituents. Thus, in addition to having a given chemical molecular structure or range of structures, polymeric fibers and films also have what is termed a fine structure (16) or a supermolecular structure (14). This structure describes the relative co-existing ordered-disordered lateral position or placement of the linear polymer chains or groups of chains in the particular polymer structure, and relates to the general over-all crystallinity of that structure (Fig. 2). The relative degree of molecular chain order to disorder in a film varies with such factors as the method of film application, the solvent system from which the film was cast, and the stresses in the film; and affects such physical properties as film strength, solubility, and miscellaneous mechanical properties (14). The toughness and rigidity of films are favored by a high chain order (14). Crystallinity, independent of molecular weight, is the single effective determinate which is directly related to film stiffness and yield point, and also affects film permeability, flexibility, and brittleness (17–19). In general it is more difficult to correlate the physical properties of a polymer film to a lateral order parameter (crystallinity) for derivatives of a parent polymer, such as cellulose derivatives, than for the parent polymer itself, *i.e.*, cellulose (14).

Solvent Effects (Solvation).—Pharmaceutical polymeric film coatings, almost without exception, are applied to the substrate from colloidal solution in an organic solvent system. During the dissolution of a macromolecular substance, the cohesive forces between the solute macromolecules are neutralized by unions with the solvent molecules (solvation). The more crystalline a polymer, the greater the intermolecular cohesive forces, and the more difficult it will usually be to dissolve such a polymer.

The polymers used in pharmaceutical film coating and other operations, so as not to be inert in the human gastrointestinal tract, are generally polyfunctional polyelectrolytes, containing an aliphatic polymeric nonpolar carbon chain with polar substituents along the chain. Depending on the aqueous pH at which the functional groups ionize and on the rate of ionization, the coating polymer may produce a fast, an enteric, or a prolonged disintegrating or dissolving coating. Polymers which are completely aliphatic (polyethylene), substantially crystalline (pure cellulose), or which contain only nonionizable functional groups or a very high proportion of such groups to ionizable groups (highly esterified carboxyl containing polymers) will be insoluble in water regardless of pH.

The functionality of the polymer also relates to solution properties and to film characteristics. As the functional groups on a linear polymer become ionized during dissolution, the charged groups will repel each other, producing a stretching of the poly-

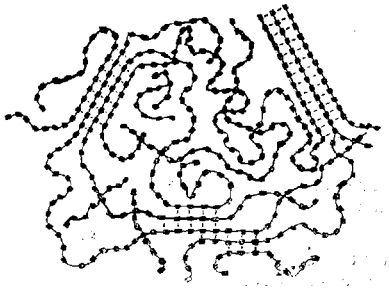


Fig. 2.—The fine structure or supermolecular structure illustrating the laterally ordered (crystalline)–disordered (amorphous) placement of linear polymer chains in a film matrix.

mer chain. The greater the degree of dissociation and the more extensively the chain is charged, the more the chain will uncoil. Simultaneously, the solvation effect, or the interaction between the charged polymer molecules and the molecules of the polar solvent, will increase with the increasing charge on the chain. With increased solvation there will usually be a viscosity increase due to the envelop of solvent surrounding each chain more effectively, keeping the chains from coming into contact and aggregating, and due to the extended configuration and greater spatial requirements of the more completely solvated system.

Polar solvents (Table I) tend to be solvents for polar substances including macromolecular polyelectrolytes (Table II), and nonpolar solvents are generally poor solvents for such polymers. A given polymer is most soluble in solvents that best match the polymer or its solvated derivative in cohesive energy density (Tables I and II) (20). Solubility properties of colloidal macromolecules in organic solvents are complex and depend on chemical, electrical, structural, and steric effects, which lead to mutual interactions between solute and solvent.

Copolymers present a special solubility problem,

TABLE I.—RELATIVE POLARITY AND INTERMOLECULAR ATTRACTION OF COMMON ORGANIC SOLVENTS AND WATER

Solvent Class	Solvent Examples	Dielectric Constant of Solvent (70)	Cohesive Energy Density of Solvent, cal./ml.[a]
Water		78.5[b]	551.1
Glycols		35–50[b]	...
	Glycerol	42.5[b]	...
	Ethylene glycol	37.7[c]	...
	Propylene glycol	35.0[c]	...
Alcohols		15–30[b]	...
	Methanol	32.6[b]	210.3
	Ethanol	24.3[b]	163.4
	1-Propanol	18.3[b]	...
	n-Propanol	20.1[c]	147.5
	1-Butanol	17.8[c]	...
	n-Butanol	17.1[b]	131.8
Ketones		10–20[b]	...
	Acetone	20.7[b]	98.5
Ethers, esters,		0–10	...
aliphatic, and	Dichloromethane	9.1[c]	...
chlorinated	Ethyl acetate	6.0[b]	83.0
hydrocarbons	Chloroform	4.8[c]	85.4
	Dichloroethylene	4.5[c]	...
	Ethyl ether	4.3[c]	54.1
	Trichloroethylene	3.4[c]	...
	Carbon tetrachloride	2.2[c]	73.6
	n-Hexane	1.9[c]	52.4

[a] The cohesive energy densities were calculated from latent heats of vaporization and molecular volumes at 20°. C.E.D. is a measure of the intermolecular attraction and molecular cohesion of a substance and is particularly useful in predicting solvent power for polymers. (See *Reference 72*). [b] Dielectric constants determined at 25°. [c] Dielectric constants determined at 20°. As the dielectric constant decreases, the relative polarity, solvent to solvent, decreases. With a decrease in temperature the dielectric constant increases, and a polar organic solvent may become a better solvent for a polar polymer.

TABLE II.—RELATIVE POLARITY AND SOLUBILITY PARAMETERS (COHESIVE ENERGY DENSITY) OF POLYMERIC MATERIALS

Polymer or Polymer Class	Dielectric Constant (60 cycle) (71)	Cohesive Energy Density, cal./Gm. (73)
	Nonpolar	
Polyethylene	2.3	62
Polystyrene	2.5–2.7	75
Acrylates	3.4–3.6	85
Polyvinyl chloride	3.2–3.6	90
	Intermediate Polarity	
Polyvinyl acetate	...	85–95
Acrylics	3.5–3.8	95
Ethylcellulose	3.2–4.0	...
Polyamides	4–5	...
Cellulose acetate	3.5–7.5	130
Cellulose acetate phthalate	3.5–6.4	...
Cellulose nitrate	6.7–7.3	110
	Polar (soluble in water and polar organic solvents)	
Polyvinyl alcohols	Carboxyvinyl polymers	
Maleic acid copolymers	Hydroxyethyl celluloses	
Polyvinyl pyrrolidone	Carboxymethyl celluloses	
Polyethylene glycols	Methylcelluloses	
	Gelatin	

since virtually all pharmaceutically employed copolymers contain a substantially nonpolar comonomer plus a polar comonomer. Examples of such copolymers, with the nonpolar component given first are: styrene, vinyl alcohol or acrylic acid copolymers; ethylene, maleic anhydride or acid copolymers; acrylate, acrylic acid copolymers.

The solubility of copolymers is generally low in solvents for either homopolymer, but may be high in mixtures of these solvents wherein maximum solvation and extension of the polar and nonpolar comonomer units occur in a polar–nonpolar mixed solvent system. A single solvent of intermediate polarity will not usually be as effective for such copolymers or for a mixture of 2 or more homopolymers which differ widely in polarity as the mixed polarity compound solvent system.

Not only the degree of substitution (D.S.) and the polarity of substituents, but also the space requirements of substituents, may affect the solubility properties of some polymers. The water solubility of cellulosics has been related to the wedging apart of the cellulose ring structure chains by the substituent groups to bare the remaining hydroxyls for hydration (21). Bulky substituent groups, being more effective in wedging the chains apart, enable water solubility to occur at lower degrees of substitution. Methylcellulose is soluble at D.S. 1.3 (22, 23), ethylcellulose at D.S. 0.7, and sodium carboxymethylcellulose at D.S. 0.3 (24). Solubility of cellulosics in organic solvents stems from a predominance of alkyl ether substituent groups over the remaining hydroxyl groups.

As a rule, maximum coating solution solvation and polymer chain extension will produce the most superior films showing the greatest combined strength and cohesiveness. Since solvation and polymer chain extension is reflected in the viscosity of the sol, viscosity provides a useful control measure to (*a*) compare the relative effectiveness of various solvents for a particular polymer or polymer system, and (*b*) appraise the adequacy of solvation and chain extension of a polymer system in a given solvent according to formulation and method of preparation prior to film application. Other physical methods have been used to measure solvation making use of birefringence measurements, vapor pressure data, heats of solution, and infrared absorption spectra (25–27), but the usefulness of such measurements varies with the polymer structure, and the results are often difficult to interpret. As a control procedure of the polymeric sols used in film coating operations, viscosity remains the most simple and direct, although empirical, method of comparing solvation.

Plasticization.—A plasticizer is defined as a substantially nonvolatile, high boiling, nonseparating substance, which when added to another material changes certain physical and mechanical properties of that material. Plasticizers (28–31) are added to polymeric substances for a variety of reasons, but they are especially necessary adjuncts to most polymeric films in order to reduce brittleness, improve flow, impart flexibility, and increase toughness, strength, tear resistance, and the impact resistance of the film coating. The mechanism by which the plasticizer achieves these changes is theorized to be a decrease in the cumulative intermolecular forces along the polymer chains (reduction in cohesion), which generally produces a decreased tensile strength, a lower softening temperature, and a decrease in the glass transition temperature. The plasticizer and polymer are generally thought to be held together by intermolecular secondary valence forces forming a complex or molecular aggregate (31). The lowering of the glass transition temperature below room temperature by plasticization changes a hard, brittle, glass-like material at room temperature to a soft, flexible, and tough material.

Two types of plasticization are recognized (3). External plasticization is the process, thus far described, by which a substance is added to the polymer structure and may be physicochemically associated to it, reducing cohesion in the structure to effectively extend, dilute, and soften the structure. Similar changes can be accomplished by altering the internal chemical structure of the polymer, as by copolymerization, which is known as internal plasticization. The method of plasticization employed with cellulosic and other comonomer polymeric pharmaceutical films is external plasticization. Acrylic, vinyl, styrene, and other polymers which may be readily copolymerized will require little, if any, external plasticization after copolymerization.

The basic requirements of any plasticizer in a polymer system are compatibility and permanence. To be compatible the plasticizer must be miscible with the polymer, indicating similar intermolecular forces in the 2 components. The most effective plasticizers will generally resemble most closely in structure the polymers they plasticize. Thus, water-soluble cellulose ethers (retaining a high ratio of hydroxyls to such ethers) are best plasticized by hydroxyl containing compounds such as glycerin, glycols, and other hydroxy containing compounds. Substantially aliphatic nonpolar polymers are best plasticized by chemically similar materials such as nonsolvent oils. However, since the intermolecular forces in nonpolar polymers, such as polyethylene, are low, it is difficult to find a plasticizer for such materials. Likewise, since the intermolecular forces of strongly crystalline polymers are so high, plasticizers cannot be found with adequately high intermolecular forces to satisfy such polymers and be compatible with them. Effective plasticization is, thus, usually limited to amorphous polymers or to amorphous-crystalline polymers in which the crystalline phase is not predominant.

It cannot be emphasized too strongly that the total film coating formulation of polymer-plasticizer-solvent, plus other components, such as insoluble additives or surfactants to promote spreading, must be considered as primarily affecting, in consort, the nature and properties of the film that is formed. Thus, in considering the 3 fundamental elements of polymer-plasticizer-solvent, not only must the polymer and plasticizer be compatible and the polymer be effectively solvated in the solvent, but the plasticizer must approximately match the solubility properties of the polymer in the solvent system used. In simple systems, since the polymer and plasticizer probably possess common functional groups, solvents can usually be readily selected in which the components are equally readily soluble, thereby preventing premature plasticizer or polymer separation during film dep-

osition and drying. In compound systems in which two or more polymers differing in polarity and chemical type are used with one or more plasticizers in a mixed solvent system, the problem of compatibility becomes complex. If a satisfactory mixed solvent system may be formed which is an azeotrope, the problems of changes in solvent composition during evaporation and premature separation of film components during drying will have been overcome. In nonazeotropic mixed solvent systems, drastic changes in solvent composition during drying with accompanying heterogeneous film component separation may be avoided if solvents can be selected which have somewhat similar vapor pressures at the film-drying temperature.

The permanence requirement of plasticizers in pharmaceutical operations is very important, since it relates to the physical and mechanical stability of the film with time and under stress environmental conditions. External plasticizers, which are physicochemically associated with the polymer by miscibility and by primary or secondary forces, may not readily be leached out of the film matrix and may resist losses by evaporation. The use of the higher molecular weight plasticizers of an effective plasticizer series, which have a lower vapor pressure and lower diffusion rate in the film matrix, will produce a plasticized film of greater permanence. The plasticity of an unmodified as well as of a plasticized polymer film is related to the chemical composition of the polymer (and plasticizer) and to the arrangement, stereochemistry, and forces acting between the chain macromolecules, including intermolecular and internuclear distances, and to the effect of the regularly interposing plasticizer molecules within the molecular polymeric network. These relationships and effects will dictate the proportion in which the plasticizer must be used to produce the desired film properties. Cellulosic polymeric films commonly require 30 to 60% plasticizer, relative to polymer weight for adequate plasticization. Less rigid polymers and copolymers will rarely require the addition of more than 10–20% of an external plasticizer.

Plasticizer efficiency, stability, compatibility, and permanence may be evaluated by a number of semiempirical tests, including measurement of the amount of nonsolvent required to cause phase separation of each component from the polymer-plasticizer solution, viscosity studies, polymer-solvent interaction constants of the solutions, depression of the glass transition temperature, and other physical and mechanical properties of plasticized free film samples. (See under *Mechanical Properties of Films.*) Since these tests are semiempirical they will not all rate a series of plasticizers in the same order. The ultimate selection of a particular plasticizer may also depend on selected physical properties of the plasticizer, *e.g.*, hygroscopicity (which may affect moisture uptake by the film and its effectiveness as a moisture barrier) and water solubility. Plasticizer water solubility is often important in pharmaceutical applications especially when higher plasticizer concentrations are required. A soluble plasticizer may be needed for a soluble coating, and an insoluble plasticizer may be required to produce an enteric or slow release coating.

Plasticizers having pharmaceutical applications include (15, 32): (*a*) phthalate esters, which account for over half of all the plasticizers used industrially; (*b*) phosphate esters, chiefly tricresyl phosphate, which may be restricted to topical film use; (*c*) adipates, azelates, oleates, and sebacates, especially useful for vinyls; (*d*) epoxy plasticizers produced by reacting hydrogen peroxide with unsaturated vegetable oils and fatty acids; (*e*) fatty acid esters from natural sources, which are also useful as extenders to reduce cost or to produce a slowly soluble coating; and (*f*) glycol derivatives which are particularly useful for cellulosics and poly(vinyl alcohol).

Addition of Dispersed Solids.—Frequently the most expensive aspect of film coating is the cost of the organic solvent carrier of the coating polymer. Recovery systems for these solvents in the exhaust air outlets of coating equipment have been prohibitively costly to date. Depending on the molecular weight of the polymeric materials and on the viscosity produced in the organosols, 2–10% w/v of polymer is the usual range of polymer which can be applied as a coating solution. When coating powders, beads, or pilules containing drug, the increased surface of the smaller particles may require that 25–100% of uncoated particle weight be added as film coating material on a dry weight basis. This means that for 1 Gm. of product to be coated from 2.5–50 ml. of coating solution may be required. In such cases solvent costs become extremely high and coating times are unduly long. To combat this problem pharmaceutical scientists have borrowed an approach of the paint industry by adding insoluble particulate fillers or extenders to the film compositions, either dispersed in the film coating polymer solution or dusted on the drug containing particles during coating. Alternate deposition of film coating polymer and dusting powder leads to the deposition of alternate polymer-powder monolayers with relatively low adhesional energies and a consequent loss of film durability (33, 34). The effects of dispersed added solids on polymer film structures, with the dispersed solids having been added from a coat solution containing suspended filler, are generally predictable according to the effect on properties wrought by a decrease in molecular order in the film. The decrease in order may result in plasticization-like effects, while the increased density and solids content in the film may actually increase film strength. Properly formulated fillers and extenders may greatly enhance film dimensional stability, impact resistance, tensile and compressive strength, abrasion resistance, and thermal stability (35, 36).

Desolvation (Gelation).—As the solvent of the applied polymer solution evaporates, an organogel commonly forms at some critical solids concentration during film formation. The most important property, which gives information about the onset of such gelation during drying, is the increase in viscosity with time at constant temperature and during increasing concentration of the colloid. Gelation involves structuration of the organosol system. In the less concentrated organosols prior to solvent evaporation, Brownian movement will be adequately intense and unhindered to keep the aggregates in a continually dispersed condition by the molecular impacts. Upon cooling or with an increasing concentration of polymer in the solution

via solvent evaporation, the power of the impacts diminishes, and the particles can stick together or form a network type of structure. If the chains are asymmetric, as are most of the pharmaceutical polymeric polyelectrolyte materials, they will be joined into a substantially random three-dimensional mesh on gelation, which will immobilize the organic solvent liquid. Another way of visualizing the gel as it forms is by desolvation of the colloidal polymeric material by evaporation of the organic solvent. Since the number of molecules of solvent per molecule of polymer decreases as evaporation proceeds, desolvation of the polymer is continuously affected, and at some point gel growth will occur, and the system will go through an organogel or semigel phase. The dry film will, therefore, represent the final stage of a gel-like aggregate resulting from the progressive evaporation of the volatile solvent.

The rate of desolvation and gelation is a primary function of the evaporation rate of the solvent or compound solvent system (37). A rather rapid rate of desolvation and gelation is usually indicated in pharmaceutical film coating operations to permit more rapid application of the coat, to reduce the duration of the tacky state of the coat during drying on continuous or intermittent application, and to promote simultaneous desolvation of the various coat components. If the coating is being applied from a spray system, the solvent must not evaporate prematurely so as to deposit a spray dried or gelatinized coating on the matrix. The major advantage of the airless spray technique is in circumventing this last problem.

MECHANICAL PROPERTIES OF FILMS

Polymeric films possess mechanical and stress-strain (rheological) properties which are comparable to the viscosity properties of liquids. These film properties relate to such characteristics of film coatings as impact strength, flexural strength, coat stability to temperature change, peel strength, flexibility, and coat resistance to many types of environmental and physical stresses. The stress-strain properties are determined by measuring the linear expansion of standard free test film strips under increasing load forces (38). From the stress-strain curve (40) (Fig. 3) the following physical characteristics of the film samples may be determined: tensile strength, yield point, breaking strength, modulus of elasticity, plastic deformation, and other properties.

The modulus of elasticity, also known as Young's modulus, is the constant of proportionality of stress to strain, and is equal to the slope of the straight-line portion of the stress-strain curve. This parameter is a measure of the "stiffness" of the film, or the ability of the film to withstand a high stress while undergoing little elastic deformation. The greater the slope of the curve and the higher the modulus of elasticity, the stiffer and stronger the film, and the more stress will be required to produce a given amount of deformation.

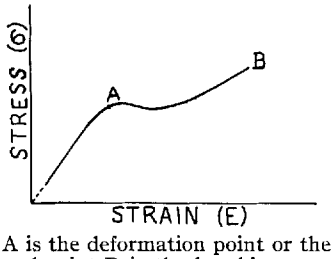


Fig. 3.—Stress (Gm. load/cm.^{2} of surface)—strain (cm. or per cent elongation). Curve of a typical pharmaceutical thermoplastic polymer film structure. Point A is the deformation point or the lower yield point, and point B is the breaking stress, breaking point, or the upper yield point.

Tensile stress is the load per unit area of the original cross section at any instant, usually expressed as force/unit area (39). The maximum tensile stress during a test (A of Fig. 3) is termed the tensile strength. Point A of the stress-strain curve also represents a stress at which the film first undergoes a marked increase in strain without a corresponding increase in stress, representing permanent deformation. This stress value for a standard film sample is termed the "yield point." Sometimes the stress at the deformation point, A, is called the "lower yield point," and the stress at point B, the film break point, is called the "upper yield point." Some materials have no lower yield point since they fail or break before they deform materially. A yield strength may then be determined, which can either be the maximum stress above which the material is damaged or, in comparing a series of films, is the stress of some fixed strain.

The distance between A and B along the stress-strain curve (Fig. 3) indicates the degree of plastic deformation the sample undergoes before breaking. If the film sample has a low yield point and undergoes little deformation before breaking (the distance A to B is short), the film is weak and brittle. Lever and Rhys (40) classify polymeric materials into 4 categories according to the relative height of the yield point on the stress axis and according to the difference between the yield point and the breaking point along the strain axis.

Ordinary substances are little affected by changes in the external environment such as pressure, temperature, humidity, or physical stress, relative to their ordering or crystallinity. Polymers behave quite differently. Environmental factors may appreciably affect the mechanical properties and the stress-strain relationships of certain polymer films, especially polar polymers such as the cellulosics, due to the moisture sorption and swelling of the film structure (41, 42). The effect of such moisture sorption is similar to plasticization; elongation (strain) begins at a lower stress (load), elongation is much greater for given load forces, and the presence of a yield point may not be detectable. The amount of strain (elongation) under stress (load) is also generally significantly increased with a temperature increase (43, 44). Polymer structures also tend to increase greatly in crystallinity with stress. The reasons for this difference lie in the filiform shape and the high internal flexibility of the individual molecules in the macromolecular compounds.

Stress-strain data may be used in comparing film samples as a function of formulation factors, *e.g.*, polymer combinations used, plasticizer, solvent system or surfactant employed, the effect of dispersed solids; or the data after correlation to coat mechanical stability properties on a given substrate may provide a direct indication of coat friability resistance, impact strength, abrasion resistance, and other properties. Munden *et al.* (45)

and Utsumi (46) have recently reported the modulus of elasticity, tensile strength, and per cent elongation of plasticized and unplasticized free films. Kabre (47) reports that the plasticizer is the most important formulation factor affecting mechanical properties of films, and he compared a series of plasticizers for their effect on the mechanical properties of a cellulosic polymer system.

An important mechanical effect of polymeric films, from a pharmaceutical coating standpoint, relates to their surface friction. Hardy (48) has reported on the property of polar organic compounds lowering the coefficient of friction of rubbing solids. Langmuir reported early on the effectiveness of fatty acid films in reducing frictional coefficients (49) and also found that multimolecular layers of film did not significantly reduce the coefficient of friction beyond the reduction produced by a monomolecular film (50). The mechanism of the lowered surface friction is related to the polar film forming groups adhering to the substrate, leaving the more aliphatic and less polar polymer structure exposed to comprise a surface of lower free surface energy, with the film coating initially deposited therefore acting like a boundary lubricant (51). The problems encountered in tablet and particle film coating in conventional equipment due to the lowered coefficient of friction of the coated material, the sliding of the polymer coated contents in the coating pan, and the common necessity for baffling the coating pans is well known. As polymer coat buildup proceeds beyond the monolayer, the problem involved with slipping and sliding of the coating pan contents often diminishes. The reason for this is that as the substrate is covered and the coat builds, there is less polymer orientation to the substrate, polymer deposition in the film is more random, and there is a consequent increase in the coefficient of friction of the coating.

PERMEABILITY PROPERTIES OF FILMS

The permeability of polymeric film coatings involves 3 processes of interest to the pharmaceutical scientist: (*a*) gas diffusion processes, notably oxygen permeation, through the film; (*b*) water and water vapor sorption and permeation (liquid permeation processes); and (*c*) dialysis processes concerned with the dialysis and permeation of soluble components across the intact or modified film.

Munden (45) found an oxygen permeability range of 10^{-4}–10^{-3} Gm. cm./cm.^{2} 24 hr. for the oxygen permeability through free unplasticized films. With a few exceptions, he found an inverse relationship between water vapor transmission and oxygen permeability. Water vapor permeability has been shown to be dependent on the relative polarity of the polymer (52). The more polar films tend to be more ordered and less porous, hence less oxygen permeable; while the less polar films are more porous, permitting the permeation of oxygen but not necessarily of the larger water molecules; and, being more lipophilic, the less polar films have less affinity for moisture and water sorption. Gas permeation through a completely crystalline polymer is known to be negligible. Bent (53) reports that gas permeation is proportional to the volume fraction of the amorphous phase of a film structure,

$$P = P_a X_a$$

where P_a is the premeability for the amorphous phase, and X_a is the volume fraction of the amorphous phase in the film structure.

Lamonde (54), in studying a series of substantially insoluble films, found that the addition of phthalate plasticizers increased water permeability rates with an increasing concentration of plasticizer, and with a decrease in molecular weight of the plasticizers of a homologous series at a constant concentration level. Figure 4 shows the latter relationship for Lucite 46, a 50/50 *n*-butyl/isobutyl methyl acrylate copolymer. The water permeability decrease is related to a water solubility decrease in the plasticizer as one goes up in molecular weight in a homologous series of plasticizers. Even though the difference in water solubility in the plasticizers within the series is small, the slight water sorption by the plasticizer as described by solution theory (55) promotes further sorption, frequently nonideal (56) by the film, related to a clustering tendency of the penetrant molecules in the film (57–59). The addition of other additives, such as surfactants, to a film of ethylcellulose has been reported to produce clustering centers for water sorption (60).

The relationship between the assorted formulation factors and oxygen, and water vapor or water permeability and actual stability parameters of coated products remains to be established.

Dialysis and liquid permeability properties may be important to both fast and slow releasing film coating formulations. Some present commercial pharmaceutical tablet film coatings, presumably composed of cellulose acid phthalate and polyethylene glycol, release medicament substantially completely within 1 hr. through an intact film. An obvious application with marked advantages of

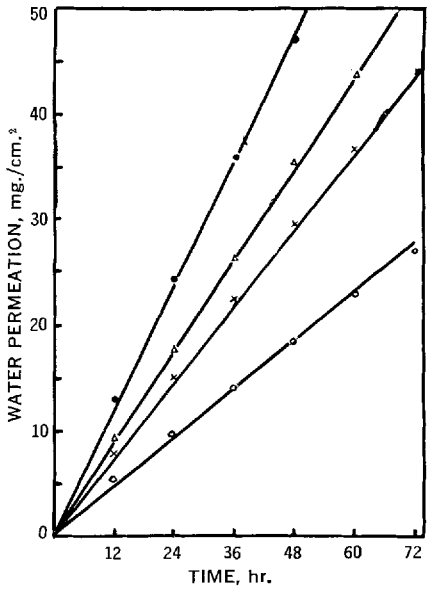


Fig. 4.—The effect of plasticizer composition (molecular weight and substitution) on the water permeability of *n*-butyl/isobutyl methacrylate (Lucite 46) copolymer films containing 15% plasticizer. Key: ○, unplasticized; ×, dioctyl phthalate; △, dibutyl phthalate; ●, diethyl phthalate.

liquid permeable and dialyzing film coatings of controlled release rates is in the sustained-release field.

Glass powder beads are reported to increase the glass transition temperature (Tg) of polyisobutylene, polyurethane (61), polystyrene, and poly (methyl methacrylate) (62), and titanium dioxide is reported to have the same effect on the Tg of other polymers (63). The increase in glass transition temperature appears to depend on the volume fraction of the filler in the system with the effect being attributed to the immobilization by adsorption of the polymer segments close to the surface of the filler particles. Carbon black did not affect the Tg of several polymers studied (64). Polar titanium dioxide as well as nonpolar fillers substantially reduce the sorption of organic vapors by poly (vinyl acetate), even at very low vapor pressures (65). The absorption isotherms of filled polymer films closely resembled that of the pure polymers below their glass transition temperatures, which also was attributed to immobilization of polymer segments at the solid surface.

As more is learned about methods of controlling the film membrane diffusion coefficients of pharmaceutical films as functions of added dispersed solids, film structure and orientation, salt concentration, ion ratios, film membrane–solution interactions (65–67), acid and base concentrations (68), and liquid boundary layer thickness (69), and according to formulation and methods of application, this physicochemical approach to controlled drug release will certainly advance, and the utility of polymeric films as more effective protective coatings may be exploited more completely.

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Some Spray-Dried Formulations of Sulfaethylthiadiazole for Prolonged-Release Medication

By AHMED F. ASKER* and CHARLES H. BECKER

Aqueous and organic solvent solutions containing sulfaethylthiadiazole and dissolution-retarding materials were spray dried with the purpose of preparing sulfaethylthiadiazole in prolonged-release form. Shellac, cellulose acetate phthalate, Glycowax S-932, Castorwax MP 80, aluminum monostearate, and glyceryl monostearate were used as the dissolution-retarding materials. Microscopic examination and infrared spectral analysis were carried out on some of the spray-dried products. An *in vitro* evaluation of the antibacterial activity of spray-dried sulfaethylthiadiazole was compared with an original sample. Dissolution characteristics of the products were studied *in vitro,* and one product was evaluated *in vivo* by urinary excretion data.

THE PHARMACEUTICAL industry has in the last decade become increasingly active in the manufacture of oral dosage forms intended to impart prolonged, sustained, or long-acting therapeutic effects. Spray drying as a method of processing such products has received little attention. Although the process is mentioned in the patent literature, detailed information is not given.

Spray drying usually results in the production of free-flowing monodispersible particles which could be directly compressed into tablets, filled into capsules, or made into suspensions.

The technique of spray drying suspensions, as shown by literature reports, necessitates the use of micronized drug, the maintenance of stirring the feed throughout the operation, and the use of suspending agents to maintain a uniform suspension. There also appears to be a limitation concerning the amount of solids which can be suspended to give a satisfactory spray. Therefore, solutions rather than suspensions were used in this study. The use of solutions also adds simplicity to the process of manufacture, thus insuring better reproducibility of product.

Aqueous ammonia has been widely used as a solvent for shellac. Ammonia was reported to volatilize when an ammoniacal solution of shellac was used in coating tablets (1). For some sulfonamides, such as sulfadiazine and sulfathiazole, Higuchi *et al.* (2) demonstrated that, when their ammonium salt solutions were spray dried, ammonia volatilized, and the drug reverted to its original form. Use was made, therefore, of the solvent action of aqueous ammonia on shellac and sulfaethylthiadiazole to prepare clear solutions, which were then spray dried.

Malm *et al.* (3) reported the formation of soluble sodium and triethanolamine salts of cellulose acetate phthalate. Its ability to form an ammonium salt was utilized in this study for the same reasons mentioned for shellac.

In the case of Glycowax S-932,[1] Castorwax MP 80,[2] and glyceryl and aluminum monostearate, a solvent mixture of 1 part by volume of absolute alcohol and 3 parts of chloroform was used to dissolve both the drug and the dissolution-retarding material with the aid of gentle heat.

EXPERIMENTAL

Materials.—Sulfacthylthiadiazole (SETD) was obtained from the American Cyanamid Co. The dissolution-retarding materials used included white dewaxed shellac,[3] cellulose acetate phthalate,[4] Glycowax S-932, Castorwax MP 80, aluminum monostearate U.S.P., and glyceryl monostearate N.F.

Preparation of Solutions.—In the case of shellac and cellulose acetate phthalate, their ammonium salt solutions were mixed with solutions of the ammonium salt of SETD prepared by essentially the same method described by Higuchi *et al.* (2). Glycowax S-932 and Castorwax MP 80 were dissolved in warm solutions of a mixture of 1 part by volume of absolute alcohol and 3 parts of chloroform in which the SETD was previously dissolved.

Preparation of Spray-Dried Materials.—The solutions were spray dried in a Bowen table model spray dryer. The solutions were fed by gravity and the feed rate controlled by means of a glass stopcock. The resulting products were screened through a 100-mesh sieve, transferred to tight containers, and stored at room temperature. Table I

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[1] Glycowax S-932 is a product of Glyco Chemicals, Williamsport, Pa.
[2] Castorwax MP 80 is a product of the Baker Castor Oil Co., Bayonne, N. J.
[3] Bradshaw-Praeger and Co., Chicago, Ill.
[4] Marketed as C-A-P by Eastman Kodak Co., Rochester, N. Y.

TABLE I.—OPERATING CONDITIONS FOR SPRAY DRYING THE SOLUTIONS OF SETD AND DISSOLUTION-RETARDING MATERIALS

Soln.	SETD-Dissolution-Retarding Material Ratio	Feed Solids, % w/v	Feed Rate, ml./min.	Temp. Ranges, °C. Inlet	Outlet	Cooling Ports
SETD–shellac	1:1	3.8	12	210–216	38–40	Partially open
	1:2	4.3	18	210–216	38–40	Partially open
	1:3	4.0	13	210–216	37–39	Partially open
SETD–cellulose acetate phthalate	1:1	5.7	30	220–223	36–38	Partially open
	1:2	5.0	30	216–221	37–38	Partially open
	1:3	4.7	42	216–221	38–40	Partially open
SETD–Castorwax MP 80	1:1	8.0	16	138–149	36–38	Open
	2:3	8.0	18	143–149	37–38	Open
	1:2	6.8	20	143–149	37–38	Open
SETD–Glycowax S-932	1:1	6.7	12	171–174	38–40	Open
	2:3	7.5	20	171–173	37–39	Open
	1:2	4.7	18	171–175	36–39	Open

shows feed concentrations and operating conditions for spray drying the solutions made.

Microscopic Examination of the Spray-Dried Materials.—Microphotographs were taken of the spray-dried formulations which consisted of 1 part of the drug and 2 parts of the dissolution-retarding material. The characteristic particle shape and size are seen in Figs. 1–4.

In Vitro Evaluation of the Antibacterial Activity of SETD.—The agar cup-plate method of assay (4) on samples of original SETD and spray-dried ammonium salt of SETD was carried out against *E. coli* and *S. aureus*. A microstatistic advocated by Dixon and Massey (5) was utilized in the evaluation of the results at $\alpha = 0.05$. The results obtained are shown in Table II.

Infrared Spectral Analysis.—Infrared spectral analysis of original SETD, shellac, cellulose acetate phthalate, and their spray-dried ammonium salts was performed by the potassium bromide pelleting technique on a Perkin-Elmer Infracord model 137.

In Vitro Dissolution Studies.—The *in vitro* release patterns of the spray-dried products were determined in 0.1 *N* HCl (pH 1.1) and alkaline pancreatin solution (pH 8.3). The method used is essentially the same as that given by Robinson and Swintosky (6). The rotating-bottle apparatus described by Souder and Ellenbogen (7) operating at 40 r.p.m. in a 30 ± 0.5° water bath was employed. The amount of SETD released at various time intervals was determined in the filtrate by the Bratton and Marshall (8) colorimetric assay.

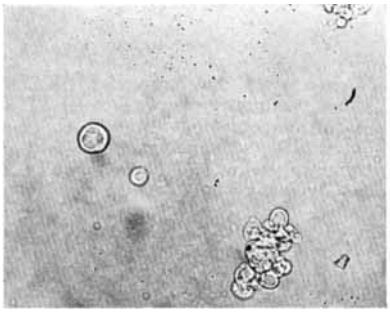

Fig. 1.—Spray-dried SETD (1 part)–cellulose acetate phthalate (2 parts).

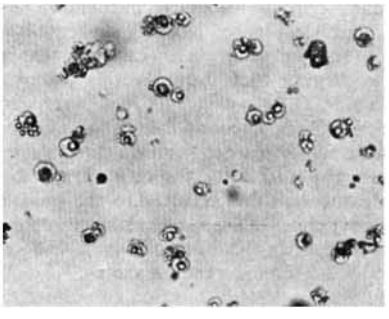

Fig. 3.—Spray-dried SETD (1 part)–Glycowax S-932 (2 parts).

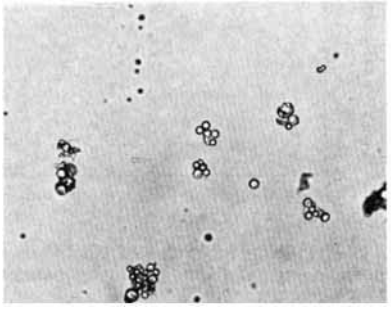

Fig. 2.—Spray-dried SETD (1 part)–shellac (2 parts).

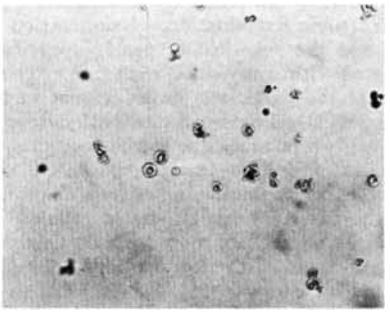

Fig. 4.—Spray-dried SETD (1 part)–Castorwax MP 80 (2 parts).

TABLE II.—COMPARATIVE INHIBITORY ACTI OF ORIGINALVITY AND SPRAY-DRIED SETD USING *E. coli* AND *S. aureus*—EXPERIMENTAL DATA AND STATISTICAL EVALUATION

Sample	*E. coli* Zones of Inhibitions, mm.	*E. coli* Av. Diam., mm.	*E. coli* Range, mm.	*S. aureus* Zones of Inhibition, mm.	*S. aureus* Av. Diam., mm.	*S. aureus* Range, mm.
SETD	37 35 39 39 38	37.6	4	40 40 39 38 39	39.2	2
SETD (spray-dried)	37 36 37 35 38	36.6	3	37 38 39 39 38	38.2	2
	$t_d = 0.286$			$t_d = 0.5$		
	Critical region $t_d > \pm 0.613$			Critical region $t_d > \pm 0.613$		
	Significant difference No			Significant difference No		

The *in vitro* release curves of products consisting of 1 part of SETD by weight and 2 parts of the dissolution-retarding materials are shown in Figs. 5 and 6.

In Vivo Evaluation.—The method is basically the same as that described by Nicholson *et al.* (9) for the clinical evaluation of sustained-release tablets of SETD using urinary excretion studies. Four healthy adult male subjects were utilized in the evaluation of the spray-dried formulation of SETD–Glycowax S-932 which gave a satisfactory *in vitro* release pattern as compared with a sustained-release powder of SETD.[5] Urine samples were collected at various time intervals, and the amounts of free SETD excreted were determined by the Bratton and Marshall method (8).

RESULTS AND DISCUSSION

Preparation of Spray-Dried Materials.—Solutions of the dissolution-retarding materials were spray dried in order to study their behavior during the operation. The spray-dried material also served as blanks for the *in vitro* dissolution studies.

Shellac, cellulose acetate phthalate, Glycowax S-932, and Castorwax MP 80 presented no difficulties in spray drying. This behavior was reflected in spray drying their formulations containing the drug (Table I).

Difficulty was encountered in spray drying an aluminum monostearate solution due to its gelatinization in the feed line. Spray drying of a glyceryl monostearate solution resulted in the production of sticky particles. These difficulties were not overcome by operating the dryer over a wide range of feed rates and inlet gas temperatures.

The ammonium salt solution of sulfaethylthiadiazole was spray dried to serve as a control. The spray-dried material was found to have a melting point of 185–186.5° which is in agreement with the literature value for sulfaethylthiadiazole.

Microscopic Examination.—Examination of Figs. 1–4 shows the spherical or nearly spherical shape associated with spray-dried materials. The porous nature of the particle is quite evident in the case of the SETD–cellulose acetate phthalate combination. This is possibly due to the good film-forming properties of cellulose acetate phthalate with the result that a case-hardening effect was produced.

In the case of formulations of cellulose acetate phthalate and shellac, more isolated particles were obtained than with formulations of Glycowax S-932 and Castorwax MP 80, of which a greater portion was in the form of aggregates. This may be expected from wax or wax-like materials because of the tendency of wax particles to adhere to each other.

In Vitro Evaluation of the Antibacterial Activity of Spray-Dried SETD.—Although it is accepted that heat degradation does not occur during spray drying, Smith (10) pointed out that the spray of the smallest particles is dried faster than the main portion of the spray; therefore, the smallest par-

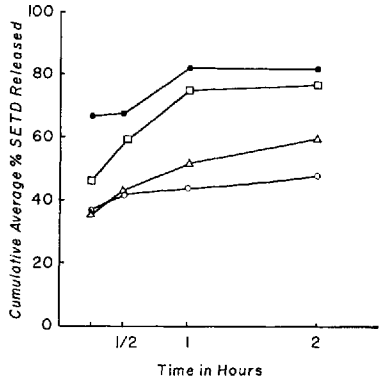

Fig. 5.—*In vitro* dissolution of SETD in 0.1 *N* HCl from products consisting of SETD (1 part) and dissolution-retarding materials (2 parts). Key: ○, SETD–shellac; ●, SETD–cellulose acetate phthalate; □, SETD–Castorwax MP 80; △, SETD–Glycowax S-932.

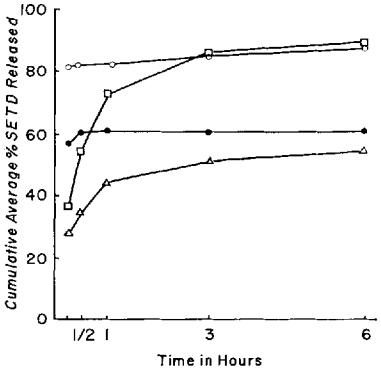

Fig. 6.—*In vitro* dissolution of SETD in alkaline pancreatin solution from products consisting of SETD (1 part) and dissolution-retarding materials (2 parts). Key: ○, SETD–shellac; ●, SETD–cellulose acetate phthalate; □, SETD–Castorwax MP 80; △, SETD–Glycowax S-932.

[5] Supplied by Smith Kline & French Laboratories, Philadelphia, Pa.

TABLE III.—PHYSIOLOGIC AVAILABILITY OF SETD BASED ON FREE SETD FROM SETD–GLYCOWAX S-932 (1:2) COMBINATION IN FOUR SUBJECTS

Collection Time Interval, hr.	Plain SETD — SETD Excreted, mg. Subj. A	B	C	D	SETD–Glycowax S-932 — SETD Excreted, mg. Subj. A	B	C	D
0–3	486	676	150	495	8	...	...	8
3–6	810	323	414	931	22	247	36	53
6–9	429	1001	544	522	23	635	223	275
9–12	293	707	359	343	88	[a]	354	125
12–15	214	[a]	297	203	95	128	244	63
15–24	386	233	477	358	552	328	376	445
24–48	387	321	386	184	1129	383	202	481
48–72	52	...	101	37	289	120	...	224
Total	3057	3261	2728	3073	2206	1841	1435	1674
Physiologic availability based on the 72-hr. period					72.2%	56.5%	52.6%	54.5%

Av. physiologic availability = 59%
S. D. ± 8.97%

[a] No urine was voided.

ticles approach the inlet gas temperature and could be damaged by heat. In order to study the effect spray drying might have on the antibacterial activity of SETD, the agar cup-plate method was carried out. The results obtained appeared to show no significant difference between original SETD and spray-dried ammonium salt of SETD when tested against *E. coli* and *S. aureus* (Table II).

Infrared Spectral Analysis.—The infrared spectrum of the spray-dried ammonium salt of SETD compared well with that of the original sample of SETD, suggesting no apparent changes in the chemical constitution of the drug during spray drying. This was also confirmed by the melting point determination. Spectra of the spray-dried ammonium salts of shellac and cellulose acetate phthalate, however, were not comparable with the original materials. The complex constitution of shellac, the possibility of its polymerization during spray drying, and the polymeric nature of cellulose acetate phthalate may account for these differences.

The spectral analysis provided by this study was not sufficient to support a conclusion of more significance, but it gives some insight of the apparent changes produced by spray drying.

In Vitro Dissolution Studies.—The *in vitro* screening test was carried out in order to identify products worthy of *in vivo* evaluation (Figs. 5 and 6).

SETD–shellac formulations gave substantial retardation of release when tested in 0.1 *N* HCl. The higher the proportion of shellac to the drug, the more retardation was obtained. However, no significant retardation was demonstrated by formulations of SETD–shellac in alkaline pancreatin solution, regardless of the amount of shellac to drug used in this study. This may be attributed to the formation of highly soluble salts of shellac in alkaline pancreatin solution. This insignificant retardation was amplified by the use of dewaxed shellac and the small porous particles obtained in spray drying.

SETD–cellulose acetate phthalate formulation demonstrated more release of the drug in 0.1 *N* HCl as compared to those of SETD–shellac combinations. However, less dissolution of drug was obtained in alkaline pancreatin solution.

No appreciable retardation of drug release was demonstrated by SETD–Castorwax MP 80 in either 0.1 *N* HCl or alkaline pancreatin solution. This may be attributed to the slightly hydrophilic nature of Castorwax MP 80 as compared to other waxes due to hydroxy acid esters present.

SETD-Glycowax S-932 formulations showed a significant retardation of release of the drug in 0.1 *N* HCl and in alkaline pancreatin solution especially at a concentration of 1 part of the drug and 2 parts of the wax. The *in vitro* release pattern of this combination compared within a reasonable range with that of a sustained-release control sample (supplied by Smith Kline & French Laboratories) consisting of 2 parts of SETD and 3 parts of Castorwax MP 80. This formulation was therefore utilized in the *in vivo* evaluation.

In Vivo Evaluation.—Evaluation of SETD–Glycowax S-932 (1:2) by urinary excretion data showed that an insignificant amount of drug appeared to be released in the first 3 hr. after drug administration (Table III and Fig. 7).

Results also showed that less drug appeared to be released *in vivo*, contrary to what was expected from the *in vitro* screening results. This emphasizes the fact that *in vitro* testing does not necessarily reflect *in vivo* response. This was also reported by Blythe (11), who found that although sulfaethylthiadiazole in sustained liquid or tablet form

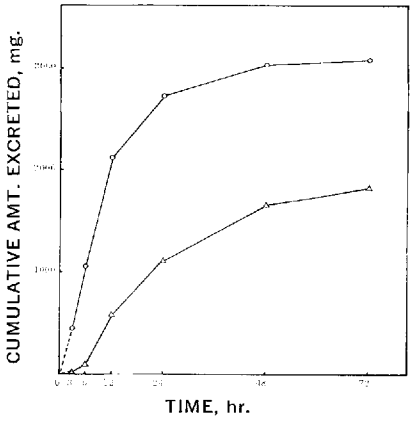

Fig. 7.—Average cumulative urinary excretion of free SETD for 4 humans receiving a 3.9-Gm. oral dose of SETD in plain form (○) and SETD–Glycowax S-932 combination (△).

gave substantially different release rates *in vitro*, yet both gave practically identical blood levels when tested in humans.

The fact that little or no drug was released in the first 3 hr. after drug administration, suggests that plain SETD can be added to the initial dose of the SETD–Glycowax S-932 combination without complications as far as product design is concerned.

Since less drug was released from the SETD–Glycowax S-932 (1:2) combination, a lower proportion of the wax appears necessary to obtain a medication that would meet the requirements for a more desired prolonged-release product.

The average physiologic availability of the drug from SETD–Glycowax S-932 combination tested *in vivo* was 59% after 72 hr. This low physiologic availability would reflect the incomplete release of the drug as indicated by urinary excretion data.

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Automated Differential Amperometric Analyzer

Application to Penicillin Determination

By JOSEPH BOMSTEIN, J. M. SHEPP, S. T. DAWSON, and W. J. BLAEDEL*

An automated, differential amperometric analyzer has been constructed, based on the tubular platinum electrode and a differential signal detection system. Its chief advantages are sensitivity to species which are oxidizable or reducible at the platinum electrode, and a linear relationship between concentration and signal. As applied to the iodimetric determination of several penicillins, precision is 0.7–2.6 per cent and accuracy ranges from 1.0 to −3.0 per cent.

CONSIDERABLE progress has been made in recent years in automating analytical laboratory processes. A number of reports deal with automation of 1 or more steps of analytical procedures, and outstanding success has been achieved industrially with the AutoAnalyzer (Technicon Instruments Corp., Chauncey, N. Y.), generally applying spectrometric techniques. A greater range of applications might be handled by combining the instrument's continuous-flow and chemical-physical processing capabilities with an electrometric detector. Redox analyses could then be performed. Such a system has been designed, constructed, and evaluated for the iodimetric determination of penicillins.

The method involves degradation of the penicillin with alkali or penicillinase, followed by iodimetric determination of the resulting penicilloic acid (1). A published procedure, in which a penicillin stream after hydrolysis and dialysis reacts with iodine to cause an absorbance decrease (2), has been modified and used to prove the practicality of an amperometric technique.

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EXPERIMENTAL

Differential Electrode Detection System.—The detector is a differential electrode system based on the tubular platinum electrode (TPE) (3, 4). Figure 1 shows the pair of platinum electrodes and the saturated calomel reference electrode. The sample stream enters one side of the Y-tube, flowing past a pulse suppression overflow tube, then through 0.5 in. length of platinum tubing (i.d. 0.06 in., o.d. 0.07 in.) which serves as 1 arm of a Wheatstone bridge. (See TPE_1 in Fig. 2.) The reference stream, which serves the purpose of blank compensation, flows past a second overflow tube, then through a second TPE identical to the first. This is the arm of the bridge TPE_2, in Fig. 2. The two streams combine to give a conducting path for current flow through the saturated-calomel reference electrode (SCE), physically separated from the flowing stream by a porous glass wall (code 7730 Vycor, Corning). Impurities introduced by diffusion into the SCE are displaced by a flow of saturated KCl/Hg_2Cl_2. The ground connections are added to improve electrical stability.

Bridge Circuit.—Figure 2 shows the schematic diagram of the bridge to which the signal from the electrodes is applied. Except for the replacement of the microvoltammeter and recorder by the VOM-6 recorder (Bausch & Lomb, Rochester, N. Y.) alone, it is identical to the bridge discussed by Blaedel and Olson (3). Initial balance is obtained

gave substantially different release rates *in vitro*, yet both gave practically identical blood levels when tested in humans.

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By JOSEPH BOMSTEIN, J. M. SHEPP, S. T. DAWSON, and W. J. BLAEDEL*

An automated, differential amperometric analyzer has been constructed, based on the tubular platinum electrode and a differential signal detection system. Its chief advantages are sensitivity to species which are oxidizable or reducible at the platinum electrode, and a linear relationship between concentration and signal. As applied to the iodimetric determination of several penicillins, precision is 0.7–2.6 per cent and accuracy ranges from 1.0 to −3.0 per cent.

CONSIDERABLE progress has been made in recent years in automating analytical laboratory processes. A number of reports deal with automation of 1 or more steps of analytical procedures, and outstanding success has been achieved industrially with the AutoAnalyzer (Technicon Instruments Corp., Chauncey, N. Y.), generally applying spectrometric techniques. A greater range of applications might be handled by combining the instrument's continuous-flow and chemical-physical processing capabilities with an electrometric detector. Redox analyses could then be performed. Such a system has been designed, constructed, and evaluated for the iodimetric determination of penicillins.

The method involves degradation of the penicillin with alkali or penicillinase, followed by iodimetric determination of the resulting penicilloic acid (1). A published procedure, in which a penicillin stream after hydrolysis and dialysis reacts with iodine to cause an absorbance decrease (2), has been modified and used to prove the practicality of an amperometric technique.

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EXPERIMENTAL

Differential Electrode Detection System.—The detector is a differential electrode system based on the tubular platinum electrode (TPE) (3, 4). Figure 1 shows the pair of platinum electrodes and the saturated calomel reference electrode. The sample stream enters one side of the Y-tube, flowing past a pulse suppression overflow tube, then through 0.5 in. length of platinum tubing (i.d. 0.06 in., o.d. 0.07 in.) which serves as 1 arm of a Wheatstone bridge. (See TPE_1 in Fig. 2.) The reference stream, which serves the purpose of blank compensation, flows past a second overflow tube, then through a second TPE identical to the first. This is the arm of the bridge TPE_2, in Fig. 2. The two streams combine to give a conducting path for current flow through the saturated-calomel reference electrode (SCE), physically separated from the flowing stream by a porous glass wall (code 7730 Vycor, Corning). Impurities introduced by diffusion into the SCE are displaced by a flow of saturated KCl/Hg_2Cl_2. The ground connections are added to improve electrical stability.

Bridge Circuit.—Figure 2 shows the schematic diagram of the bridge to which the signal from the electrodes is applied. Except for the replacement of the microvoltammeter and recorder by the VOM-6 recorder (Bausch & Lomb, Rochester, N. Y.) alone, it is identical to the bridge discussed by Blaedel and Olson (3). Initial balance is obtained

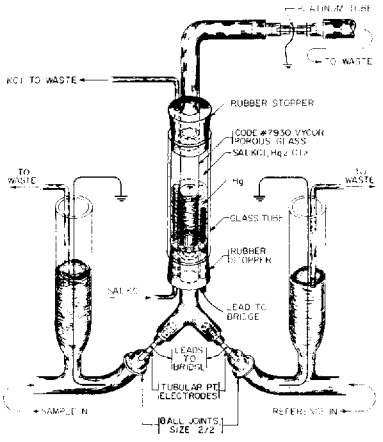

Fig. 1.—Electrode system.

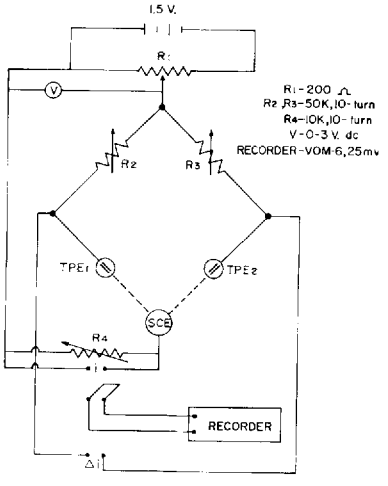

Fig. 2.—Bridge circuit. Key: —, wiring; – – –, solution (conducting) path.

by adjusting R_2 and R_3 while the blank solution is flowing through both TPE's. R_1 is set to a potential suitable for the amperometric measurement of iodine, as determined by the nature of the chemical system. When the sample replaces the blank in TPE_1, the balance between the 2 diffusion currents is disturbed, resulting in a displacement of the recorder pen when the switch is closed in the Δi-position. Closing the switch in the i position provides a convenient way to measure total current flow in order to select the proper recorder range. Impedance matching with the VOM-6 recorder requires the use of a millivolt range.

Pulse Suppression.—The reagent pump in this automated analyzer is peristaltic, and capable of long-term operation with excellent reproducibility over a wide range of speeds. The peristalsis, however, causes a pulsing flow of the reactive species through the electrodes, inducing a corresponding pulsing electrical signal at the recorder. Even careful matching of blank and sample channels is not adequate to remove the pulse effect when high recorder sensitivity is used. In order to provide a smooth base-line and sample record, a pulse suppression system is introduced. A flow-control pump (model PA-56, New Brunswick Scientific Co., New Brunswick, N. J.) is installed at the exit end of the system, pumping out at a rate less than the combined rate of the reagent, blank, and sample influent streams. The resulting difference in pressure causes an overflow from each channel into a chamber (10-ml. syringe body) which is open to the atmosphere at the other end. The liquid head is held at a predetermined level by pumping off the excess liquid to waste through the same pump used to supply electrolyte to the reference electrode. (See Figs. 1, 3, 4.)

Application to Penicillins.—The flow system for determining penicillins is shown in Fig. 3, and the relationship among the basic units is shown in Fig. 4. For test purposes, samples were fed manually, omitting any programming device. The reagent pump is the Harvard model 600-1200 (Harvard Apparatus Co., Dover, Mass.) having dual peristaltic channels pumping 180° out of phase. To accommodate the 8 (standard instrument) pump tubes required, an adjustable slotted rack is mounted on top and is positioned to hold 4 tubes tautly in each channel. (See Fig. 5.) Penicillin solution is continuously aspirated in a single tube and is split at a tee before passing through sample and blank lines in the Harvard pump. Successive samples are separated by allowing air to enter the pick-up tubing. In order to insure equal pump rates in blank and sample streams, the reagents are paired in each channel, *e.g.*, both KIO_3 streams are pumped in the same channel, etc. Despite this precaution, a small time difference may arise in the arrival of the streams at the TPE's. This difference

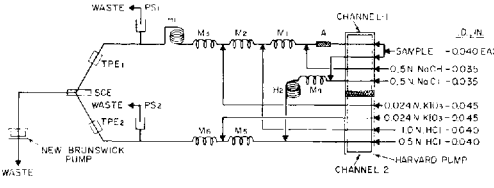

Fig. 3.—Iodimetric determination of penicillins.

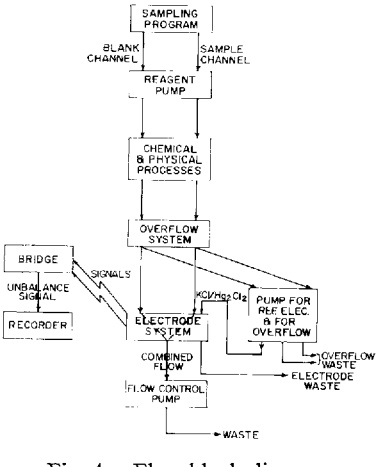

Fig. 4.—Flow block diagram.

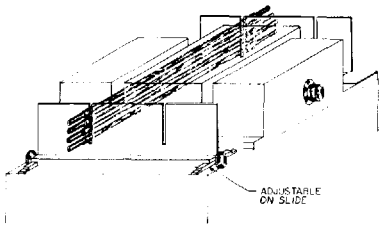

Fig. 5.—Pump modification.

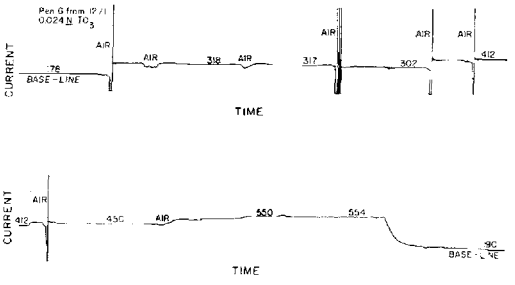

Fig. 6.—Typical amperometric recording.

can be minimized by extending or shortening 1 path as required, by means of a glass-in-Teflon telescoping sleeve arrangement at A (Fig. 3).

On the sample side, NaOH is added, and 3 large mixing coils at M_1 permit hydrolysis to proceed for 15 min. HCl is added to neutralize the alkali and to provide enough of an excess to liberate all available iodine from the KIO_3 which is added next. M_2 and M_3 are small mixing coils. The stream passes through a 12-min. holding coil H_1 to permit iodination, and is finally pumped through TPE_1, and SCE to waste. On the blank side, the sample is first mixed (mixer M_4, holding coil H_2) with NaCl equivalent to that produced on the sample side by the reaction of alkali with acid. Excess acid is added next, mixed at M_5, and KIO_3 is added finally to produce the same concentration of iodine at TPE_2 that would exist at TPE_1 in the absence of hydrolyzed penicillin. M_4, M_5, and M_6 are single mixing coils, and H_2 is made long enough to equalize the total path length of both streams. All mixers and holding coils are standard instrument parts. Tubing up to M_3 on the sample side and M_6 on the blank side is standard instrument transmission tubing; beyond these points the system is built of glass and Teflon to minimize absorption of iodine, except for the portions carrying the streams to waste.

The TPE potential is set at −50 mv. with respect to the SCE, recorder range at 25 mv. full-scale, and resistors R_2, R_3 at about mid-range. Reagents are those indicated in Fig. 3, except that the iodate contains 13.3 Gm. of KI per liter, required for the reaction $IO_3^- + 5\ I^- + 6\ H^+ = 3\ I_2 + 3H_2O$. Standards are prepared to cover the range 200–500 mcg. penicillin/ml. (Other operating conditions could have been chosen to accommodate lower or higher concentrations.) Adjustment of the pumps is important, and is accomplished as follows. The Harvard pump is set for 24 r.p.m., and the platen is moved toward the pumping fingers until flow through the tubing is just perceptible. The platen control knob is tightened 2 divisions more and is then locked in place. The New Brunswick pump is operated at 56 r.p.m., and the backing plate is tightened until the liquid levels in the pulse-suppressors are barely rising. The plate is then locked in position. The sampling pattern for penicillin determinations was chosen to provide maximum stability at the electrode and to minimize mixing between successive samples. Data reported here were obtained with the following manual program: sample aspiration, 2 min.; air, 15 sec.; sample, 5 min.; air, 15 sec. These 4 steps are repeated for each succeeding sample.

RESULTS

Penicillin G, oxacillin, phenethicillin, and ampicillin were determined successfully by the amperometric technique. Data were obtained by measuring the differences between diffusion current differentials recorded for samples and base-line, the latter recorded when samples were replaced by water in both channels. A typical trace is shown in Fig. 6.

Table I shows good precision and accuracy for several determinations on penicillin G, oxacillin, phenethicillin, and ampicillin, compared to Auto-Analyzer values determined colorimetrically (2). Instrumental response is linear (Fig. 7), plots of diffusion current against sample concentration giving straight lines within experimental error. Under these conditions, sensitivity is about 10–15 mcg./ml., and a range of 1–2 mcg./ml. appears attainable.

Data for the other penicillins show respective precision and accuracy of 1.0 and −0.2% for oxacillin, 0.7 and 1.0% for phenethicillin, and 2.6 and −3.0% for ampicillin. Methicillin could not be analyzed, probably because of its instability in acid solutions (5).

DISCUSSION

Advantages and Disadvantages.—Two major advantages accrue to the amperometric system: (*a*) linearity, the signal arises from the diffusion current, which is linear with concentration, and this eliminates the spectrometric problem of working with a logarithmic function; (*b*) measurement of spectroscopically inactive species which are reducible or oxidizable at the platinum electrode. Specificity may be obtained by proper choice of applied potential, electrode material, masking reactions, and solvent systems.

The measurement of a difference signal also permits simultaneous handling of the sample and its blank. In principle, the same advantage may be utilized in spectrophotometric analysis, although in practice this technique has not yet been widely applied in routine determinations.

Several difficulties have also been encountered with the amperometric system. Some are due to the choice of iodimetry for the application, but some are inherent in the electromechanical system. Chief among the latter are: (*a*) peristaltic pulsing. As discussed earlier, suppression of pulsing results in some experimental complexity. Considerable improvement in sensitivity was available for the

TABLE I.—PRECISION AND ACCURACY

Precision			
Penicillin G, u./mg.	Oxacillin, mcg./mg.	Phenethicillin, mcg./mg.	Ampicillin, mcg./mg.
1345, 1400	894, 894	897, 920	826, 826
1319, 1325	833, 826	879, 878	820, 852
1337, 1343	874, 872	883, 878	853, 803
1375, 1375	914, 914	883, 878	776, 758
1391, 1388	871, 871	862, 861	824, 815
1315, 1361	878, 874	847, 858	860, 831
1296, 1299	852, 813	870, 865	908, 888
1322, 1365	827, 848	883, 884	841, 903
1380, 1293	852, 850	811, 816	880, 875
1368, 1313	871, 854	820, 830	...
1351, 1283	852, 843	805, 815	...
Rel. S. D. 2.3%	1.0%	0.7%	2.6%

Accuracy[a]							
Penicillin G, u./mg.		Oxacillin, mcg./mg.		Phenethicillin, mcg./mg.		Ampicillin, mcg./mg.	
Found	AutoAnal.	Found	AutoAnal.	Found	AutoAnal.	Found	AutoAnal.
1373	1385	894	835	909	885	816	860
1322	1380	828	835	879	860	844	875
1340	1410	872	865	883	885	813	845
1375	1445	912	875	880	885	761	835
1390	1430	873	890	861	850	825	885
1338	1450	878	875	853	850	851	835
1298	1320	833	855	868	860	904	885
1344	1340	838	855	883	870	881	895
1337	1335	851	850	...	...	882	895
1341	1335	863	885	...	...	...	...
1317	1345	846	890	...	...	...	...
Mean error −37		−2		+9		−26	
Rel. error −2.7%		−0.2%		+1.0%		−3.0%	

[a] Averages of duplicates.

penicillins, but it is likely that the ratio of pulse-height/signal imposes a limitation. (*b*) Sample diffusion. In extended systems, such as is required in the determination of penicillins, diffusion occurs between adjacent samples unless some form of mechanical separation is introduced. When no separation is used, a steady state cannot be achieved, even with 10 to 15-min. sampling times. When air is introduced, a steady state is reached in about 4 min. This period, added to the 2-min. scrub, limits the throughput for the penicillin determinations to not more than about 8 samples/hr. This compares with the effective rate of 20/hr. for the colorimetric method (2). (*c*) Timing. Careful control is required to insure that sample and appropriate blank reach the electrodes simultaneously. The extent to which they are out-of-phase has a strong influence on the time required to reach a plateau in the diffusion current.

Effects of Iodate Concentration.—Although 0.004 *N* iodate is sufficient to react stoichiometrically with the highest penicillin concentration used (500

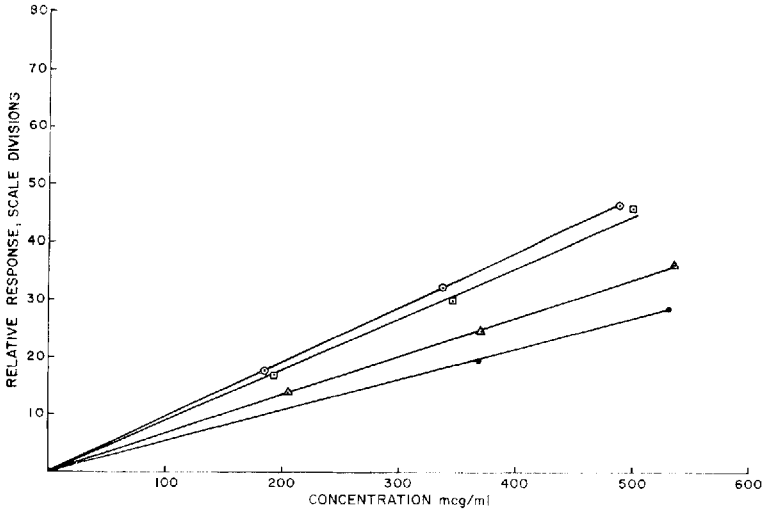

Fig. 7.—Instrument response for penicillins. Key: ⊙, phenethicillin; ⊡, ampicillin; ⧋, penicillin G; ●, oxacillin.

mcg./ml.), a deviation from linearity between response and concentration is observed at this concentration. All published iodimetric methods for penicillin require a greater-than-stoichiometric iodine-to-penicillin ratio; the reason has not been reported in the literature.

For the 4 penicillins studied, best linearity is obtained with 0.016 *N* iodate for penicillin G and oxacillin, and 0.024 *N* iodate for phenethicillin and ampicillin. At much higher iodate concentrations, deviations from linearity become apparent, possibly due to the increased influence of substitution reactions.

Iodine absorption on the tubing walls is minimized by using an all-glass-Teflon system beyond the point of acidification of iodate. Nevertheless, without air-scrubbing, visible deposits of iodine accumulate on the glass. Deposits also build up in the porous glass wall isolating the calomel electrode, but these can be washed out at the end of each working day. When more extensive washing appears desirable, 3 *N* HNO_3 is pumped through the entire system for 3 hr.

The influence of iodide ion on the penicillin method is minimized by holding a high concentration of excess iodide in all streams. In view of the current belief that the platinum electrode has an adsorbed I^- layer (6), the iodide is considered important to avoid electrical drift.

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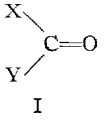
Notes

Molecular Orbital Localization Energies and Carbonyl Nucleophilic Reactivity

By LEMONT B. KIER

Molecular orbital anion localization energies have been calculated for a number of carbonyl group-containing compounds using the LCAO–MO Hückel procedure and the ω-technique. These energies have been found to reflect very adequately the summation of electronic effects from neighboring atoms to the carbonyl group as evidenced by the excellent correlation of the energies calculated with the rate constants of base catalyzed hydrolysis.

THE RELATIVE rate of nucleophilic reactivity of a carbonyl group-containing system such as I can be of great importance from the standpoint of



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drug activity and drug stability, as well as contributing to structural and mechanistic theory. Classical resonance theory has dictated that a balance of mesomeric and inductive effects derived from atoms X and Y in I should facilitate or retard the ease of hydroxyl ion attack on the carbonyl carbon, this becoming the rate-determining step in the reaction sequences. The contributions of X and Y manifest themselves in the form of an energy barrier referred to as the free energy of activation, $\Delta F^{\ddagger}$. It has been assumed that, as a good approximation, $\Delta F^{\ddagger}$ is composed chiefly of $\Delta E^{\ddagger}_{\pi}$, the change in π electronic energy between the unreacted molecule and the transition state (1). In comparing the relative rates of reaction in a closely related set then the relationship holds

$$-RT \log \frac{k_2}{k_1} = [(\Delta E^{\ddagger}_{\pi})_1 - (\Delta E^{\ddagger}_{\pi})_2] \quad \text{(Eq. 1)}$$

The evaluation of $\Delta E^{\ddagger}_{\pi}$ demands a consideration of the nature of the transition state, or a reasonable model of it, in order to base the calculations on physical reality. Among several models proposed for the transition state, the σ complex or the Wheland intermediate has appeared to be the most satisfactory simple approach in the quantum mechanical study of chemical reactivity (2). In the case of the carbonyl carbon subjected to nucleophilic attack, the Wheland intermediate is a carbon surrounded by 4 bonds, each containing 2 electrons, III.

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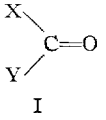

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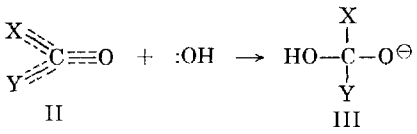

II III

Justification for this model is found in the tests of Hammond's postulate; if the σ complex is an unstable intermediate in the reaction, the transition state should closely resemble it in energy (3).

A means of estimating the energy difference between II and III, known as the localization procedure (2), has been proposed by Wheland. It is based on the concept that the essential difference in π electronic energy between the 2 states is the energy required to perturb the delocalized π electrons to permit the nucleophile to attack the carbon atom. The electron contributed by the carbon atom in the ground state must now be delocalized over the remaining π system or specifically in the case of the carbonyl group system II, it must be localized on the oxygen atom, with the accompanying disruption of the π system due to loss of participation of the central carbon atom. The problem then reduces to one of calculating the energies of the ground state, E_π, and the transition state $E_\pi^\ominus$, and calculating the anion localization energy

$$L_c^\ominus = E_\pi - E_\pi^\ominus \qquad \text{(Eq. 2)}$$

The means of calculating these energies is available through the method of molecular orbitals.

The simple LCAO–MO procedures are well suited for calculations involving heteroatoms because of the relative simplicity of these calculations, and because the present crude nature of heteroatom treatments emphasizes only compatibility with as many observed properties as possible, rather than correct numerical values. In the course of quantum mechanical studies on drug molecules, the author and co-workers have arrived at a set of parameters for heteroatoms which give calculated quantities mirroring physical experience (4). These values, listed in Table I, have been derived from ionization potentials, hence, they bear a consistent relationship to each other as well as the ionization phenomena (5). In other studies in this laboratory these values have been used to calculate successfully π dipole moments and carbonyl oxygen charge densities (4).

TABLE I.—VALUES OF h_x AND k_{cx} FOR HETEROATOMS FOR $\alpha_x = \alpha_c + h_x\beta_{cc}$

$\beta_{cx} = k_{cx}\beta_{cc}$

Atom X[a]	h_x	k_{cx}
Ȯ	1.3	0.8
Ö	2.7	0.6
N̈	1.7	0.7
C̈l	2.8	0.5
C̈H$_3$	3.0	0.7

[a] The designation Ȯ refers to oxygen with a core charge of 1, *i.e.*, an oxygen donating 1 electron to the π system. In this study this would be used for the carbonyl oxygen. Similarly, Ö and N̈ refer to heteroatoms donating 2 electrons to the π system, hence, having core charges of 2. This is used for ester- and amide type atoms in the C—X—C linkage. The values for Cl and CH$_3$ reflect the concept of 2 electron donation to the π system as discussed and used by Streitwieser (7).

The values listed in Table I are modifying parameters used in the expressions for the Coulomb and resonance integrals, α and β, for the heteroatom, X. The methyl group is treated as a heteroatom.

$$\alpha_x = \alpha_c + h_x\beta \qquad \text{(Eq. 3)}$$

$$\beta_{cx} = k_{cx}\beta_c \qquad \text{(Eq. 4)}$$

The integrals for the heteroatoms are commonly expressed in terms of a standard aromatic carbon atom.

There has been a further attempt to treat the failure of the simple Hückel method to account for electron correlation by using the ω technique, first suggested by Wheland and Mann (6) and later studied by Streitwieser (7). The technique employs a disposable parameter, ω, to modify the Coulomb integral based on the calculated charge density, q_i, so that

$$\alpha_i = \alpha_0 + q_i\omega\beta_0$$

The process is reiterated to a self-consistent value of the Coulomb integral. The use of this parameter effectively increases the Coulomb attraction between a π electron and a donating atom. The example of Streitwieser (7) and others (8) has been followed in using a value of 1.4 for ω.

The carbonyl-containing compounds calculated in this study (Table II) were selected to represent a wide diversity of heteroatoms for X and Y in I. They were also selected so as to minimize as far as possible any steric interaction with the carbonyl group. Finally, the set selected is known or thought to undergo base-catalyzed hydrolysis by a second-order nucleophilic attack of hydroxyl ion on the carbon atom (9–14). It was further thought that success in this correlation would constitute further proof of the value of the parameters in Table I and justify their use in other studies.

TABLE II.—SECOND-ORDER BASE HYDROLYSIS RATE CONSTANTS AND ANION LOCALIZATION ENERGIES FOR SEVERAL CARBONYL COMPOUNDS

Compd.	log k + 5 (moles^{-1}, min.$^{-1}$, 25°)[a]	$L_c^\ominus$ (in β)
Urea	0.250	1.144
Acetamide	1.352	1.002
Urethan	2.079	0.966
Methyl acetate	3.945	0.804
Ethyl carbonate	5.447	0.766
Ethyl chloroformate	6.380	0.643

[a] See *References 9–14* for the respective kinetic data.

Calculation of Ground State π Energies.—The value of the π energy of the ground state of each compound is obtained by the solution of a secular determinant of the Coulomb and resonance integrals of participants in the π network. The eigenvalues so derived are expressed in terms of the Coulomb and resonance integrals and each represents an energy level. Filling each level according to aufbau principles, and summing the occupied levels, the total energy of the ground state in terms of α and β is obtained.

Calculation of $L_c^\ominus$ and Correlation with Base Hydrolysis Rate Constants.—From Eq. 2 the value of $L_c^\ominus$ is computed in terms of β (Table II). As a test of the relative value of these energies, the

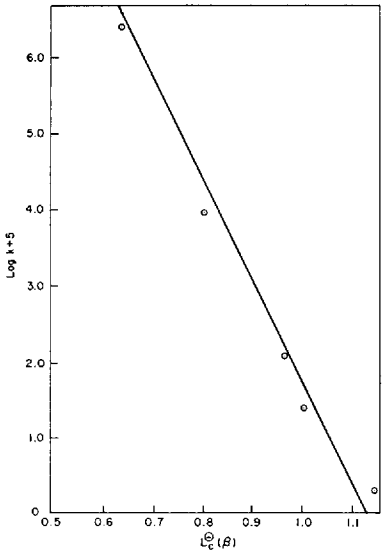

Fig. 1.—Correlation line for log $k + 5$ *vs.* $L_c^{\ominus}$. Correlation coefficient = 0.986. Equation for line: log $k + 5 = 15.25 - 13.57\ L_c^{\ominus}$.

values calculated have been plotted in Fig. 1 along with the least squares equation. The correlation is excellent (correlation coefficient of 0.986) fully justifying the use of parameters to reflect the participation of the atoms X and Y, adequately, in the reaction studied. Also corroborated by the correlation is the use of the transition state model and accompanying mechanism for each compound studied. The parameters used and the techniques employed may further permit a prediction of relative base hydrolysis rates within the framework of the approximations made and limitations imposed.

Numerical Computations.—The numerical computations were performed on an IBM computer. The program used for the ω-Hückel calculations was translated from Fortran to Scatran.[1] The program determines the eigenvalues and eigenvectors of a real symmetric matrix, using an HDIAG subroutine, according to the method of Jacobi. The program has been further modified with the help of Douglas Fleckner of this computation laboratory, to accept core charges other than 1, for use with some heteroatoms. This is necessary in the use of the ω technique. For example, the oxygen of a carbonyl group would have a core charge of 1 while the oxygen of an ester C—O—C would have a core charge of 2.

The calculation of correlation coefficients, least squares regression line, and other statistical data were computed using standard subroutines from this numerical computation laboratory.

Summary.—The anion localization energies have been calculated for a number of simple substituted carbonyl systems undergoing hydroxyl ion-catalyzed hydrolysis. The localization energies calculated have been assumed to be, as a first approximation, a relative measure of the free energy of activation of the reactions, using the Wheland intermediate as a model for the transition state. An excellent correlation is exhibited between these calculated values and the log of the rate constants for the compounds studied.

It has thus been shown that the LCAO–MO Coulomb and resonance integral modifying parameters for the heteroatoms were well chosen and truly mirror their relative electronic interaction within the delocalized electron framework. It is also apparent that the model for transition state and the proposed mechanism of action were correctly reflected by the reaction model chosen. The correlation of the calculated localization energies with the rate constants attests to the utility of quantum chemical methods in studying and predicting drug molecule reactions, stability, and possibly even pharmacological activity.

[1] The Ohio State Computer Center language, by Evan L. Brill of the Numerical Computation Laboratory staff. The program was originally written by G. Pettit, the University of Minnesota, and modified for use with the ω technique by D. Lazdins, the University of Minnesota.

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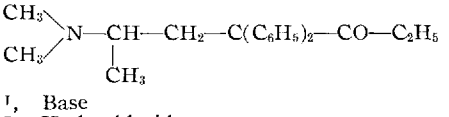
Molecular Asymmetry of Methadon

By LELAND L. SMITH

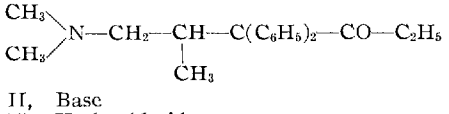
Proton nuclear magnetic resonance spectra of methadon hydrochloride, isomethadon hydrochloride, and related derivatives are recorded and interpreted in terms of a molecular asymmetry and a preferred conformation concept.

SUGGESTIONS relating the potent analgesic activity of methadon (6-dimethylamino-4, 4-diphenylheptan-3-one) (I) and isomethadon (6-dimethylamino-5-methyl-4, 4-diphenylhexan-3-one) (II) with stereochemical considerations of restricted rotation and subsequent molecular asymmetry have been made (1–6) based on indirect evidence of chemical unreactivity of the hindered carbonyl group (7), ultraviolet light absorption and dipole moment measurements (8), dissociation constants (3), and differential analgesic activity of the optical antipodes (9). Proton nuclear magnetic resonance spectra in Table I for several methadon and isomethadon derivatives provide independent evidence for molecular rigidity and for a preferred conformation of the molecule.

These proton spectra have certain features in common, including an acetyl methyl proton triplet, an acetyl methylene quartet, a secondary C-methyl doublet, a 10-proton aromatic signal, and 3 unanalyzed 1-proton multiplets. Whereas the dimethylamino protons of I base appeared as sharp 6-proton singlets at 2.14 p.p.m., the dimethylamino proton signals of salts of I were shifted characteristically downfield (0.6–0.7 p.p.m. except for I*c*) and involved greater multiplicity dependent on salt form, solvent composition, concentration, and temperature.



I, Base
I*a*, Hydrochloride
I*b*, Deuterochloride
I*c*, Sulfur trioxide compound
I*d*, Hydrogen sulfate



II, Base
II*a*, Hydrochloride

Spectra of I*a* were dominated by an unexpected 6-proton 1:2:1 triplet pattern centered about 2.77 p.p.m. The same pattern was observed in 40-Mc. spectra (center at 2.75 p.p.m.). From the slightly different spacings of the 60-Mc. triplet an overlapping doublet of doublets pattern was suspected, and indeed, spectra of solutions cooled in dry ice–acetone exhibited the clearly resolved doublet of doublets pattern. The center of the low-field doublet (J = 5.5 c.p.s.) was separated from the center of the highfield doublet (J = 5.0 c.p.s.) by 10 c.p.s., and as the cooled solution warmed to room temperature the downfield doublet shifted upfield toward the stationary doublet, so that at room temperature a triplet signal was obtained. The triplet was not collapsed by recording the spectra at 60°.

The dimethylamino proton doublet of doublets pattern in I*a* spectra collapsed to a sharp singlet on equilibration of the deuterochloroform solutions with deuterium oxide or by dilution with pyridine. Deuterium oxide solutions of I*a* likewise showed a 6-proton singlet. In contrast, the deuterochloride I*b* exhibited a doublet pattern in suitably dilute deuterochloroform solution. These solvent dependent effects on the dimethylamino proton signal form were not accompanied by other significant changes in spectra, and integration of spectra established that no carbon-bound proton in I*a* exchanged with deuterium oxide in deuterium oxide–deuterochloroform media, or had any exchange of carbon-bound proton for deuterium occurred in the preparation of the deuterochloride I*b*.

The dimethylamino protons of the sulfur trioxide compound I*c* appeared as two well-separated 3-proton singlets at 2.44 and 2.85 p.p.m., but the dimethylamino protons of the hydrogen sulfate I*d* appeared as a 6-proton broad signal.[1]

Proton spectra of II*a* were also characterized by a well-resolved doublet of doublets pattern centered at 2.79 and 3.12 p.p.m. (also reproduced at 40 Mc. at 2.73 and 3.10 p.p.m.). Equilibration of deuterochloroform solutions of II*a* with deuterium oxide again collapsed the dimethylamino proton doublet of doublets pattern to a sharp singlet, and integration of the spectra established that no carbon-bound proton in II*a* had exchanged with deuterium.

Concentration effects were observed in spectra of I*b* and II*a* in deuterochloroform, initial 15% solutions of both salts exhibiting singlet signals for the dimethylamino protons, which were split into doublet of doublets patterns, respectively, on 1:1 dilution with deuterochloroform.

The observed complexities of the N-methyl proton signals in I*a*, I*b*, I*c*, and II*a* must arise through magnetic nonequivalence on the 2 N-methyl groups in each case.[2] Thus, in I*a* and II*a* the 2 nonequivalent N-methyl groups appear as two 3-proton signals further split into a doublet of doublets pattern by coupling between the N-methyl protons and the ammonium proton.[3] The absence of an

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[1] Similar in appearance to the broad N-methyl signal of dimethyl-3-chloropropylammonium chloride (11).

[2] Temperature dependence and dilution effects in spectra of I*a*, I*b*, and II*a* deny spin–spin interactions as a reasonable basis for the observed multiplicities; also long-range coupling constants are generally smaller than the 5–6 c.p.s. (10 c.p.s. at dry ice temperatures) separations found.

[3] The field independence of the 5 c.p.s. spacings in the doublet of doublets pattern in I*a* and II*a* spectra as well as their magnitude support this assignment. Spin–spin interactions between N-methyl protons and an ammonium proton giving rise to coupling constants of 4.5–6.17 c.p.s. in various solvents have been observed (12–14).

TABLE I.—60 MC. PROTON SPECTRA OF METHADON DERIVATIVES AND ANALOGS[a]

Compd.	Acetyl Protons	Aromatic Protons	C_5- and C_6-Methylene/Methine Protons	C-Methyl Protons	*N*-Methyl Protons
I	3H: 0.84 (t), J = 7 2H: 2.27 (q), J = 7[b]	10H: 7.38 (m)	3H: 1.7–3.1 (m)	3H: 0.48 (d), J = 6	6H: 2.14
I*a*	3H: 0.83 (t), J = 7 2H: 2.27 (q), J = 7[b]	10H: 7.43 (m)	1H: 2.43 2H: 2.95–3.35 (m)	3H: 0.72 (d), J = 6.5	3H: 2.72 (d), J = 6 3H: 2.82 (d), J = 5
I*b*	3H: 0.83 (t), J = 7 2H: 2.27 (d), J = 7[b]	10H: 7.50 (m)	1H: 2.44 2H: 2.95–3.35 (m)	3H: 0.72 (d), J = 6.5	3H: 2.73[c] 3H: 2.83[c]
I*c*	3H: 0.86 (t), J = 7 2H: 2.16 (q), J = 7[b]	10H: 7.48 (m)	1H: 2.33 2H: 2.8–3.3 (m)	3H: 0.61 (d), J = 6.5	3H: 2.44 3H: 2.85
I*d*	3H: 0.80 (t), J = 7 2H: 2.20 (q), J = 7[b]	10H: 7.25 (m)	1H: 2.38 2H: 2.95–3.25 (m)	3H: 0.60 (d), J = 6.5	6H: 2.81 (b)
II*a*	3H: 0.76 (t), J = 7 2H: 1.7–2.8 (m)[d]	10H: 7.50 (m)	1H: 3.3–3.8 (m) 2H: 1.7–2.8 (m)[d]	3H: 1.25 (d), J = 6.5	3H: 2.79 (d), J = 5[c] 3H: 3.12 (d), J = 5[c]
III	3H: 0.81 (d), J = 6	10H: 7.30 (m)	[e]	3H: 0.74 (d), J = 6	6H: 2.18
IV	...	10H: 7.20 (m)	3H: 2.0–2.3 (m)	3H: 0.92 (d), J = 5.5	6H: 2.11
V	...	10H: 7.20–7.80 (m)	1H: 2.8 (m) 2H: 2.2–2.65 (m)	3H: 1.18 (d), J = 6.5	6H: 2.22

[a] Spectra were obtained on 15% (w/v) deuterochloroform solutions using a Varian Associates A-60 spectrometer. Chemical shifts (δ) are measured in p.p.m. downfield from an internal reference of tetramethylsilane. Data are recorded in order: chemical shift in p.p.m., multiplicity (in parenthesis), coupling constant in c.p.s. Signals are singlets except as noted. Abbreviations used are: b, broad; d, doublet; m, multiplet; t, triplet; q, quartet. [b] Fine splitting of about 1.5 c.p.s. was also observed. [c] Observed on 1:1 dilution of the initial 15% solution. [d] Two water protons are also observed in this range on 15% solutions. On 1:1 dilutions the water protons are consolidated as a 2 proton singlet at 2.42 p.p.m. The sample of II*a* used was a hydrochloride monohydrate (10). [e] Five unassigned protons appear: 1H, 1.88; 2H, 1.0–1.7 (m); 2H, 2.15–3.0 (m). A single proton geminal to hydroxyl is at 3.90 p.p.m. (q), J = 9.5 and 3.0 c.p.s. by first-order analysis.

ammonium proton in the deuterochloride I*b* simplified the *N*-methyl proton signals so that only a doublet is present. In the sulfur trioxide compound (I*c*) there likewise is no ammonium proton; however, in this instance the 2 *N*-methyl singlets are separated by 0.4 p.p.m.

The magnetic nonequivalence of the 2 *N*-methyl groups of these derivatives is not a consequence of restricted rotation about the N—C_6 bond. Such restricted rotation and consequent *N*-methyl nonequivalence occurs in *N*-methylamides (15), *N*-methylamidinium compounds (16), and in certain heterocyclic *N*-methyl compounds (17–19) where partial planar character of the nitrogen–carbon bond is involved. A very slow rate of amine inversion might be a basis for nonequivalence of the *N*-methyl protons, and some similarities exist between the present instance and spectra of dibenzylmethylammonium chloride (20) in aqueous acid. However, persistence of the nonequivalence of 60° does not support this explanation. Were slow inversion the case, however, some of the solvent effects could be readily understood as resulting from increased nitrogen inversion rates.

Of the conditions under which unexpected nonequivalence of protons obtain, as summarized by Bible (21), more favored explanation of the instant nonequivalence derives from inherent molecular asymmetry attributed to the nearby asymmetric carbon atom in the methadon and isomethadon molecules (C_6- and C_5-, respectively). This inherent asymmetry could manifest itself in nonequivalence of the *N*-methyl protons, of the C_5- or C_6-methylene protons, or of both sets of protons. The concept has been used to explain similar spectral complexity for other dimethylamino compounds (22, 23).

However, presence of dilution effects in the spectra of I*b* and II*a*, equivalence of the *N*-methyl protons of the related nitriles *dl*-4-dimethylamino-2,2-diphenylvaleronitrile hydrochloride (IV) and *dl* - 4 - dimethylamino - 3 - methyl - 2,2 - diphenylbutyronitrile hydrochloride (V), and identity of effect in both methadon (where the asymmetric C_6-carbon is 3 bonds separated from the *N*-methyl protons) and isomethadon (where the asymmetric C_5-carbon is 4 bonds separated), as measured by the coupling constants J = 5–6 c.p.s., suggest other factors may be involved. These several observations are consistent with a molecular asymmetry associated with a preferred conformation of the molecules I and II, the preferred conformation being formed through hydrogen bonding between cationic nitrogen and the carbonyl group as postulated by Beckett (2–4).

The dilution effects of the spectra of I*b* and II*a*, together with the simplification of spectra on equilibration with deuterium oxide suggest that the preferred conformations of the I and II molecules in solution giving rise to the magnetically nonequivalent *N*-methyl groups are not assumed in every instance of concentration and solvent choice, but that such asymmetric conformations may exist in special situations. Extension of these considerations to the complex media of living animals must therefore be done with caution.

The increased shielding (0.50 p.p.m.) of the C_6-methyl protons of I*a* in comparison with that of the C_5-methyl protons of II*a* may also be considered in terms of a preferred conformation of the molecules involving close approach of the carbonyl oxygen and the ammonium proton. In such conformation the C_5-methyl group of II*a* is very nearly in the plane of the C_4-benzene ring (using Dreiding models), whereas the C_6-methyl group of I*a* is more nearly above the plane of the C_4 benzene ring and thereby is subject to increased long-range diamagnetic shielding of the benzene ring.

The three C_5- and C_6-methylene and methine protons of I and II derivatives are magnetically nonequivalent. One proton appeared as a singlet overlapping the acetyl methylene quartet, and 2 protons appeared as a series of lines overlapping one another and/or the *N*-methyl signals. One of these latter

multiplets must be the C_6-proton (in I*a*), since it would be spin-spin coupled to the C-methyl protons, the ammonium proton, and probably one or both C_5-protons, and being adjacent to nitrogen, must be at low field. The other low field proton is then one of the C_5-methylene protons, the other being at higher field and overlapping the acetyl methylene quartet.

The nonequivalence of these methylene protons in I and II derivatives is most readily explained in terms of the nearby amsymmetric carbon atom C_6- and C_5,-, respectively (21), and in this matter, the precursor nitrile salts IV and V exhibit methylene proton nonequivalence also.

Attempts at further analysis of the methylene/methine proton signals on spectra of α-methadol (*dl* - α - 6 - dimethylamino - 4,4 - diphenylheptan-3-ol) (III) or of the nitrile precursors of I and II, IV and V, respectively, were thwarted by overlapping of these signals with one another and with those of the dimethylamino protons. It is to be noted that the dimethylamino proton signals in both nitriles IV and V fall in a high field relative to their ketone analogs I*a* and II*a*.

EXPERIMENTAL[4]

6 - Dimethylamino - 4,4 - diphenylheptan - 3-one Deuterochloride (Ib).—Ten grams of commercial methadon hydrochloride was recrystallized 3 times from 99.8% deuterium oxide (Stuart Oxygen Co., San Francisco, Calif.) to constant melting point, m.p. 232–234°.

6 - Dimethylamino - 4,4 - diphenylheptan - 3 - one Sulfur Trioxide Compound (Ic).[5]—A mixture of 1.5 Gm. of I*a*, 1.8 ml. of isopropenyl acetate, and 2 drops of concentrated sulfuric acid was distilled slowly over a 7-hr. period. The mixture was filtered from insoluble material (0.32 Gm. of recovered I*a*, m.p. 230–234°), and the filtrate was concentrated. A red resinous precipitate formed and crystallized over several weeks, yielding 0.37 Gm. of product I*c*. Recrystallization from benzene gave crystals, m.p. 139.0–141.0°, and from ethanol–ether gave the analytical sample, m.p. 140.0–142.5°. $\lambda_{max.}$ 255 mμ (ϵ 579, inflection), 260 mμ (ϵ 646), 266 mμ (ϵ 629), 292 mμ (ϵ 624); $\lambda_{min.}$ 250 mμ (ϵ 526), 264 mμ (ϵ 624), 277.5 mμ (ϵ 502).

Anal.—Calcd. for $C_{21}H_{27}NO_4S$: C, 64.75; H, 6.99; N, 3.59; S, 8.23. Found: C, 64.89; H, 6.98; N, 3.57; S, 8.54.

6 - Dimethylamino - 4,4 - diphenylheptan - 3 - one Hydrogen Sulfate (Id).—A solution of 1.0 Gm. of methadon in absolute ethanol was treated with an ethanolic sulfuric acid solution. The solution was evaporated under vacuum and the oily residue was crystallized from chloroform–ether, yielding 0.30 Gm. of salt, m.p. 147.0–149.0°. An additional 0.21 Gm., m.p. 144.0–146.5°, was recovered from the filtrate. After further recrystallizations from methanol–ether and from ethanol–ether, the salt melted at 146.0–148.0°.

Anal.—Calcd. for $C_{21}H_{27}NO.H_2SO_4$: N, 3.44; S, 7.87. Found: N, 3.39; S, 8.13.

[4] Melting points were determined using a calibrated thermometer and a heated oil bath. Ultraviolet light absorption spectra were recorded on methanol solutions using a Beckman model DU spectrophotometer.

[5] The minor by-product I*c* was obtained in otherwise unsuccessful attempts at enolacetylation of I*a*. The structure of I*c* as a sulfur trioxide derivative of I is supported by elemental analysis, ultraviolet light absorption essentially identical with spectra of I*a* (8), water solubility (from which water solutions barium chloride precipitated barium sulfate), and nonidentity with the hydrogen sulfate salt I*d* prepared with sulfuric acid in the usual way.

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Identification of Pyrethrol with Taraxasterol

By WERNER HERZ and R. N. MIRRINGTON

The insecticidally inactive substance pyrethrol from pyrethrum flowers has been identified as taraxasterol.

THE OLEORESIN obtained by extraction of pyrethrum flowers (*Chrysanthemum cinerariaefolium* Vis.) contains not only the active insecticidal principles pyrethrin I and II,[1] but also comparatively large quantities of inactive material, including such compounds as the monocarbocyclic sesquiterpene lactone pyrethrosin (2) and an unidentified substance called "pyrethrol." The latter, first isolated by Fujitani (3) and referred to briefly by Staudinger and Ruzicka (4) had m.p. 199°, $[\alpha]_D$ + 73°, and was assigned (3) the molecular formula $C_{21}H_{34}O$. More recently (5), pyrethrol was stated to be a triterpene alcohol isomeric with lupeol.

The authors received a sample of crude pyrethrol[2] isolated a number of years ago (6) during the purification of pyrethrosin and have investigated its properties. Purification and conversion to a number of derivatives established its identity as taraxasterol.

In this laboratory, pyrethrol had m.p. 217–219° after several recrystallizations from chloroform–ethanol and $[\alpha]_D$ +92°.

The presence of 1 secondary hydroxyl group was indicated by the infrared and NMR spectra (complex signal at 3.2 p.p.m., H_3) and confirmed by the formation of a monoacetate, m.p. 246–248°, $[\alpha]_D$ +105°, NMR signal at 4.6 c (H_3), a monobenzoate, m.p. 244–247°, and by mild oxidation to a ketone, m.p. 179–182°, infrared band at 1710 cm.$^{-1}$, positive Zimmermann test, which contained no other hydroxyl groups. Furthermore, an exocyclic methylene group was clearly indicated in all four of the above compounds by infrared bands at 1650 and 890 cm.$^{-1}$ and, in the NMR spectra, by a broad singlet, $W_{1/2}$ 4 c/s, intensity 2 protons, at 4.65 p.p.m. The NMR spectra also indicated the presence of 7 methyl groups.

The melting points and rotations of pyrethrol and its derivatives bore a reasonable resemblance to those most widely accepted (7) for taraxasterol, m.p. 226–227°, $[\alpha]_D$ +97°; acetate, m.p. 256–257°, $[\alpha]_D$ +100°; benzoate, m.p. 242–244°; taraxastenone, m.p. 184–185°. The spectral data were also in harmony with the supposition that the 2 substances might be identical.

This supposition was verified by direct comparison (mixed melting point and infrared spectra) of pyrethrol with an authentic sample of taraxasterol.[3] The 2 samples were undistinguishable. Pyrethrol is therefore identical with taraxasterol and the former name should be stricken from the literature.

EXPERIMENTAL[4]

Pyrethrol.—Crude pyrethrol crystallized from chloroform–ethanol as colorless needles, m.p. 217–219°, $[\alpha]_D$ +92°, infrared bands at 3400, 1050 (hydroxyl), 1650, 890 cm.$^{-1}$ (exocyclic methylene), NMR signals at 4.65 (broad singlet, $W_{1/2}$ 4 c/s, 2 protons, exocyclic methylene), 3.2 p.p.m. (multiplet, 1 proton, H_3). The infrared spectrum was superimposable on that of an authentic sample of taraxasterol, m.p. 219–221°, mixed m.p. 219–221°.

Derivatives of Pyrethrol.—*Acetate.*—Acetylation of pyrethrol with acetic anhydride and pyridine at 80° for 2 hr. gave a monoacetate which crystallized from chloroform–ethanol as lustrous plates, m.p. 246–248°, $[\alpha]_D$ +105°, infrared bands at 1735, 1250 (acetate), 1650, 890 cm.$^{-1}$ (exocyclic methylene), no hydroxyl absorption, NMR signals at 4.65 (as in pyrethrol above), 4.5 (multiplet, 1 proton, H_3), 2.02 p.p.m. (singlet, 3 protons, acetate).

Benzoate.—Benzoylation of pyrethrol with benzoyl chloride and pyridine at 50° for 2 hr. gave a monobenzoate which crystallized from ethanol as colorless needles, m.p. 244–247°, infrared bands at 1730, 1280 (benzoate), 1650, 890 (exocyclic methylene), 1605, 1595, 715, 690 cm.$^{-1}$ (monosubstituted benzene ring).

Dehydropyrethrol.—A suspension of 0.5 Gm. of pyrethrol in 50 ml. of acetone was treated with Jones reagent at 25° until a brown color persisted. Dilution with water gave a flocculent precipitate which was collected and crystallized from ethanol to furnish 0.41 Gm. of dehydropyrethrol, m.p. 179–182°, infrared bands at 1710 (cyclohexanone), 1650, 890 cm.$^{-1}$ (exocyclic methylene), no hydroxyl absorption, NMR signal at 4.65 (broad singlet, 2 protons, exocyclic methylene).

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[1] For a review of the chemistry of the natural pyrethrins, see *Reference 1*.

[2] Supplied through the courtesy of Drs. M. S. Schechter and B. H. Braun, Agricultural Research Center, U. S. Department of Agriculture, Beltsville, Md.

[3] Kindly supplied by Dr. K. Takemoto, Tohoku University, Sendai, Japan, through the courtesy of Dr. M. Matsui, Institute for Physical and Chemical Research, Tokyo, Japan.

[4] Rotations were run in chloroform, infrared spectra in chloroform and as Nujol mulls, NMR spectra in deuterochloroform with tetramethyl silane serving as internal standard.

Blood Nitrite and Nitrate Concentration After Oral and Intravenous Administration of Glyceryl Trinitrate in Rabbits

By O. J. LORENZETTI*, ARTHUR TYE, and JOHN W. NELSON

Blood nitrite and nitrate concentrations were measured after oral and intravenous administration of glyceryl trinitrate at 1.0 mg./Kg. in rabbits. Nitrate and nitrite blood concentrations attained peak concentrations in 90–120 min. after oral administration and slowly returned to control levels. Nitrate and nitrite blood concentrations decreased slowly after intravenous administration of glyceryl trinitrate, then fell rapidly after 60 min. Blood nitrate concentrations were twice the blood nitrite concentrations 60 min. after either oral or intravenous administration of glyceryl trinitrate.

THE ABSORPTION of glyceryl trinitrate from the gastrointestinal tract has been questioned for over a half century. Most investigators believe the absorption to be poor on the basis of resulting physiological effects observed. Sollman states that when glyceryl trinitrate is ingested it is absorbed into the portal circulation and destroyed by the liver (1). Oral and intravenous administration of organic nitrates to dog and man have provided evidence that there is no correlation between blood nitrite and nitrate concentration and the observed physiological activity (2, 3). Recent investigations utilizing the observation of the vascular bed of the rabbit ear have shown that glyceryl trinitrate is absorbed in the gastrointestinal tract after oral administration (4).

The following study was undertaken to determine the blood nitrite and nitrate levels after oral and intravenous administration of glyceryl trinitrate in the rabbit.

METHODS

White New Zealand male rabbits weighing 3.5–4.5 Kg. were used in this study. The rabbits were lightly anesthetized with pentobarbital (25 mg./Kg., i.p.). One group of rabbits received glyceryl trinitrate orally (1.0 mg./Kg. from a stomach tube) in 1.5 ml. of water. A second group received glyceryl trinitrate intravenously (1.0 mg./Kg.) from a needle cannula in the marginal ear vein. The intravenous injection was made in 0.5 ml. of solution over a 60-sec. period. Blood samples were taken over a 2–3-hr. time period.

Each group of rabbits was used for only 3 successive blood samples, because of the large blood sample required (4 ml.) for the complete assay. A total of 30 rabbits was used for the assay. Blood samples were taken from an indwelling catheter (PE50) in the femoral artery.

Each 4-ml. blood sample was laked with 4 ml. of deionized water in a 125-ml. conical flask, and the following were added: 10 ml. of 0.2% solution of sulfanilamide, 2 ml. of 50% concentrated hydrochloric acid solution, and 2 ml. of 5% aqueous solution, prepared from mercuric chloride that had been sublimed 3 times. The resulting brownish mixture was thoroughly mixed by swirling and then centrifuged for 15 min. at 6000 r.p.m. in a horizontal centrifuge. The clear, deproteinated, decanted filtrate was quantitatively divided into two 10-ml. portions. One portion was used for the nitrite assay and the other for the nitrate assay (5).

Nitrite Assay.—To the 10-ml. portion of the deproteinated filtrate was added 1.0 ml. of 0.1% aqueous solution of *N*-(1-naphthyl)-ethylene diamine dihydrochloride to act as a coupling agent. Then the solution was allowed to stand for 10 min. and the color read on a Beckman DU spectrophotometer at the 540-mμ wavelength. The nitrite concentration was read from a previously determined standard curve prepared by plotting absorbance *versus* known concentrations of nitrite.

Nitrate Assay.—The remaining 10 ml. of the deproteinated filtrate was transferred to a 25-ml. test tube and hydrogen sulfide was bubbled through to precipitate unreacted mercury ions as mercuric sulfide. The excess hydrogen sulfide was removed by boiling the solution carefully to avoid bumping and subsequent loss of the solution. When all trace odor of hydrogen sulfide was removed, the murky solution was transferred to a 75-ml. conical flask, and 5.0 Gm. of Amberlite IR 120 AA (Mallinckrodt No. 3323), a strongly acidic, sulfonic cation exchange resin, was added.

The mixture was swirled for several minutes and centrifuged at 4000 r.p.m. for 10 min., or until all nitrite was absorbed, which was confirmed by placing 1 drop of coupler upon 1 drop of the decanted filtrate on a spot plate and by noting the absence of a color change. To the nitrite-free filtrate contained in a 125-ml. conical flask was added 5.0 ml. of 12% concentrated ammonium hydroxide solution and 1.0 ml. of 1% manganous chloride tetrahydrate to catalyze the reduction of nitrate ions. The solution was immediately placed in a water–ice bath maintained at 14 to 15° for 10 min. A 0.2 Gm. portion of zinc dust was added and the mixture stirred on a magnetic stirrer for 10 min. The slurry was filtered through a 3-in. funnel. To 5 ml. of the collected filtrate was added 1.0 ml. of *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and the resulting solution was allowed to stand for 10 min., after which the absorbance was read at the 540-mμ wavelength. The nitrate concentration was read from a previously determined standard curve prepared by plotting absorbance *versus* known concentration of nitrate.

RESULTS

The results are presented in Fig. 1. By plotting the blood nitrate concentration and the blood nitrite concentration after intravenous injection, *versus* time, 2 curves running similarly were obtained. On the other hand, after oral administration the

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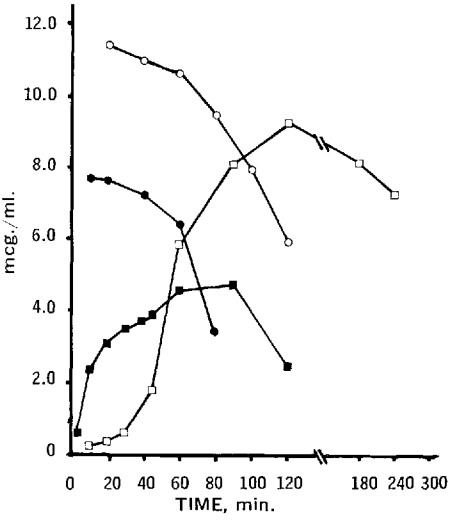

Fig. 1.—Micrograms of nitrate and nitrite per milliliter of blood in rabbits after oral and intravenous administration of glyceryl trinitrate, 1.0 mg./Kg. Key: O—O, nitrate, intravenous; ●—●, nitrite, intravenous; □—□, nitrate, oral; ■—■, nitrite, oral. Control blood nitrate level, 1.05 mcg./ml. ±0.54, as determined in 8 rabbits; control blood nitrite level, less than 0.50 mcg./ml., as determined in 6 rabbits.

initial nitrite concentration was higher than the nitrate concentration but was followed by a higher nitrate-to-nitrite concentration ratio of approximately 2:1 at 90 min.

DISCUSSION

The fact that blood nitrate and nitrite concentrations do not correlate with the time of peak effect of vasodilative action is well documented for glyceryl trinitrate and other organic esters of nitric acid (6). It is interesting to observe the prolonged high blood concentration of inorganic nitrate following either the intravenous or oral route of administration. Although the observed dilatation reported in the literature is said to last 60 min., these investigations show the blood concentration to remain significantly elevated 2–3 hr. after ingestion of glyceryl trinitrate at the dose reported. The possibility of buccal absorption is eliminated since the glyceryl trinitrate was placed directly in the stomach by intubation. Recently, DiCarlo, Hartigan, and Phillips have shown quantitative absorption of pentaerythritol tetranitrate by the gastrointestinal route (7). Crandall reported the enzymatic degradation of glyceryl trinitrate and related organic esters by erythrocytes in dogs (8). This enzymatic degradation was further correlated to the action of glyceryl trinitrate reductase in hog liver and heart (9). A similar enzyme system has been reported for pentaerythritol tetranitrate in human erythrocytes (10).

SUMMARY

Data for the blood nitrate and nitrite concentration after oral and intravenous administration of glyceryl trinitrate have been presented. After oral glyceryl trinitrate, the peak blood concentrations of nitrate were approximately twice that of nitrite. The blood nitrate and nitrite concentrations decreased similarly after intravenous administration of glyceryl trinitrate.

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Chlorpromazine: Effect on Food Intake and Glucose Distribution in Obese and Nonobese Mice

By P. D. CULLEN*, D. C. ARNEY†, and H. A. SWARTZ

Chlorpromazine, in doses of 1 mg./Kg., caused a significant reduction of food intake, weight loss, and oxygen consumption in both gold thioglucose-obese and normal nonobese mice as compared to saline-treated controls. Tissue activities after glucose-1-C-14 administration were reduced, but the blood levels were increased.

Gold thioglucose-obese mice have been employed in numerous studies involved with the etiology of obesity and hyperphagia. A summary of these studies, presented in a previous report (1), included evidence of the role and relationship of the lateral and ventro medial hypothalamic nuclei in food-intake regulation and satiety. The ventro medial nuclei, in response to blood glucose, have been shown to inhibit a lateral hypothalamic feeding center (2, 3) which results in an inhibition of food intake (4, 5). Stimulation of the feeding center results in hyperphagia and its destruction in a temporary cessation of food intake or aphagia. It is probable that any agent that could increase or block glucose uptake by the ventro medial nuclei could influence food intake. The role of centers in the frontal lobes cannot be overlooked, and is discussed in a review by Andersson and Larsson (6).

Selected anorectic agents were observed to reduce food intake in both normal nonobese and gold thioglucose-obese mice, with *d*-amphetamine and phenmetrazine the most effective of the agents employed (7). Reduction was much higher in the normal nonobese mice. These agents also caused a significant increase in residual hypothalamic activity following the administration of labeled glucose in the nonobese mice (1).

Interest arose over the effects of central depressants, and chlorpromazine was selected on the basis of its reported actions on hypothalamic centers, such as depression of emotional centers, sedative action, and tissue distribution (8–10).

EXPERIMENTAL

Food Intake and Weight Loss.—Gold thioglucose-obese mice were prepared as previously described (1, 7), and the treated and nontreated mice were maintained in separate groups for 3 to 4 months until a weight plateau was observed. At this time the mice were placed into individual cages with free access to food and water. Food was placed in hoppers suspended from the cage lid. After a period of 2 weeks to allow for adjustment to environment and isolation, the normal body-weight change and average daily food intake for a 7-day period was determined for both groups of mice. Food intake was determined by accurately weighing the food on the first and final day, and weight changes were determined from the initial and final body weights. The control values for both groups of mice were obtained from animals administered saline i.p. daily in volumes equivalent to those employed for the administration of chlorpromazine. Chlorpromazine,[1] as the hydrochloride, was administered in saline i.p. daily in a dose of 1 mg./Kg. (2.83 μ moles/Kg.). The observed values for both groups of mice are tabulated in Table I.

Oxygen Consumption.—Oxygen consumption for both groups of mice was determined by means of a spirometer (Minute Oxygen Uptake Spirometer, V68808 Aloe Scientific). Saline and chlorpromazine were administered as previously described 10 min. prior to placing the animals in the spirometer chamber. Following a 20-min. equilibration period, the oxygen consumption was determined for a 60-min. period, the values corrected to S.T.P. and the ml./hr./Gm. determined. From these values the Gm.-cal./hr./cm.^{2} were calculated. The values for both groups of mice are tabulated in Table II.

Glucose-1-C-14 Distribution.—The effect of chlorpromazine on residual tissue activity after the administration of glucose-1-C-14 was determined in both groups of mice. Saline was employed for the controls. The glucose-1-C-14 dose was 0.05 μc./Gm. and was administered i.p. in an aqueous solution of glucose 20 mg./ml., which gave a dose of 100 mg./Kg. Chlorpromazine and saline were administered as previously described 30 min. prior to the administration of the labeled glucose. The animals were sacrificed by decapitation 30 min. after the administration of the labeled glucose. Blood was collected in a porcelain container which was previously smeared with a saturated solution of sodium citrate to prevent coagulation, and a sample was obtained by means of micropipets. Tissue samples of adrenal, kidney, liver, and muscle were obtained, wiped free of external blood, and accurately weighed. The intact brain was removed, chilled in a deep freeze, and dissected so as to separate the cerebrum, diencephalon (thalamus and hypothalamus), and the hind brain. Weighed samples of each area were obtained and placed, along with the blood and other tissue samples, into individual counting vials containing 1 ml. of 1 *M* benzethonium chloride[2] base in methanol (11). The vials were sealed with screw caps and incubated at 55° for 24 hr. to facilitate solution, then cooled to room temperature, and 10 ml. of a liquid scintillation solvent system added (2,5-diphenyloxazole 0.4%, naphthalene 5.0%, cellosolve 300 ml., 1,4-dioxane 300 ml., and toluene to 1000 ml.) (12). The vials and contents were dark-adapted and temperature adjusted to −20° for 48 hr. A standard of the labeled glucose solution employed for the administration of the glucose-1-C-

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[1] Chlorpromazine HCl supplied by Smith Kline & French Laboratories, Philadelphia, Pa.

[2] *p*(Diisobutylcresoxyethoxyethyl) - dimethylbenzyl - ammonium chloride. Marketed as Hyamine by the Rohm and Haas Co.

TABLE I.—WEIGHT LOSS AND DAILY FOOD INTAKE IN GOLD THIOGLUCOSE-OBESE AND NONOBESE MICE[a]

	Obese Mice		Nonobese mice	
	Saline	Chlorpromazine	Saline	Chlorpromazine
Init. wt. Gm. $\bar{X}$	56.83	53.33	30.5	32.66
Wt. loss Gm. $\bar{X}$	0	1.5	+0.83	0.72
S_x	1.49	0.96	0.55	0.92
Wt. loss[b] Gm./Gm. $\bar{X}$	0	0.028	+0.027	0.022
S_x	0.026	0.018	0.018	0.028
Food intake daily Gm. $\bar{X}$	6.21	4.36	4.38	3.93
S_x	0.83	0.27	0.28	0.27
Food intake[b] Gm./Gm. $\bar{X}$	0.109	0.081	0.14	0.12
S_x	0.014	0.005	0.009	0.008
Food intake[c] reduction %	...	29.8	...	10.3
Gm./Gm.[b] $\bar{X}$	...	0.028	...	0.02

[a] Values are arithmetical means of 6 animals for a 7-day period. [b] Gm./Gm. values calculated from Gm. initial body weights. [c] Food intake reduction values calculated from saline controls.

TABLE II.—OXYGEN CONSUMPTION AND ENERGY OUTPUT[a]

		Obese Mice		Nonobese Mice	
		Saline	Chlorpromazine	Saline	Chlorpromazine
Init. wt. Gm.	$\bar{X}$	44.3	46.08	28.5	26.1
Body surf. cm.^{2}[b]	$\bar{X}$	125.1	128.9	93.1	88.1
O_2 ml./Gm./hr.	$\bar{X}$	3.4	2.8	3.5	2.86
S_x		0.41	0.29	0.17	0.37
Gm.-cal./hr./cm.^{2}[c]	$\bar{X}$	5.94	4.93	5.4	4.04
S_x		0.66	0.65	0.26	0.46

[a] Values are arithmetical means of 6 animals. [b] Body surface calculated from $A = \frac{W^{2/3}}{K}$ (K assumed to be 10) (15). [c] Calculated from $\frac{\text{ml./Gm./hr.} \times \text{init. wt.} \times 4.8}{\text{body surf. in cm.}^2}$ (15).

TABLE III.—PER CENT RESIDUE OF ADMINISTERED RADIOACTIVE DOSE OF GLUCOSE-1-C-14 PER GRAM OF DRY TISSUE AND MILLILITER OF BLOOD[a]

Tissue	Saline Treated[b] $\bar{X}$	S_x	Chlorpromazine Treated[b] $\bar{X}$	S_x
Gold Thioglucose-Obese				
Blood	1.87	0.03	2.36	0.07
Adrenal	8.89	1.15	7.73	1.43
Kidney	11.53	0.47	12.63	1.09
Liver	19.41	0.64	18.56	0.93
Muscle	3.86	0.75	2.14	0.79
Cerebrum	18.25	2.25	16.31	2.41
Hind brain	12.97	1.32	10.21	1.92
Hypothalamus	19.92	1.33	17.20	2.17
Normal Nonobese				
Blood	0.94	0.1	2.16	0.31
Adrenal	7.84	0.74	7.82	3.80
Kidney	18.83	1.46	14.21	1.21
Liver	18.27	1.08	16.30	1.31
Muscle	11.04	1.75	6.60	2.40
Cerebrum	23.60	2.45	20.61	3.12
Hind brain	22.73	0.98	16.87	2.71
Hypothalamus	11.63	0.88	9.81	1.60

[a] Each value is the arithmetical mean of 5 individual animals. [b] Administered 30 min. prior to administration of glucose-1-C-14.

14 was prepared by adding an aliquot of the solution to 10 ml. of the liquid scintillation solvent system. The activity of each blood and tissue sample and the glucose-1-C-14 standard was determined by means of a liquid scintillation detector and associated β spectrometer (Ekco Detector model N664 and Scaler model N610A). Quenching of the true count rate of each sample was corrected by use of an internal standard of hexadecane-1-C-14 (13). Blank samples were prepared to determine the background count. The per cent of the administered radioactive dose per Gm. of dry tissue and per ml. of blood was calculated for each animal. The mean values are tabulated in Table III. The dry tissue weights were calculated from the known moisture contents, determined as previously described (1).

RESULTS AND DISCUSSION

The administration of chlorpromazine caused a significant ($p > 95$) loss of body weight, reduction of food intake, oxygen consumption in ml./Gm./hr., and lowered Gm.-cal./hr./cm.^{2} in both the gold thioglucose-obese and the normal nonobese mice, as compared to their respective controls.

The actual weight loss was greater in the obese mice than with the nonobese mice (1.5–0.72 Gm.), but when calculated as Gm./Gm. of the initial body weight or per cent, the values were 0.028 Gm./Gm. or 2.8 % of the obese and 0.022 Gm./Gm. or 2.2 % for the nonobese mice. Food intake was reduced 1.85 Gm. (29.8%) or 0.028 Gm./Gm. initial body weight for the obese mice and 0.45 Gm. (10.3%) or 0.02 Gm./Gm. initial body weight for the nonobese mice.

Oxygen consumption in ml./Gm./hr. was similar in both groups of mice, but when calculated on the basis of body surface and expressed as Gm.-cal./hr./cm.^{2}, the obese mice had higher values than the non-

obese and in both groups the administration of chlorpromazine caused a significant ($p > 95$) reduction in the observed values. This effect is not expected with weight loss and is indicative of a reduced metabolic rate and/or depression of the C.N.S. The latter was not observed, and in fact, there was no visual difference in activity between the saline controls and the chlorpromazine-treated animals in either groups of mice. It is known, however, that the release of TSH to affect the secretion of the thyroid hormone is influenced by hypothalamic centers (14), and the action of chlorpromazine to lower the metabolic rate could be due to an action on this sequence, and in particular on the hypothalamic nuclei involved.

The mechanism by which chlorpromazine affected food-intake reduction was of sufficient magnitude to result in significant weight losses in both groups of mice even with the reduction of caloric output. As both the weight losses and food intake reduction values were similar in the obese and nonobese mice when compared on the Gm./Gm. initial body weight basis, it is probable that the mechanism was similar in both groups of mice. The effect of chlorpromazine on the residual tissue activity following administration of labeled glucose was studied as a possible clue to the mechanism of food-intake reduction and in general, a slight reduction in residual tissue activity was observed in all tissues as compared to the controls, with the exception of kidney tissue from the gold thioglucose-obese mice. Blood levels were significantly higher ($p > 95$) in both groups of mice. The lowered tissue levels, coupled with the increased blood levels are in agreement with the observed reduced oxygen consumption and energy output; and the increased blood levels, undoubtedly associated with elevated blood sugar, would be expected to have activated the ventro medial nuclei to inhibit the lateral hypothalamic feeding center (4, 5). This action is not supported, however, by the brain residual tissue activities, as chlorpromazine was observed to cause lower levels in all the brain tissues examined, and if activation of the ventro medial nuclei had occurred, the hypothalamic levels should have been higher than in the controls. Furthermore, the gold thioglucose-obese mice with destroyed or partially destroyed ventro medial nuclei had a similar food-intake reduction as the nonobese on the Gm./Gm. basis. The lower tissue activities in all brain tissue indicate a general mild depression, and this could include the lateral hypothalamic feeding center or centers in the forebrain. Sedation would not be expected to cause sufficient reduction in food intake to cause weight loss as observed, particularly with the reduction in the metabolic rate. Thus, it would appear that the action of chlorpromazine to reduce food intake involves a specific central action. The data presented do not permit a conclusion as to centers involved, but do exclude action through activation of the ventro medial nuclei and increased glucose uptake by these cells. Hypothalamic activity can be postulated, however, for both food intake reduction and lowering of the metabolic rate, on the basis of previous reports as to the tissue distribution of chlorpromazine and action on other hypothalamic centers (8–10).

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Nuclear Magnetic Resonance Spectra of Amines I. Identification of *N*-Methyl Tertiary Amines

By W. E. THOMPSON, R. J. WARREN, J. E. ZAREMBO, and I. B. EISDORFER

The effects of strongly acidic solvents on the chemical shift and spin-spin splitting of the methyl protons in *N*-methyl tertiary amines are found to be unique for this particular functional group. These effects provide the basis for the simultaneous identification of the *N*-methyl group and the tertiary amine structure in compounds of unknown structure.

AN ANALYTICAL method using NMR has been developed for the qualitative analysis of the tertiary *N*-methyl amine function. The method is specific for aliphatic tertiary *N*-methyl amines and also distinguishes aliphatic from aromatic compounds. The method is based upon the effects of strongly acidic solvents on the NMR spectra of tertiary *N*-methyl amines.

The dependence of the NMR spectrum of *N*-methyl groups on hydrogen-ion concentration and the proton exchange rate in aqueous solutions is well known. Loewenstein and Meiboom (1) have made an extensive study of methylamine, dimethylamine, and trimethylamine in this regard. The splitting of the NMR absorption band of the methyl group in methylamine into a quartet by the 3 protons in the adjacent primary amine ion has likewise been studied and reported by Jackman (2) and Pople, Schneider, and Bernstein (3).

In this investigation the effects of protonation of the nitrogen atom and subsequent splitting of the *N*-methyl protons in aliphatic tertiary amines are illustrated. The NMR spectral changes are recommended for rapid identification of this class of amine.

EXPERIMENTAL

All spectra were recorded on a Varian A-60 spectrometer using Varian sample tubes. Deuterated chloroform and trifluoroacetic acid were used as solvents. Concentrated reagent sulfuric acid was used for diamine and aromatic amines. The spectra were obtained on samples at room temperature at a concentration of 50 mg./ml.

The simple tertiary amines were Eastman Organic Chemicals as purchased from Distillation Products Industries, Rochester, N. Y. The chlorpromazine base was analytical standard grade.

RESULTS AND DISCUSSION

Tertiary aliphatic amines containing 1 or more *N*-methyl groups show nuclear magnetic resonance (NMR) absorption of the methyl protons as a single peak in the vicinity of 2.2 p.p.m. downfield from tetramethylsilane as reported by Jackman (2). This single peak is characteristic of *N*-methyl tertiary amines as free base. The 2.2-p.p.m. chemical shift occurs in solvents of low dielectric constant, *e.g.*, carbon tetrachloride and chloroform. In a strongly acidic solvent such as trifluoroacetic acid the amine is transformed into the tertiary amine ion. The positive electrostatic charge on the ion increases the *N*-methyl chemical shift approximately 1 p.p.m. and the single peak is split into a doublet with a coupling constant of 5 to 6 c.p.s.

Figure 1 illustrates the NMR spectrum of an *N*-methyl tertiary amine as free amine in deuterated chloroform (spectrum A), and as the tertiary amine ion in trifluoroacetic acid (spectrum B). This phenomenon is seldom observed for an amine salt in chloroform solution due to the poor NMR spectrum resolution of ion dipoles and other ion aggregates in solvents of low dielectric constants. Solvents of medium to high dielectric constant such as water, methanol, or trifluoroacetic acid are preferred for high resolution spectra of amine salts.

The splitting of the methyl absorption band observed in trifluoroacetic acid solution is due to coupling of the methyl protons with the proton bound to the nitrogen in the tetrahedral tertiary amine ion. As mentioned previously in this paper, a similar effect has been observed in the case of primary amines. The protons adjacent to the NH_3^+ ion are split into a quartet (2, 3).

The observations from spectra A and B are general for *N*-methyl aliphatic tertiary amines. Table I lists the *N*-methyl chemical shift data for the NMR spectra of 8 *N*-methyl aliphatic amines and

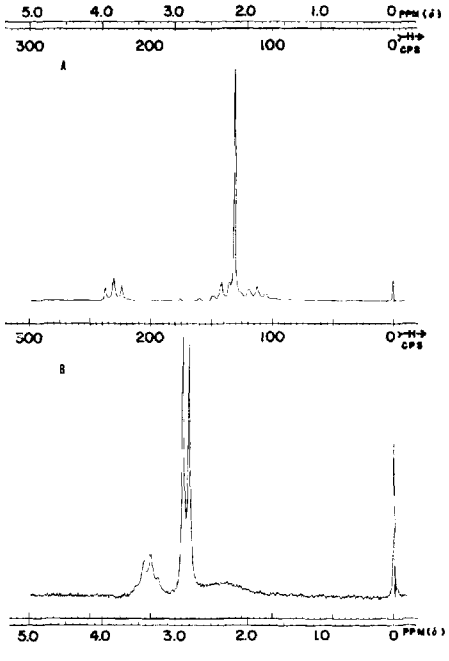

Fig. 1.—NMR spectrum of chlorpromazine (A) in $CDCl_3$ (B) in CF_3COOH.

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The authors acknowledge the assistance of Mrs. Eleanor Cherry and Mr. Richard Gillespie in obtaining the spectra presented here, and are grateful to Dr. W. D. Kumler, School of Pharmacy, University of California, for helpful discussions.

TABLE I.—NMR CHEMICAL SHIFTS FOR *N*-METHYL GROUP IN ALIPHATIC TERTIARY AMINES

Compd.	δ^a in $CDCl_3$, p.p.m.	δ^a in CF_3COOH, p.p.m.	J^b in CF_3COOH, c.p.s.
Trimethylamine	2.20	3.01	5.5
2-Dimethylaminoethanol	2.28	3.02	5.0
n-Dodecyldimethylamine	2.25	3.06	5.5
N-Methyldioctadecylamine	2.18	3.05	5.5
N-Methylpiperidine	2.22	3.01	5.5
N-Dimethylcyclohexylamine	2.32	3.03	5.5
N,N-Dimethylbenzylamine	2.22	3.05	5.5
10-(3-Dimethylaminopropyl)-2-chlorophenothiazine (chlorpromazine)	2.17	2.84	5.5

[a] δ = chemical shift downfield from tetramethylsilane internal standard. [b] J = coupling constant between proton on nitrogen and protons on adjacent methyl.

TABLE II.—NMR CHEMICAL SHIFTS FOR TERTIARY *N*-METHYL GROUPS IN AROMATIC AMINES AND ALIPHATIC DIAMINES

Compd.	δ in $CDCl_3$	δ in CF_3COOH	J in CF_3COOH	δ in H_2SO_4	J in H_2SO_4
N,N-Dimethylaniline	2.83	3.42	0	3.41	5.0
N-Methyldiphenylamine	3.27	3.90	0	3.80	5.0
N-Methylpiperazine	2.22	3.21	0	3.09	5.5
N,N-Dimethylpiperazine	2.28	3.22	0	3.07	5.0

corresponding amine ions. Aromatic amines and aliphatic diamines generally require a stronger acid (*e.g.*, sulfuric) to split the methyl absorption (Table II). In sulfuric acid the ion is stable. These weaker amines exchange protons in CF_3COOH at a high enough rate to effectively decouple the ion proton (NH^+) resulting in a J constant of zero. The larger chemical shift due to ring currents in aromatic amines can be used to differentiate an aliphatic *N*-methyl amine from an aromatic amine.

The splitting has been observed for every *N*-methyl aliphatic tertiary amine examined to date at room temperature in trifluoroacetic acid. The amine must be added to the trifluoroacetic acid as the free base. If an amine salt of a strong inorganic acid such as amine hydrochloride is dissolved in trifluoroacetic acid, the amine ion-strong acid dipole remains unionized in the acid. The NMR spectrum in this case is usually too poorly resolved to allow one to observe the 5 to 6 c.p.s. splitting.

Chloroform and trifluoroacetic acid are much more widely useful as solvents for NMR than are either acidic or basic water, since many large tertiary amines and their salts are poorly soluble in either aqueous acid or base.

CONCLUSION

The differences between tertiary amine free base NMR spectra and tertiary amine ion spectra are an extremely useful diagnostic tool. The increased chemical shift and splitting of the *N*-methyl peak in the case of the ion makes it possible to establish the presence of the *N*-methyl group and aliphatic tertiary amine structure in compounds of unknown structure.

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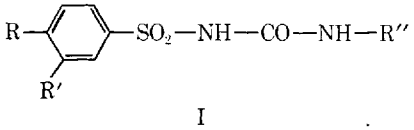
Preparation of Tritium-Labeled Compounds I. Series of Sulfonylurea Hypoglycemic Agents by Exchange with Tritium Gas

By RICHARD C. THOMAS and GEORGE J. IKEDA

A series of tritium-labeled sulfonylurea hypoglycemic agents was prepared by the tritium-gas exposure method. Sodium salts of the sulfonylureas were found to incorporate stably bound tritium more effectively than did the corresponding free compounds. Exposure to tritium gas in the presence of an electrical discharge was also found to be an effective method for preparing tritium-labeled sulfonylureas.

As part of a program to develop improved hypoglycemic agents of the sulfonylurea class (I), it became necessary to label a number of these com-



I

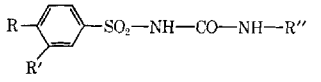
pounds with tritium for use in studying their absorption, distribution, metabolism, and excretion in humans. The tritium-gas exchange method of Wilzbach (1) was chosen because of the relatively low cost of tritium gas and the need for labeling a large number of sulfonylureas, not all of which would necessarily prove of sufficient clinical interest for continued study. An advantage of the Wilzbach method is that a large number of compounds can be exposed to tritium with minimal cost and little investment in time. If a particular compound proves of sufficient interest, the more difficult task of purification can then be undertaken without delay. Thus, in the present case, only 7 of a series of 10 sulfonylureas that had been exposed to tritium were actually purified.

Preparing this series of tritium-labeled sulfonylureas offered an opportunity to study not only the feasibility of labeling this type of compound by tritium-gas exposure but the effects of several variables on their incorporation of tritium. Thus, 15 sulfonylurea samples, representing 7 structure types within the series, were tritiated and purified. Five compounds were exposed to tritium, both as the free sulfonylurea and as the corresponding sodium salt. Two additional sulfonylureas were tritiated, both by conventional tritium-gas exposure and by exposure in the presence of an electrical discharge.

Although exposure of a compound to tritium is relatively inexpensive and easy, subsequent purification of the compound to a state of radiochemical purity can be extremely time consuming and difficult. Trace impurities of very high specific activity are often formed by reduction of an unsaturated bond or by radiation-induced reactions, such as fragmentation, isomerization, and polymerization (1–4). Such impurities, although present in only trace amounts by weight, may contain the bulk of the tritium in the crude sample. These impurities are often difficult to remove, and great care must be taken to establish the radiochemical purity of the final product. Attainment of constant specific activity following recrystallization from several solvent systems is not a sufficient criterion of radiochemical purity for a compound labeled by exposure to tritium gas. After establishing constant specific activity, it is necessary to confirm the radiochemical purity by 1, or preferably several, multistage tests such as thin-layer, paper, or gas chromatography or countercurrent distribution (1–4).

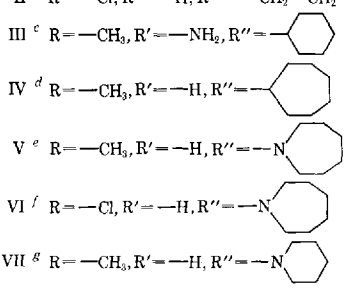
Establishing chemical purity of the final product by elemental and spectral analysis, although of little value in determining the radiochemical purity of a compound labeled by exposure to tritium, is necessary to be certain that the compound will exert its normal biological activity when used in metabolism studies. This is particularly important when the labeled compound is to be administered to humans.

Table I.—Exposure of Sulfonylureas to Tritium



I [a] R = —CH_3, R′ = —H, R″ = —CH_2—CH_2—CH_2—CH_3

II [b] R = —Cl, R′ = —H, R″ = —CH_2—CH_2—CH_3



III [c] R = —CH_3, R′ = —NH_2, R″ = —

IV [d] R = —CH_3, R′ = —H, R″ = —

V [e] R = —CH_3, R′ = —H, R″ = —N

VI [f] R = —Cl, R′ = —H, R″ = —N

VII [g] R = —CH_3, R′ = —H, R″ = —N

Exposure No.	Compd.	Form	Wt., Gm.	Exposure, curie Days
1	I	Salt	1.00	82.5
2	II	Salt	2.00	184
3[h]	II	Free	1.10	49
4	III	Salt	1.00	82.5
5	IV	Salt	1.25	84
6[h]	IV	Free	1.10	27.3
7[i]	V	Salt	1.25	42
8	V	Salt	1.25	92
9[h]	V	Free	1.10	51
10	VI	Salt	2.00	172
11[h]	VI	Free	1.10	54.5
12[i]	VII	Salt	1.25	42
13[h]	VII	Free	1.10	49

[a] Tolbutamide. [b] Chlorpropamide. [c] Metahexamide. [d] Cycloheptolamide. [e] Tolazamide. See *Reference 5*. [f] Glypinamide. See *Reference 5*. [g] See *Reference 5*. [h] Exposure made by Tracerlab, Inc. [i] Exposure made by New England Nuclear Corp.

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TABLE II.—EXPOSURE OF SULFONYLUREAS TO TRITIUM WITH ELECTRICAL DISCHARGE

Exposure No.	Compd.[a]	Form	Wt., Gm.	Tritium Gas, curies	Exposure Time, min.
14	I	Salt	1.00	2.5	20
15	III	Salt	1.00	2.5	20

[a] See Table I for structures.

TABLE III.—RESULTS OF EXPOSURE OF SULFONYLUREAS TO TRITIUM

Exposure No.	Compd.[a]	Recrystallizations, No.	Yield,[b] Gm.	Specific Activity,[b] μc./mg.	Incorp. of Stably Bound Tritium,[b] μc./curie-Day Exposure
1	I	3[c,d]	0.699	14.8	165
2	II	10[e–h]	0.597	16.1	161
3	II	6[e,f]	0.241	1.46	32.8
4	III	3[c,d]	0.618	7.27	82
5	IV	4[e,g]	0.376	10.8	150
6	IV	5[e,g]	0.255	2.76	111
7	V	3[e,g]	0.308	2.68	74
8	V	3[e,i]	0.509	5.3	67
9	V	4[e,g]	0.543	0.64	13.2
10	VI	5[e,i,j]	0.954	12.6	137
11	VI	3[e,i]	0.895	0.62	12.5
12	VII	4[e,j]	0.095	3.36	92
13	VII	4[e,j]	0.259	0.78	17.5

[a] See Table I for structures and form exposed. [b] Based on free sulfonylurea. [c] Methanol–water. [d] Ethyl acetate–heptane. [e] Ethanol–water. [f] Benzene. [g] Ethyl acetate. [h] Acetone–water. [i] Methyl ethyl ketone. [j] Benzene–heptane.

EXPERIMENTAL AND RESULTS

Preparation of Samples for Exposure to Tritium Gas.—The sodium salts of the sulfonylureas were prepared by adding a molar equivalent of 1 *N* aqueous sodium hydroxide solution to a rapidly stirred suspension of the finely ground compound in water. The resulting solution was lyophilized to obtain the desired sulfonylurea salt as a finely divided solid suitable for exposure to tritium gas. The free sulfonylureas were prepared for exposure by grinding them to a very finely pulverized state in a mortar. Particle-size determinations were not made in either case.

Exposure to Tritium Gas.—The samples were exposed to carrier-free tritium gas under the conditions noted in Table I.[1] When exposures were made in this laboratory, tritium gas was transferred by means of a Toepler pump into an ampul containing the material at a pressure of 10^{-5} to 10^{-6} mm. of mercury. The ampul and its contents under approximately 0.2 Atm. of tritium were stored in the dark at room temperature for an appropriate period of time, usually 2 to 4 weeks. At the end of the exposure period, the waste tritium gas was removed by means of the Toepler pump to leave the crude tritiated material at a pressure of 10^{-5} to 10^{-6} mm. of mercury.

Exposure to Tritium Gas with Electrical Discharge.—The discharge was carried out in a cell similar to the ones described by Dorfman and Wilzbach (6) and Jackson *et al.* (7) at a tritium pressure of approximately 15 mm. using a Tesla coil leak detector as a source of voltage. The sample was suspended in a glass cylinder between the 2 electrodes. Conditions used in the exposures are noted in Table II.

Purification.—Following exposure to tritium gas, each crude sample was precipitated from alkaline solution by addition of a slight excess of acid and then filtered and washed with water. This procedure was carried out 3 times to remove labile tritium from the sample completely. During one of the precipitations, the alkaline solution was treated with activated charcoal (Darco G-60). Some removal of high specific activity impurities was effected by this treatment. Samples were then recrystallized to constant specific activity using 2–4 solvent systems and 3–10 recrystallizations. The state of radiochemical purity was followed by paper chromatography and by scanning for radioactivity as described below. The number of recrystallizations required, solvent systems used, weight yield, specific activity, and stably bound tritium incorporated for each sample are presented in Tables III and IV.

Determination of Chemical Purity.—Ultraviolet and infrared spectra of each purified sample corresponded to authentic standards. Elemental analyses—carbon, hydrogen, nitrogen, and sulfur (chlorine for those containing this element)—were satisfactory (within ±0.3% absolute of theory) in each case.

Determination of Radiochemical Purity.—Each sample was subjected to paper chromatography in the 1-butanol–piperidine–water (81:2:17 by volume) system on Whatman No. 1 paper and to thin-layer chromatography in the chloroform–formic acid (95:5 by volume) system on silica gel GF. The compound was located in each case by fluorescence quenching under short-wave ultraviolet light. The developed paper chromatograms were scanned for radioactivity by means of a 4-π windowless, paper-strip scanner[2] having a counting efficiency of approximately 1–2% for tritium. The developed thin-layer chromatograms were first sprayed with a plastic dispersion[3] and allowed to dry at room temperature to give the silica gel a paper-like consistency

[1] Certain of the exposures, as indicated in Table I, were carried out by New England Nuclear Corp. or Tracerlab, Inc.

[2] Vanguard Instrument Co., LaGrange, Ill.

[3] Marketed as Neatan by Brinkmann Instruments, Inc., Great Neck, N. Y.

TABLE IV.—RESULTS OF EXPOSURE OF SULFONYLUREAS TO TRITIUM WITH ELECTRICAL DISCHARGE

Exposure No.	Compd.[a]	Recrystallizations, No.	Yield,[b] Gm.	Specific Activity,[b] μc./mg.
14	I	5[c,d]	0.514	3.67
15	II	5[c,d]	0.479	3.57

[a] See Table I for structures. [b] Based on free sulfonylurea. [c] Methanol–water. [d] Ethyl acetate–heptane.

and thus facilitate its handling. The silica gel film was then cut into sequential 0.5-cm. sections (usually 30) which were transferred to individual counting vials. One-half milliliter of dimethylformamide was added to each vial to dissolve the plastic dispersion,[3] releasing the silica gel, and the series of vials was counted as described below. Each purified sample showed a single radioactive zone in each chromatographic system corresponding to the zone of fluorescence quenching which in turn corresponded to the migration of the authentic sulfonylurea.

Radioactivity Measurements.—All counting was performed with a Tri-carb[4] model 314X or 314EX-2A liquid scintillation spectrometer at −8° under conditions suitable for measuring tritium. Suitable aliquots of samples were dissolved in 15 ml. of diotol scintillator{toluene–dioxane–methanol [350:350:210 by volume] containing 73 Gm. of naphthalene, 4.6 Gm. of 2,5-diphenyloxazole, and 0.080 Gm. of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter}. The absolute counting efficiency (usually 12 to 16%) for each sample was determined by addition of an internal standard of tritium-labeled toluene and results then expressed as microcuries.

DISCUSSION

As shown in Table III, the sulfonylureas were more satisfactorily labeled with tritium by exposure of their sodium salts, rather than the free compounds, to tritium gas. The sodium salts incorporated 5 times as much stably bound tritium on the average as did the free sulfonylureas. It is possible that in the case of the free sulfonylureas the tritium tends to localize at the labile —NH— position, diluting the tritium gas with hydrogen and thereby decreasing the rate of exchange of tritium with the more stable hydrogens. Blocking the labile position by formation of the sodium salt may thus allow tritium to exchange with stable hydrogens at a more favorable rate. Other explanations, such as more self-destruction of the desired labeled species when a large quantity of tritium is present in a labile position or a larger particle size or less porous surface in the cases of the free sulfonylureas, although speculative, are also possible.

Incorporation of tritium into stable positions of the sulfonylurea sodium salts was remarkably uniform for the series of 7 compounds, ranging from 67 to 165 μc. per curie-day exposure. In the single case where 2 samples of the same sulfonylurea sodium salt (compound V, Table III) were tritiated, the incorporations were in good agreement, 67 and 74 μc. per curie-day exposure, even though substantially different exposures were made in different laboratories.

Incorporation of stably bound tritium into the free sulfonylureas did not appear to be nearly so uniform, ranging from 12.5 to 111 μc. per curie-day exposure. However, except for the high value of 111 μc. per curie-day exposure (compound IV, Table III), the range of incorporations (12.5 to 32.8 μc. per curie-day exposure) was no greater than that of the sodium salts.

Compounds I and III (structures in Table I) were also tritiated by exposure to tritium gas in the presence of an electrical discharge as summarized in Tables II and IV. These tritiations were successful although the specific activities attained were not as high as those obtained by the conventional exposure method. Increasing the discharge time might have increased incorporation of stably bound tritium, but it probably would also have increased degradation (6). Even with a 20-min. discharge, more degradation appeared to take place than was the case where conventional exposure conditions were used. It is interesting to note that the specific activities of the 2 sulfonylureas prepared by the discharge method were essentially the same, whereas the specific activity of I was twice that of III when the conventional exposure method was used. These results may be due to a difference in mechanisms or a relative difference in the contributions of several mechanisms leading to incorporation of tritium by the 2 methods as discussed by Ache *et al.* (8).

[4] Packard Instrument Co., Inc., Downers Grove, Ill.

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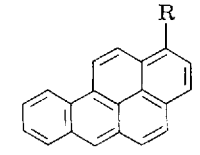
Unusual Grignard Reaction of 1-Acetylbenzo[*a*]pyrene

By O. LEROY SALERNI, JAMES F. ENGEL, and JAMES J. DOWNS

A unique dimeric alcohol, 2,4-bis(1-benzo[*a*]pyrenyl)-4-methyl-2-pentanol (IV*a*), was formed in excellent yield by the Grignard reaction of 1-acetylbenzo[*a*]pyrene (I) and methyl magnesium iodide. This unusual product was isolated when a hydrochloric acid solution was used in the work-up. Use of an ammonium chloride work-up did not give the dimeric alcohol. The preparation and some properties of other 1-substituted benzo[*a*]pyrenes are described.

In the course of the authors' program dealing with the carcinogenesis of polynuclear aromatic hydrocarbons, it became of paramount importance to prepare some 1-substituted benzo[*a*]pyrene derivatives.

Whereas methyl aryl ketones (1, 2) undergo Grignard reactions with methylmagnesium iodide to give excellent yields of carbinol or olefin, we find that 1-acetylbenzo[*a*]pyrene (3) (I) reacts with methylmagnesium iodide in an unexpected manner. The reaction of 1-acetylbenzo[*a*]pyrene (I) with methylmagnesium iodide, on subsequent acidification with hydrochloric acid, did not yield the expected product, either dimethyl-1-benzo[*a*]pyrenylcarbinol (II*a*) or 1-isopropenylbenzo(*a*)pyrene (III).



I, R = —C(=O)—CH$_3$

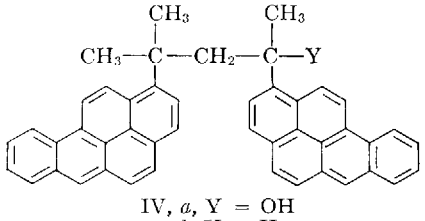
II, R = CH$_3$—C(CH$_3$)—X

a, X = OH

b, X = H

III, R = CH$_2$=C(CH$_3$)—

Rather, the reaction gave a dimeric alcohol, 2,4-bis-(1-benzo[*a*]pyrenyl)-4-methyl-2-pentanol (IV*a*), in 84% yield.



IV, *a*, Y = OH

b, Y = H

The nuclear magnetic resonance spectrum of the product is in complete agreement with the proposed structure. Integration of the NMR spectrum in aromatic and aliphatic regions showed a ratio of 22:11 of aromatic to aliphatic hydrogens. An ebulliometric molecular weight measurement in benzene gave a value of 590, in excellent agreement with the calculated value of 602. The infrared spectrum showed a small, rather broad band, centered around 3400 cm.$^{-1}$, characteristic of a hydroxyl group (4). Finally, elemental analyses supported the structure.

The structure of 2,4-bis(1-benzo[*a*]pyrenyl)-4-methyl-2-pentanol (IV*a*) was further supported by chemical evidence. Reaction of 2,4-bis(1-benzo[*a*]pyrenyl)-4-methyl-2-pentanol (IV*a*) with red phosphorus and iodine in refluxing acetic acid (5) gave the corresponding hydrocarbon, 2,4-bis(1-benzo[*a*]pyrenyl)-2-methylpentane (IV*b*), in 77% yield. The structure of IV*b* was supported by elemental analyses and infrared spectrum. The infrared spectrum of the compound did not show a peak for hydroxyl.

Mechanistically speaking, the formation of the dimeric compound can be rationalized by assuming that dimethyl-1-benzo[*a*]pyrenylcarbinol (II*a*) in acid medium forms an oxonium ion which undergoes facile cleavage to the corresponding carbonium ion and 1-isopropenylbenzo[*a*]pyrene (III). The 1-isopropenylbenzo[*a*]pyrene (III), in turn, combines with the carbonium ion in such a way as to give rise to a new stable tertiary carbonium ion (V) which is attacked in the aqueous medium by a nucleophilic water molecule with subsequent loss of a proton to afford 2,4-bis(1-benzo[*a*]pyrenyl)-4-methyl-2-pentanol (Scheme I).

A study of molecular models clearly shows the dimeric carbonium ion (V) to be sterically crowded so that reaction with another isopropenyl moiety appears all but impossible, thus explaining why further polymerization does not occur.

When the Grignard reaction of 1-acetylbenzo[*a*]pyrene (I) with methylmagnesium iodide was carried out with subsequent acidification with ammonium chloride solution, the dimeric alcohol (IV*a*) did not form. The substance isolated, on the basis of the nuclear magnetic resonance spectrum, was a 60:40 mixture of dimethyl-(1-benzo[*a*]pyrenyl)carbinol (II*a*) and 1-isopropenylbenzo[*a*]pyrene (III). The mixture was dissolved in glacial acetic acid and heated in a water bath to 50°. Almost instantly, bright-yellow needles of 1-isopropenylbenzo[*a*]-pyrene (III) began to precipitate. The infrared spectrum of the collected crystalline material was different from that of the mixture. Furthermore, the nuclear magnetic resonance spectrum indicated an 11:5 ratio of aromatic to aliphatic hydrogens and displayed peaks at δ = 5.2 and 5.6 p.p.m., indicative of an α-methylstyrene moiety (6). Elemental analyses supported the structure, and the

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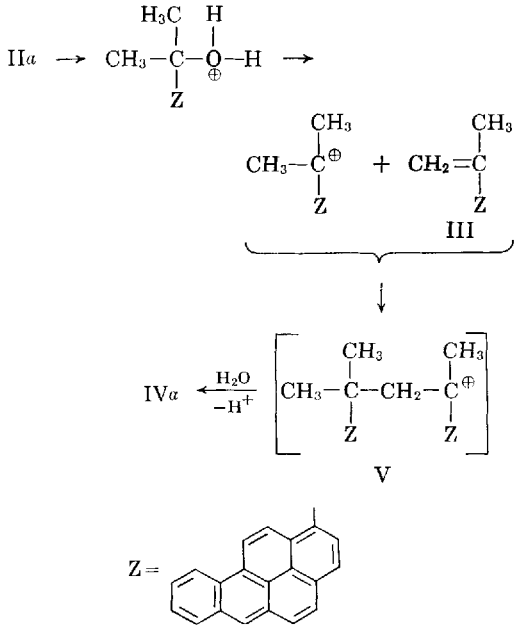

Scheme I

compound gave a m. p. 110–112° in agreement with the literature.[1]

The isopropenyl compound (III) was converted to 1-isopropylbenzo[*a*]pyrene (II*b*) in 75% yield by room temperature hydrogenation for 2 hr. using a 2:1 ratio of substrate to palladium black catalyst at 52 p.s.i. The nuclear magnetic resonance spectrum of the compound showed a characteristic 7-line pattern for the methine proton, and was void of peaks at δ = 5.2 and 5.6 p.p.m., indicating that the double bond of the α-methylstryrene moiety had been reduced to give the isopropyl derivative (II*b*).

EXPERIMENTAL[2]

2,4 - Bis(1 - benzo[a]pyrenyl) - 4 - methyl - 2 - pentanol (IV).—A Grignard reagent was prepared from 2.3 Gm. (83 mmoles) of magnesium, 6.4 ml. (100 mmoles) of methyl iodide, and 44 ml. of dry ether. When the reaction was complete, a slurry of 4.4 Gm. (15 mmoles) of 1-acetylbenzo[*a*]pyrene (I) in 420 ml. of dry benzene was added. The mixture was stirred and heated under reflux for 4 hr., cooled, and decomposed with 300 ml. of ice-cold 10% hydrochloric acid solution and worked up in the usual way. The product crystallized when triturated with cold ethanol and was purified by passing its benzene solution through an alumina column. In this way was obtained 3.6 Gm. (84%), m.p. 200–205° (gradual decomposition).

Anal.—Calcd. for $C_{46}H_{34}O$: C, 91.66; H, 5.69; mol. wt., 602. Found: C, 91.68; H, 5.64; mol. wt., 592.

1-Isopropenylbenzo[a]pyrene (III).—A Grignard reaction was carried out using the same procedure and quantities of magnesium, methyl iodide, and 1-acetylbenzo[*a*]pyrene, as above, except that the reaction was decomposed with 300 ml. of ice-cold saturated ammonium chloride solution. The oily product obtained was dissolved in 25 ml. of glacial acetic acid and heated to 50°, at which time bright-yellow needles precipitated. After chilling, the solid was collected by suction filtration and recrystallized from absolute ethanol, yielding 2.2 Gm. (50%) of bright yellow needles, m.p. 110–112.° [Lit. (3) m.p. 114.°]

Anal.—Calcd. for $C_{23}H_{16}$: C, 94.48; H, 5.52. Found: C, 94.37; H, 5.46.

1-Isopropylbenzo[a]pyrene (IIb).—A solution of 2.0 Gm. (4.3 mmoles) of III in 100 ml. of benzene was hydrogenated at a pressure of 52 p.s.i. and at room temperature using 1.0 Gm. of palladium black as the catalyst. After 2 hr., the catalyst and solvent were removed. The crude yield was quantitative. Recrystallization from absolute alcohol yielded 1.5 Gm. (75%), m.p. 128–130°.

Anal.—Calcd. for $C_{23}H_{18}$: C, 93.84; H, 6.16. Found: C, 93.76; H, 6.21.

2,4 - Bis - (1 - benzo[a]pyrenyl) - 2 - methylpentane (IVb).—In 125 ml. of glacial acetic acid and 10 ml. of water was suspended 1.0 Gm. (1.6 mmoles) of IV*a*, 200 mg. of iodine, and 200 mg. of red phosphorus. The mixture was stirred and heated under reflux for 29 hr., cooled, then extracted with three 80-ml. portions of benzene. The combined organic layers were washed with water and then dried over anhydrous sodium sulfate. Filtration and concentration yielded a crystalline solid which after recrystallization from benzene–ligroin afforded 603 mg. (77%), m.p. 210–215° dec.

Anal.—Calcd. for $C_{46}H_{34}$: C, 94.16; H, 5.86. Found: C, 93.87; H, 6.01.

[1] See *Reference 3*, p. 162. Windaus reported the synthesis of 1-isopropenylbenzo[*a*]pyrene by heating the product from the reaction of methylmagnesium iodide with 1-acetylbenzo[*a*]pyrene after an ammonium chloride workup at 230–280° under high vacuum in the presence of powdered zinc.

[2] All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. All analyses and molecular weight determinations were carried out by Galbraith Laboratories, Inc., Knoxville, Tenn. Infrared absorption spectra were taken as Nujol mulls and determined on a Perkin-Elmer model 137 spectrophotometer. The NMR spectra were determined on a Varian A-60 spectrometer with tetramethylsilane as the internal reference. Deuterochloroform solutions were used for all spectra.

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Small Tube Method for the Evaluation of Antifungal and Antibacterial Activity

By PHILIP CATALFOMO and H. WAYNE SCHULTZ

Small tubes containing a thin layer of agar medium have been used in a method for screening pure chemicals for antifungal and antibacterial activity. Employing known agents, this method provided results comparable to those obtained by other methods. In addition, the method affords the following advantages which make it suitable as a screening procedure: a no-growth end point which may be readily determined, it is effective for nonaqueous as well as aqueous solutions, it utilizes inexpensive apparatus, a minimum quantity of media and test agents are required, and a relatively small amount of working area is needed for conducting the tests.

ALTHOUGH there are numerous techniques available to test for antimicrobial activity, none of them appear suitable for the simultaneous screening of a large number of agents. The various modifications of these methods have been reviewed and evaluated by Reddish *et al.* (1), Skinner (2), and Heatley (3). Vanbreuseghem (4) recently considered the merits of those methods which determine antifungal activity.

The *in vitro* methods generally are classified as either dilution or diffusion methods. The former are best suited for assay procedures, but the methods are time-consuming for screening purposes, and many of them are not satisfactory to determine antifungal activity when filamentous fungi are used as test organisms. This is particularly so if partial inhibition is studied because it is difficult to determine the amount of growth of these fungi. Diffusion methods, such as represented by the use of filter paper disks on an agar plate, find limited value with water-insoluble agents and with organic solvents having antimicrobial activity. Also, the determination of the end point, usually the measuring of clear zones of inhibition, is tedious and somewhat arbitrary, especially with filamentous fungi.

The above factors, plus certain other innate disadvantages of existing methods, prompted this study. A technique has been developed which appears to be suitable for screening, qualitatively and semiquantitatively, large numbers of agents for antifungal and/or antibacterial properties.

EXPERIMENTAL AND RESULTS

Materials and Methods.—The fungal species used were *Candida albicans*, ATCC 10231, and *Trichophyton mentagrophytes*, ATCC 9972. The bacterial species employed were *Escherichia coli* and *Staphylococcus aureus* from the collections maintained at the Department of Microbiology, Oregon State University.[1]

The fungal organisms were maintained on Sabouraud dextrose medium Difco (liquid and agar), and the bacteria were maintained on nutrient medium Difco (broth and agar). The liquid cultures also served as a source of inocula. The experimental studies were conducted in flatbottom, screw-capped vials (17 × 60 mm.) containing 0.5 ml. of the desired medium. This volume provided a layer of medium approximately 2 mm. in depth. Antifungal activity was determined on Sabouraud dextrose agar medium and antibacterial activity on nutrient agar medium.

Actively growing 2- to 4-day-old liquid cultures (10 ml./culture tube) of bacteria and *C. albicans* were used as inocula. The small experimental tubes were inoculated by adding 1 drop of a suspension of the respective organisms utilizing a sterile standard medicine dropper (about 20 drops/ml. of water). To facilitate obtaining inocula with a medicine dropper, all organisms were transferred to sterile 15-ml. wide-mouth containers.

For studies using *T. mentagrophytes*, transfers were made from agar slants to 125-ml. conical flasks containing 30 ml. of Sabouraud medium. The mycelial growth from 5- to 7-day-old stationary cultures was homogenized in a sterile semimicro Waring blender for 30 sec. and added as inocula to the experimental tubes as described previously. All incubations were conducted at 37°.

Solutions of the agents to be tested were prepared in acetone, water, or 95% ethanol, depending on their solubility, and placed in dropper bottles. Unless otherwise indicated, dilutions of 1:100, 1:500, 1:1,000, 1:5,000, and 1:10,000 were tested for all compounds. Prior to inoculation with microorganism, 2 drops (approximately 0.04 ml.) of the various freshly prepared solutions were added to the tubes, and the solvent was allowed to evaporate or be absorbed into the agar medium.

The tubes were examined for growth after 24–48 hr. of incubation for bacteria and *C. albicans* and 48–72 hr. for *T. mentagrophytes*. In certain instances incubation was allowed to continue for 5–7 days in order to determine if the method might be suitable to indicate fungistatic *versus* fungicidal activity.

Antimicrobial Studies.—The agents used to test antifungal activity included salicylic acid, salicylanilide, griseofulvin, nystatin, tolnaftate,[2] and penicillin V. Antibacterial determinations were made with penicillin V, streptomycin sulfate, sulfadiazine, sulfathiazole, and sodium sulfathiazole. The series of dilutions previously described were prepared as follows: acetone–salicylic acid, salicylanilide, griseofulvin, penicillin V, and sulfadiazine; water–streptomycin sulfate and sodium sulfathiazole; 95% ethanol–nystatin and tolnaftate. Replicates of 3 tubes per dilution for each organism

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The authors are indebted to Mrs. D. J. Bickford for technical assistance.

[1] Obtained through the courtesy of Dr. R. Y. Morita, Associate Professor of Microbiology, Oregon State University, Corvallis.

[2] Marketed as Tinactin by the Schering Corp.

TABLE I.—ANTIFUNGAL ACTIVITY

Compd. and Dilutions	*C. albicans*	*T. mentagrophytes*
Salicylic acid		
1:100	±[a]	
1:500	+	
1:1,000	+	
1:5,000	+	
1:10,000	+	
Salicylanilide		
1:100	−	−
1:500	−	−
1:1,000	±	−
1:5,000	+	−
1:10,000	+	−
Penicillin V acid		
1:100	+	+
1:500	+	+
1:1,000	+	+
1:5,000	+	+
1:10,000	+	+
Griseofulvin		
1:100	+	−
1:500	+	−
1:1,000	+	−
1:5,000	+	−
1:10,000	+	−
Tolnaftate[b]		
1:100	±	−
1:500	+	−
1:1,000	+	−
1:5,000	+	−
1:10,000	+	−
Nystatin[c]		
1:100	−	−
1:500	−	−
1:1,000	−	−
1:5,000	±	−
1:10,000	+	+

[a] +, growth; −, no growth; ±, equivocal growth. [b] Readings taken at 72 hr. [c] After 72 hr., growth of *T. mentagrophytes* was noted at all dilutions except 1:100.

were prepared. Controls consisted of tubes inoculated with the organism only, and blanks consisted of solvent and the respective microorganism. The results are summarized in Tables I and II. Unless stated otherwise, the final results are based on 48-hr. readings. The data reported here are consistent with those well documented for the agents listed (5, 6).

At the higher concentrations (1:100 and 1:500) the compounds which were relatively water-insoluble and tested as nonaqueous solutions tended to crystallize on the surface of the medium after the solvent evaporated. To determine if the presence of surface crystals affected the growth of the organism, or the activity of the agent, a modified procedure was tested. The compounds were suspended in the modified agar media (diluted 50%) in dilutions of 1:100, 1:500, and 1:1,000. Two drops of the suspension was added to the small tubes providing them with a thin film of medium containing the uniformly dispersed compound. The compounds tested in this manner were salicylanilide, griseofulvin, and penicillin V, with fungi; and penicillin V and streptomycin sulfate against bacteria. All except the last one are not soluble in water. Since the results did not vary from those obtained in the original study, they were not recorded here.

The study utilizing nystatin (Table I), comparing the effects of this agent on both fungi, was allowed to continue for 7 days. During this period, and commencing at 72 hr., growth of *C. albicans* developed in those tubes containing the 2 lower concentrations. Growth of *T. mentagrophytes* was noted at dilutions of 1:500 and higher at 72 hr., and which appeared completely uninhibited at the seventh day. Thus, it was shown that nystatin most likely acts as a fungicide against *C. albicans* in all but the extremely diluted concentrations and as a fungistat against *T. mentagrophytes* in all but the highest concentration, while having a low order of effectiveness. These data are in general agreement with previously recorded findings (7).

Growth of the microorganisms in those tubes designated as controls and blanks was luxuriant in all but 1 instance. Alcohol appears to have a slight fungistatic action against *T. mentagrophytes*. In those cases where alcohol was used as the solvent (nystatin and tolnaftate) readings were taken only after 72 hr.

DISCUSSION

A simplified method for the simultaneous screening of a relatively large number of pure chemicals for antimicrobial activity has been devised. Using established antifungal and antibacterial agents, it was demonstrated that the method provided results consistent with those obtained by other techniques. Thus, it would appear that other pure chemicals may be screened in a similar manner. Extended studies have indicated that this method is further applicable to the screening of crude plant extracts (8).

Since a no-growth end point is used and determined by mere visual inspection of the small tubes, the tedious task of measuring clear zones of inhibi-

TABLE II.—ANTIBACTERIAL ACTIVITY

Compd. and Dilutions	*E. coli*	*S. aureus*
Penicillin V acid		
1:100	−[a]	−
1:500	+	−
1:1,000	+	−
1:5,000	+	−
1:10,000	+	−
Streptomycin sulfate		
1:100	−	−
1:500	−	−
1:1,000	±	+
1:5,000	+	+
1:10,000	+	+
Sulfadiazine		
1:100	−	−
1:500	+	−
1:1,000	+	±
1:5,000	+	+
1:10,000	+	+
Sulfathiazole		
1:100	+	−
1:500	+	−
1:1,000	+	−
1:5,000	+	+
1:10,000	+	+
Sodium sulfathiazole		
1:100	+	+
1:500	+	+
1:1,000	+	+
1:5,000	+	+
1:10,000	+	+

[a] +, growth; −, no growth; ±, equivocal growth.

tion is avoided. This factor bears particular significance with microorganisms that produce irregular defined zones, *i.e.*, many filamentous fungi. According to Cavillito (9), complete absence of visual growth is the most reproducible and satisfactory end point in an inhibition test.

The effectiveness of most diffusion methods is dependent on those factors which govern the ability of the agent to diffuse both horizontally and vertically throughout the medium. Cooper (10) indicates the importance of these factors in critical assay procedures. They are less important when applied to general screening procedures which are primarily designed to merely distinguish between active and nonactive agents. Through the use of small tubes and thin layers of media, the extent of horizontal and vertical diffusion has been limited, thus rendering diffusion factors negligible.

Those compounds which were highly water insoluble and tested as nonaqueous solutions tended to crystallize on the surface of the medium after evaporation of the solvent. The occurrence of these crystals had no apparent effect on the growth of the microorganisms or on the suspected activity of the antimicrobial agents. For example, penicillin V, having the properties of low water solubility, antibacterial but not antifungal activity, demonstrated in this experiment no effect on the growth of the fungi while it did inhibit growth of the bacteria. Furthermore, when griseofulvin was tested it was shown that the surface crystals had no effect on the growth of *C. albicans*, against which this agent is ineffective, but it did exhibit its usual inhibitory effect on *T. mentagrophytes* (11). These findings were also substantiated in the experiment where the compounds were uniformly dispersed and added to the tubes in a thin film of the respective medium. In this instance the results did not vary from those observed in the original study.

A slight antifungal effect was noted when alcohol was used as a solvent for those agents tested against *T. mentagrophytes*, but this was easily overcome by merely extending the incubation period slightly and providing adequate control tubes for comparison.

The method appears to distinguish between fungistatic and fungicidal activity. This may be accomplished by extending the incubation period to 7 days or longer but not beyond 10 days, since the medium begins to show signs of drying. It would also be a simple matter to attempt to reculture the microorganism to determine the nature of the activity of the agent being studied.

Additional advantages afforded by this method are a minimum of expenditure for materials or apparatus, and that a relatively small amount of working area is required to test a series of compounds when compared to the space needed if Petri dishes are employed.

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Inhibition of Replication of Lee Influenza Virus in Tissue Culture by Puromycin

By K. S. PILCHER and J. N. HOBBS

Puromycin, an inhibitor of protein synthesis with general growth inhibitory properties, was found to inhibit replication of influenza virus in tissue culture in low concentrations without destroying all metabolic activity of the tissue. The aminonucleoside of puromycin, reported to be as effective as the complete compound against trypanosomes, was inactive as an inhibitor of the virus. Evidence suggests the mechanism of the virus inhibition is probably interference with protein synthesis, and that the antitrypanosomal activity has a different mechanism.

PUROMYCIN (I) is an antibiotic whose structure, proved by total synthesis by Baker *et al.* (1), is that of a nucleoside bound to an unusual amino acid.

Puromycin has been found to inhibit the growth of a variety of cells, including bacteria (2), protozoa (3, 4), and animal tumors (5). This compound has been shown to inhibit protein synthesis in several biological systems, including a cell free rat liver extract (6), Ehrlich ascites tumor cells, and rabbit reticulocytes (7). It was effective in curing experimental infections of mice and rabbits with several species of trypanosomes (3, 8), and also in the therapy of human trypanosomiasis (9).

Puromycin's mechanism of action seemed to offer a possible new approach to the inhibition of virus protein formation. During the course of this study, the inhibition of poliovirus replication in tissue culture by puromycin was reported (10).

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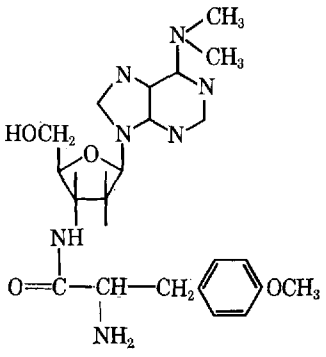
TABLE I.— INHIBITION OF REPLICATION OF LEE INFLUENZA VIRUS IN TISSUE CULTURE BY PUROMYCIN

	Concn. of Puromycin in Culture Fluid or Diluent, mcg./ml. 8.0	4.0	2.0	0
HA[a] units of virus/ml.[b,c] of tissue culture fluid	2.0	14.8	61.4	102.9
EID_{50} of virus/ml.[b,d] of tissue culture fluid	NT[e]	$10^{6.4}$	$10^{8.0}$	$10^{8.5}$
Infectivity of virus[d] exposed to puromycin 24 hr. at 35° in buffer at pH 8.0, EID_{50}/ml.	NT	$10^{7.4}$	NT	$10^{7.0}$
Effect of puromycin on outgrowth of chorio-allantoic cells in vitro	No growth	No growth	Definite cell growth	Definite cell growth

[a] HA indicates hemagglutinating units. [b] Virus hemagglutinin and infectivity measured after 44–48 hr. incubation on shaker at 35°. Initial virus concentration from inoculum about 5×10^5 EID_{50}/ml. [c] Each value is a geometric mean of 30–32 cultures from 5 experiments. [d] Values from a single representative experiment. [e] NT indicates not tested.



Puromycin (I)

MATERIALS AND METHODS

The Lee strain of influenza virus was employed in these experiments and was maintained as frozen chick embryo allantoic fluid stored at −60°. Puromycin and its aminonucleoside were obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio.

The tissue culture system has been previously described (13). Each culture consisted of about 4 cm.² of chorio-allantoic tissue from 10-day chick embryos. The tissue was suspended in 2.0 ml. of Hanks' balanced saline (BSS) in 25 × 150 mm. Pyrex culture tubes.

The method of measuring virus concentration in culture fluids by hemagglutinin titration, and the method of determining toxicity for tissue cells have also been described (13). For the measurement of infectious virus, a series of decimal dilutions of the culture fluid was made and each dilution inoculated into a group of five 10-day chick embryos. After 48-hr. incubation, the number of infected embryos in each group was determined, and the number of 50% infectious doses (EID_{50}) per milliliter of the undiluted virus calculated.

RESULTS AND DISCUSSION

The combined results of 5 experiments designed to determine the effects of puromycin on virus replication are presented in the first row of data in Table I. Puromycin was incorporated in the fluids of the tissue cultures which had been inoculated with the Lee strain of influenza virus, and the concentration of the latter after incubation was determined by hemagglutination. It is evident that a concentration of 4 mcg./ml. reduced the virus yield markedly, and even 2 mcg./ml. produced a slight reduction, which was significant in view of the relatively large number of replicate cultures. In some experiments virus concentration was measured by infectivity titrations in 10-day chick embryos. Results of a representative experiment of this type are shown in the second row of Table I. In the presence of 4 mcg. of puromycin per milliliter the titer of infectious virus was 2 log units lower than in control cultures. Relatively few virus inhibitors are active at such low concentrations.

In vitro experiments in which the virus was exposed to a puromycin concentration of 4 mcg./ml. in a phosphate–glycine buffer[1] of pH 8.0 for 24 hr. at 35° yielded results which indicated that the titer of infectious virus was essentially the same as in control preparations similarly treated, but without puromycin. Data from such an experiment are shown in the third row of Table I. Thus, the compound seemed to have no direct effect on the virus itself. It was also found to cause no interference with the hemagglutination reaction, or with adsorption of the virus to the chick chorio-allantoic tissue. These observations pointed to an effect of the compound on the infected cell as the basis for inhibition of virus replication.

It was noted that fragments of chorio-allantoic tissues exposed to a puromycin concentration of 4 mcg./ml. for 44–48 hr. appeared very similar to those in control cultures, and some metabolic processes were still active, as indicated by continuing acid production in such cultures. Thus, gross or destructive tissue toxicity was not apparent. However, employing a more sensitive criterion, it was found that outgrowth of new cells from tissue fragments in roller tube cultures containing BSS, did not occur in the above concentration of the antibiotic. Cell growth did occur, however, in a concentration of 2 mcg./ml. It is of interest to note that the concentration of 4 mcg./ml. is close to that which Yarmolinsky and de la Haba (6) found necessary for marked inhibition of leucine-^{14}C incorporation into protein in a rat liver extract.

The biological activity of the aminonucleoside of puromycin or that portion of the molecule remaining after splitting off the amino acid, has been studied by several workers. In the experimental infection of mice with *Trypanosoma equiperdum*, both puromycin and its aminonucleoside were found

[1] Concentrations of buffer components in final mixture with virus were: glycine, 0.025 *M*; NaCl, 0.025 *M*; and Na_2HPO_4, 0.0167 *M*.

TABLE II.—REPLICATION OF LEE INFLUENZA VIRUS IN TISSUE CULTURES CONTAINING PUROMYCIN AMINONUCLEOSIDE

	Concn. of Aminonucleoside in Tissue Culture Fluid, mcg./ml.			
	400	200	100	0
HA units of virus/ml.[a,b] of tissue culture fluid	136	164	137	91

[a] Virus concentration measured as hemagglutinating (HA) units after 44–48 hr. incubation on shaker at 35.° Each value is a geometric mean of 15 cultures from 3 experiments. [b] Initial virus concentration from inoculum about 5×10^5 EID_{50}/ml.

about equally effective on a molar basis in curing the disease (11), and the *in vivo* activity of each was reversed by administration of adenine.

The aminonucleoside was compared with puromycin for its ability to inhibit virus replication in tissue culture. It was found that the former compound had no inhibitory activity in concentrations comparable on a molar basis to the effective levels of puromycin shown in Table I. Much higher concentrations resulted consistently in somewhat higher yields of virus in the culture fluid than those in control cultures. The results obtained are presented in Table II. In the experiments represented, tissue culture fluids containing 200 mcg. of the aminonucleoside per milliter were found to yield virus titers nearly twice that of controls. These experiments showed conclusively that the amino acid moiety of the puromycin molecule was essential for inhibition of influenza virus formation. They also indicated that the mechanism of action of puromycin against trypanosomes is apparently different from that responsible for the virus inhibition. A further difference was revealed by experiments in which attempts were made to block or reverse the virus inhibition by means of adenine, guanine, cytosine, uracil, and the corresponding nucleosides and nucleotides added to the tissue culture medium. In no case could any significant or reproducible reversal be demonstrated.

Preliminary experiments were performed to determine the activity of puromycin in mice infected with minimal doses of Lee influenza virus. These animals were given intraperitoneal injections of 1 mg. at 12–14-hr. intervals for 3 days. This treatment had no effect on the virus concentration in the lungs at the end of that period. Larger doses and more frequent injections should be tried, as well as administration by aerosol inhalation.

Yarmolinsky and de la Haba found that while puromycin inhibited protein synthesis in their rat liver extract system, the aminonucleoside showed only slight activity in equivalent molar concentrations (6). Rabinovitz and Fisher found the complete antibiotic molecule a potent inhibitor of protein synthesis in Ehrlich ascites tumor cells and in rabbit reticulocytes, while the aminonucleoside was inactive (7).

Several characteristics of the influenza virus inhibition by puromycin suggest that the mechanism probably depends on inhibition of protein synthesis. The concentrations required for the virus inhibition are very similar to those found necessary by the above investigators to inhibit incorporation of amino acids into protein. The aminonucleoside has been found to have little or no inhibitory activity for either virus replication or protein synthesis. In the virus tissue culture system described, when puromycin was present in inhibitory concentrations, multiplication of the host cells was also inhibited, without destruction of metabolic activity as measured by acid production. The amino acid moiety of puromycin, which was essential for virus inhibition, is *p*-methyoxyphenylalanine. *p*-Fluorophenylalanine is an antimetabolite which has also been found to interfere with protein metabolism (12). When combinations of puromycin and the latter compound were studied in the influenza virus system as previously reported for other inhibitor combinations (13), it was found that the degree of virus inhibition was no greater than that produced by either agent alone. This suggested a similar site of action for the 2 inhibitors.

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Fractionation of the Black Snake Venom (*Walterinnesia aegyptea*) by Sephadex Gel Filtration

By O. A. ZAKI*

By the use of a column of Sephadex G-200, it was possible to separate from venom 7 protein components which showed different patterns in electrophoretic mobility. Only 2 of the separated fractions have toxic effects on albino rats.

SNAKE VENOMS are known to contain a number of enzymes. Attempts have been made to correlate the toxicity of snake venoms with their enzymatic activity (1). Most of these studies have been carried out on whole venoms (2) or after elimination of some components by heat treatment (3). A number of methods, such as electrophoresis, use of ion exchange resins such as Amberlite, and cellulose ion exchangers have been used for fractionation. All the methods used were not quite efficient for the complete separation of the different factors in the venom. Yang *et al.* (4) used electrophoresis on potato starch for the separation of the components of Hyopoda venom, and they found that the proteases and phosphatases occurred in the same fractions as the toxic components. Master *et al.* (5) stated that the use of paper and agar gel electrophoresis did not give a sufficiently good separation of the components of Indian cobra and Russel's viper venom.

Mohammed and Zaki (6) studied the properties of the black snake venom, and their chemical studies proved that the venom is a protein mixture. Sephadex gels act as molecular sieves allowing separation of protein mixtures (7). These gels are composed of small granules prepared by the crosslinking of dextran. Sephadex G-200 retains materials of molecular weight up to 200,000. Molecules larger than the pore size pass directly through the gel-packed column, whereas those small enough to enter the grains are retained until replaced by the eluant.

The present study was undertaken to see if any useful separation of the various protein factors in the black snake venom could be obtained by gel filtration.

METHODS

For protein, Sephadex G-200 is the most suitable type since the available capacity is high even for relatively large molecules. Gel filtration was performed by the method of Porath and Flodin (8). A column 7.5 × 41 cm. (volume of 1800 ml.) with Sephadex G-200 was used. Before being packed into the column, Sephadex G-200 was allowed to swell until equilibrium was attained. Forty-eight hours was allowed for swelling. The swelling of Sephadex was in an excess of water containing the buffer. The column was filled to about one-third of its height with the buffer. This consisted of 0.163 *M* NaCl in 0.0004 *M* trihydroxyaminoethane (Tris) previously adjusted to pH 7.4 with HCl. A ball of glass wool was placed at the bottom of the column, and a layer of small glass beads, sufficient to cover the glass wool to a depth of about 1 cm., was added. The suspension of Sephadex G-200 was poured into the column, filling an extension tube of about 20 cm. joined to the top of the column. The gel particles were allowed to sediment until a layer a few centimeters thick had formed. The outlet of the column was then gradually opened, and if the effluent was clear, packing was continued. When packing was finished, the extension tube was removed and the buffer reservoir connected.

The bed, stabilized by washing overnight, was ready for use. In each experiment the column was washed by NaCl–buffer solution (100:20). The venom (10 mg. in 2 ml. of buffered solution) was added to the column by a tip pipet. Before introduction of the sample, all the liquid above the surface of the bed was removed with care. After the sample had been layered on the bed, the bottom outlet was opened. A small volume of the buffer was added in the same manner and was allowed to enter the bed. The column was then filled with the buffer and the buffer reservoir was connected. In each experiment 32 fractions of 2 ml. each were collected. The rate was that each fraction was collected in 15 min. Then 0.4 ml. of each fraction was diluted by 3.6 ml. of buffered solution. The proportion concentration of each fraction was estimated from measure-

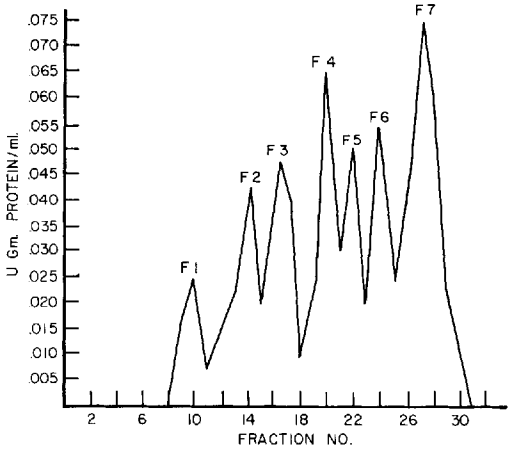

Fig. 1.—The position of the different factors in the black snake venom according to their protein extinction when the venom is fractionated with Sephadex G-200. The extinction of each fraction, of 32 fractions, is plotted on the curve. The summit of each fluctuation in the curve is taken as a definite fraction and these fractions are represented on the curve as F_1, F_2, F_3, F_4, F_5, F_6, and F_7. Eluant, 0.163 *M* NaCl in 0.004 *M* Tris buffer. Fraction size, 2 ml.

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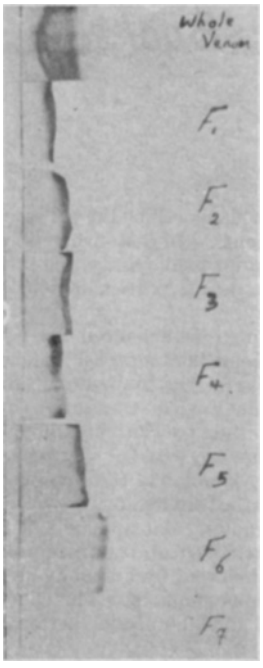

Fig. 2.—The electrophoretic pattern of the Egyptian black snake venom and that of the 7 fractions (F_1, F_2, F_3, F_4, F_5, F_6, and F_7) obtained by fractionation of the venom using a column of Sephadex G-200.

ments of the extinction 280 mμ using a Unicam S.P. 500 spectrophotometer.

Paper strips electrophoresis was carried out on the whole venom as on each of the fractions. A horizontal Shandon tank was used, with constant current and constant voltage. Oxoid 2.5 × 12 cm. strips were used. The volume of venom added to each strip was 0.005 ml. of 1:1000 venom solution. The buffer used in the electrophoresis had pH 8.6 and was of the following composition: sodium acetate, 6.5 Gm.; sodium barbital, 8.87 Gm.; barbital, 1.13 Gm.; in 1 L. of distilled water.

The electrophoretic run was continued for 2 hr. using a current slightly below 0.4 ma./cm. width of the strip. After electrophoresis, the strips were removed with forceps and were dried by suspending them in a hot air over for 20 min. at 100°. The dry strips were left overnight in the staining solution (0.001% nigrosine in 2% aqueous acetic acid). After staining, the strips were washed by running tap water and then dried.

The protein fractions collected from the column were tested for their toxicity. This was done by the subcutaneous injection of 0.5 ml. of the diluted fraction into albino rats weighing about 100 Gm. The fraction which caused the death of the rats within 24 hr. was assumed to contain the toxin. This experiment was repeated several times to confirm the position of the toxic components.

RESULTS AND DISCUSSION

Considerable separation of the protein venom factors was achieved by Sephadex G-200 filtration. Seven fractions showed definite protein extinctions (Fig. 1). By studying the electrophoretic pattern of the whole venom, 6 separate components could be recognized (Fig. 2), indicating that 2 of the gel filtration fractions had the same electrophoretic mobility. The paper strips electrophoresis carried out on each fraction showed that factors 2 and 4 (F_2 and F_4) had the same mobility.

When the toxicity of the fractions was tested, it was found that the fraction corresponding to F_4 and F_6 killed the rats. The death in case of F_6 injection had the same typical symptoms of paralysis as that which occurred when the whole venom was injected (6). This suggested that presence of a neurotoxin in this fraction.

When the animals were injected by F_4, they showed severe itching, excessive salivation, difficult breathing, and then respiratory failure followed by circulatory failure. There were no paralytic symptoms.

The results showed that Sephadex gel filtration may be of considerable value in the separation of the components of the snake venoms.

Probably the most useful application will be in the preparation, physiological study, and the study of enzymes in each factor of the venom separately. Such experiments are being conducted in these laboratories.

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REVIEWS

Pharmacognosy. 5th rev. ed. By E. P. CLAUS and V. E. TYLER, JR. Lea & Febiger, 600 Washington Sq., Philadelphia, Pa. 19106. 1965. 572 pp. 16 × 26 cm. Price $15.00

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NOTICES

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Review Article

A Survey of Diabetes Mellitus

By GEORGE N. HOLCOMB

REFERENCE to the disease known to us as diabetes mellitus goes back almost as far as recorded history itself. The Ebers Papyrus, an Egyptian document dating from about 1500 B.C., has outlined the symptoms of diabetes as well as a number of "cures" for the disease. Diabetes was described in some detail by a physician known as Aretaeus the Cappadocian as early as the first century A.D., but it was not until the seventeenth century that Thomas Willis, an English physician, observed that the urine of diabetics had an extremely sweet taste. This appears to have been the first evidence to connect diabetes mellitus with the metabolism of sugars.

A recent study on the prevalence of diabetes (1) indicates that there are somewhat over 2,000,000 known diabetics in the United States. Other evidence cited by the same workers (1) shows that the frequency rate for undiagnosed cases of diabetes in this country is about 8 per 1000 persons. Taking these two figures into account leads to an estimate that there are close to 3,500,000 diabetics in the U. S. alone. This represents nearly 20 diabetics per 1000 population and qualifies the disease as a major medical and public health problem.

THE METABOLIC EFFECTS OF DIABETES

Although more is known about the biochemical basis of diabetes mellitus than for most other diseases, too little is known to give an accurate definition of the disease. Some of the characteristic symptoms of diabetes are hyperglycemia, glycosuria, polyuria, polydipsia, and possible loss of weight and strength. Ketone bodies (acetoacetic acid, acetone, and β-hydroxybutyric acid) may be present in the blood and urine of diabetic subjects.

Diabetes may be classified into at least 2 types (2): the growth-onset (juvenile) type and the maturity-onset (adult) type of diabetes. The juvenile type of diabetes which is contracted early in life is characterized by a greatly diminished capacity to produce insulin (3) which approaches zero within a few years. Maturity-onset diabetics are frequently obese and develop symptoms quite gradually, usually after age 40. These individuals often have substantial quantities of pancreatic insulin (4) and normal or near normal levels of circulating insulin (3).

THE DEVELOPMENT OF INSULIN

The first evidence that the pancreas is concerned with diabetes came as early as 1889 when von Mering and Minkowski showed that pancreatectomy in laboratory animals produced the same symptoms as diabetes. This led workers to propose that the pancreas secretes a substance which regulates blood sugar as well as other factors concerned with diabetes. In 1909 de Meyer coined the name *insuline* to refer to the then hypothetical substance secreted by the islets of Langerhans of the pancreas.

It was not until 1922, however, that the biological activity of insulin was finally demon-

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strated by Banting and Best (5), who showed that a pancreatic extract could be used to alleviate the symptoms of diabetes. For this monumental discovery, Dr. Banting received a Nobel Prize. Four years later Abel and his associates (6) obtained crystalline insulin by the extraction of animal pancreases, and proposed that insulin was a protein.

Sanger was awarded the Nobel Prize in chemistry in 1958 in recognition of his work on the elucidation of the chemical structure of the insulin molecule. His work involved the cleavage of insulin into 2 peptide chains by performic acid oxidation (7), followed by degradation studies on the 2 individual chains to determine the amino acid sequence of each (8, 9). This work has been summarized by Sanger (10). As a result of the work of Sanger and others, it is known that insulin has a molecular weight of approximately 6,000 and is composed of 2 peptide chains. The A, or glycyl chain, is composed of 21 amino acid residues while the B, or phenylalanyl chain, is composed of 30. The 2 chains are connected by disulfide bridges between A 7 and B 7 and between A 20 and B 19, with a third disulfide bond found in the A chain from A 6 to A 11.

The absolute proof of the structure of insulin must come from the preparation of a synthetic insulin which exhibits a significant amount of insulin activity in a variety of biological assays. Because of this, a great deal of recent research has been directed toward this goal. Katsoyannis and his associates (11–13) prepared the A and B chains of sheep insulin by synthetic procedures requiring over 200 steps. Zahn, Meienhofer, and their co-workers have also reported the synthesis of A and B chains of insulin (14–16).

It can be readily seen that the combination of synthetic A chain with synthetic B chain to produce insulin is no mean task. The number of ways in which the two chains can combine *via* disulfide bonds is extremely high, so it is no surprise that the yields of synthetic insulin prepared in this manner have been extremely low. It has been reported (12) that Dr. G. H. Dixon, working in cooperation with Katsoyannis' group, was able to combine the synthetic A chain with the synthetic B chain to produce a product which showed insulin activity, but the details of the combination experiments have not yet been reported. Zahn and his associates (17) have combined synthetic A and B chains to produce a preparation containing 0.5% of the activity of crystalline insulin. Their calculations are based on a natural insulin preparation containing 26.7 I.U./mg.

A recent publication by a group of Chinese workers (18) describes the cleavage of insulin with sodium sulfite and sodium tetrathionate to produce the *S*-sulfonates of A and B chains. The chains were separated and then recombined by reduction and subsequent reoxidation. The insulin produced in these experiments showed an activity of 12 I.U./mg. when measured by the mouse convulsion test. This represents nearly 50% of the activity of natural insulin. Zahn and Brinkhoff have recently combined natural A chain with natural B chain to produce a product exhibiting 40% of the activity of natural insulin (19). These results make it seem quite likely that the yields for the combination of synthetic A and B chains can be improved significantly in the future.

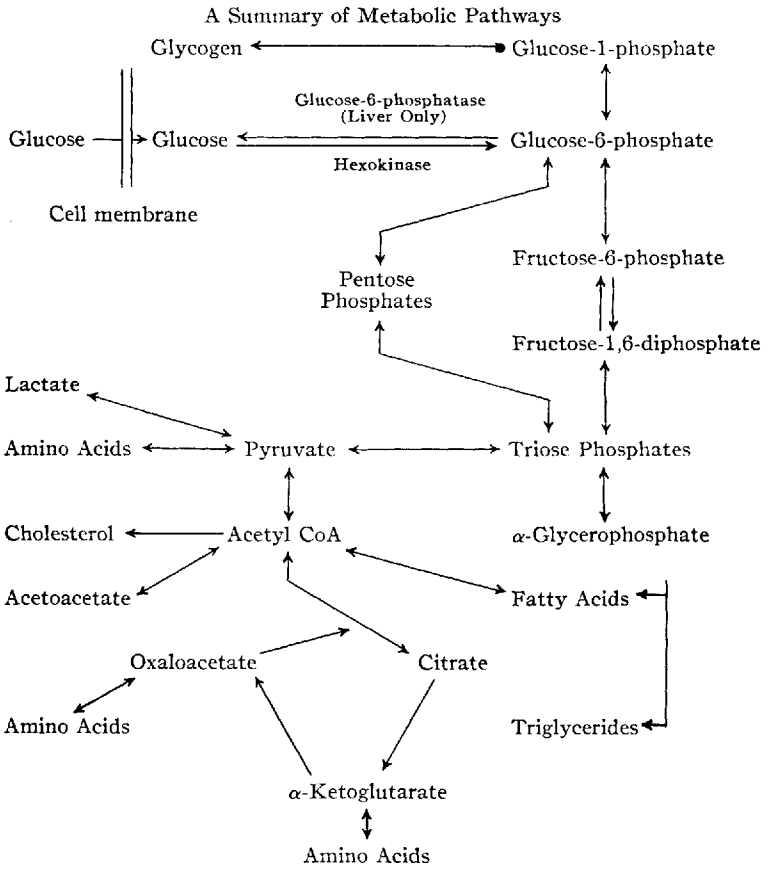
THE BIOLOGICAL EFFECTS OF INSULIN

It is well known that insulin exerts a profound effect not only on carbohydrate metabolism but also on the metabolism of fats and proteins. The biological effects of insulin have been extensively reviewed by numerous authors (20–25) and will not be treated in great detail here. The following discussion will simply summarize the effects of insulin on the metabolism of carbohydrates, fats, and proteins. Scheme I is included to allow the reader to follow the general metabolic pathways of these materials and to emphasize the interrelationship between the metabolism of all 3 classes of compounds.

Effects on Carbohydrate Metabolism

The important sites of carbohydrate metabolism which are sensitive to insulin are the liver, where glycogen is formed, stored, and broken down, the skeletal muscles, where glucose is oxidized to produce energy, and adipose tissue, where glucose may be converted to fatty acids, glycerol phosphate, and triglycerides. An appreciable quantity of glucose is also metabolized in the brain, but this tissue does not respond to insulin stimulation. The effects of insulin on carbohydrate metabolism are therefore different in the various tissues.

Effects in Skeletal Muscle.—Insulin is known to increase the uptake of glucose by skeletal muscle. Gemmil (26, 27) was the first to show that insulin stimulates glucose consumption in the isolated rat diaphragm. This system has been studied thoroughly by Vallance-Owen and has been developed as an assay for insulin in blood and other fluids (3). The increased glycogen synthesis promoted under these conditions is accounted for by Larner and Villar-Palasi (28, 29) by an insulin stimulation of uridine diphosphoglucose glycogen transglucosylase, an enzyme which catalyzes the rate-determining step in glycogen synthesis.



Scheme I

The glucose uptake of most peripheral tissues in humans has been shown to be stimulated by insulin. Evidence for this has been obtained by measuring the difference in glucose concentrations of venous and arterial blood in the limbs of humans (30).

The actual mechanism or mechanisms by which insulin promotes glucose utilization by skeletal muscle are not understood in biochemical terms. It does, however, seem reasonable to suppose that since insulin appears to promote all known pathways of glucose disposal and utilization (31), it probably exerts its primary action early in the metabolic sequence. This observation led to a number of investigations concerning the effect of insulin on the hexokinase reaction, a reaction which must occur before glucose can be metabolized by any route (32–35). Workers have been unable to show that insulin stimulates this reaction, nor have they been able to show consistently that it counteracts inhibitors of the reaction (36). It is, therefore, difficult to connect this reaction with the biochemical mechanism of action of insulin.

More recent studies have been concerned with the effect of insulin on the transfer of glucose and other sugars across cell membranes. Much of the research in this area has been carried out by Levine and his co-workers and has recently been summarized (31). These workers studied the effect of insulin on the transport of galactose, a sugar which is transported in the same manner as glucose but which is not metabolized in skeletal muscle. They observed that insulin definitely stimulates galactose transfer in most tissues. Narahara and Ozand (37) made similar observations in connection with their studies on the transport of 3-*O*-methyl-D-glucose, another non-utilizable sugar. Park and his associates have found that insulin also stimulates glucose transport in isolated muscle (38).

This evidence has led Levine (31) to propose that the primary effect of insulin on carbohydrate metabolism is at the level of the cell membrane. According to this theory, insulin increases the permeability of the cell membrane to glucose, thus allowing glucose to pass freely in either direction. Since the glucose inside of the cell is

rapidly phosphorylated, the process is essentially unidirectional. This idea appears to be in agreement with the available facts, but insulin might also have a specific action on other steps involved with the utilization of glucose.

Effects in Adipose Tissue.—In adipose tissue, carbohydrate metabolism is somewhat different than in muscle. In adipose tissue a large portion of the glucose-6-phosphate is oxidized through the pentose phosphate pathway and much is converted to glycerol phosphate, fatty acids, and triglycerides, whereas in the muscle the major synthetic product is glycogen.

Insulin has been shown to increase glucose oxidation by rat epididymal adipose tissue *in vitro* (39) and also increases the synthesis of fatty acids from glucose in this tissue (40, 41). The work of Winegrad and Renold (42), who worked with the oxidation of glucose-U-^{14}C, glucose-1-^{14}C, and glucose-6-^{14}C in isolated rat epididymal adipose tissue, indicates that in this tissue insulin stimulates catabolism by the pentose phosphate pathway to the same extent as by the Embden-Meyerhof pathway. This further suggests that the major effect of insulin is at a point early in the metabolic pathway. It is believed that the increased oxidation of glucose by adipose tissue in the presence of insulin is a result of an increased transport of the sugar across cell membranes (43).

Effects in the Liver.—Less is known about the effects of insulin on carbohydrate metabolism in the liver than about its effects in skeletal muscle and adipose tissue. It is exceedingly difficult to ascertain which of these effects are direct and which are secondary, thus making it difficult to present a detailed discussion of the exact effects of insulin on carbohydrate metabolism in the liver.

The liver is an important organ in the regulation of blood sugar levels. When blood glucose levels are high the liver takes up glucose and when they are below normal it produces glucose from glycogen. It has been shown (44) that the rate of removal or production of glucose by the liver is directly proportional to the degree of hyper- or hypoglycemia. Unlike skeletal muscle and adipose tissue, the cell membrane in the liver is freely permeable to glucose and other sugars (45) which indicates that transport is not rate limiting and does not account for the effect of insulin on net glucose uptake by the liver.

The fate of glucose in the liver must be considered in searching further for a possible effect of insulin on carbohydrate metabolism in the liver. Glucose is first phosphorylated by ATP to form glucose-6-phosphate; the enzyme catalyzing this reaction in the liver is quite specific and is referred to as "glucokinase." Once it is formed, glucose-6-phosphate may be reconverted to glucose under the influence of glucose-6-phosphatase, an enzyme present in appreciable quantities only in the liver, or it may be converted to glycogen and stored. The glucose-6-phosphate may also be oxidized in the liver, either by Embden-Meyerhof glycolysis or *via* the pentose phosphate pathway (45). The glucokinase reaction limits glucose utilization by the liver (46), and the glucose-6-phosphatase reaction regulates hepatic glucose output (47).

In severe diabetes hepatic glucokinase activity falls to one-fifth of normal, while glucose-6-phosphatase activity doubles (45). Both the decreased glucokinase activity (45) and the increased glucose-6-phosphatase activity of diabetics can be corrected by the administration of insulin over a period of time (45). The effect of insulin on carbohydrate metabolism in the liver then appears to be to decrease net hepatic glucose output.

Initially, it was found to be quite difficult to demonstrate an immediate decrease in hepatic glucose output after the administration of insulin to intact animals, and many workers were unable to produce a prompt effect (48–50). On the other hand, Madison and his associates (51) showed that insulin has a prompt effect on hepatic glucose output in unanesthetized dogs, providing the insulin is administered by slow intravenous infusion. More recently, other workers have shown that insulin promptly depresses net hepatic glucose output in intact animals quite rapidly (52–54). These workers have found that animals on a high carbohydrate diet respond better than those on a high protein diet, presumably because of the higher plasma insulin levels produced by the high carbohydrate diet. Their studies also indicate that significant hypoglycemia must be avoided in order to produce an insulin effect. This may be accomplished by the slow intravenous infusion of insulin and by administering small quantities of glucose to the animals.

Bishop and his co-workers have recently studied the effect of insulin on glucose output in intact dogs quite extensively (55) and have found that the first effect of insulin on the liver is to decrease glycogen breakdown, thereby decreasing the release of free glucose into the bloodstream. A later effect (after 2 hr.) is an increased glucose utilization for glycogen synthesis. The over-all result is then an immediate and sustained depression of net hepatic glucose output.

Effects on Fat Metabolism

The presence of ketone bodies in the blood and urine of uncontrolled diabetics indicates that

these individuals exhibit abnormal fat metabolism as well as abnormal carbohydrate metabolism. Stadie and his group (56) observed that liver slices from pancreatectomized cats produced 5 times more ketone bodies than liver slices from normal cats. This increased production of ketone bodies by diabetic animals results from an increased oxidation of fatty acids in these animals.

The work of Brady and Gurin (57) has shown that liver slices from alloxan-diabetic rats have a reduced ability to synthesize long chain fatty acids from acetate. It has also been demonstrated that insulin is able to stimulate the incorporation of acetate into longer chain fatty acids *in vitro* (58). Insulin has been shown to inhibit the release of free fatty acids (FFA) from glyceride stores in adipose tissue (59). Since insulin is able to stimulate fat synthesis and inhibit fatty acid mobilization in adipose tissue, it comes as no surprise that it is able to lower the elevated FFA levels observed in diabetic subjects (60).

The impaired carbohydrate metabolism of diabetics could be partially responsible for the decreased rate of fatty acid synthesis. This is indicated by the fact that fatty acid synthesis in liver slices is promoted by the presence of glucose in the medium. The problem is, however, probably much more complex than this and insulin undoubtedly has some more direct effect on fat synthesis. Chernick and Chaikoff (61) have demonstrated that the diabetic liver can oxidize lactate, pyruvate, and acetate but cannot incorporate these substrates into fatty acids. This malfunction in fatty acid synthesis can be corrected by treatment of the animal with insulin for several days (24), which is indicative of a direct effect on fatty acid synthesis.

Effects on Protein Metabolism

It is well established that diabetes results in a loss of body weight, a depletion of body protein, and a negative nitrogen balance (22). This nitrogen loss can be prevented by the administration of insulin (62), which suggests that insulin has some effect on the synthesis and/or degradation of proteins.

It is difficult to study the effects of insulin on protein metabolism in intact animals, although certain pertinent observations have been made in this manner. It has been demonstrated (63) that insulin lowers plasma amino acid levels, and it has also been shown (64) that it promotes the uptake of amino acids by skeletal muscle.

The best evidence that insulin affects protein metabolism comes from studies carried out in a number of *in vitro* systems. Numerous workers have used the isolated rat diaphragm to show that insulin stimulates the incorporation of labeled amino acids into protein (65–68). These studies show that insulin concentrations as low as 50 microunits/ml. will stimulate the incorporation of amino acids into proteins in this system. Since it is effective at this low concentration, the response is probably a physiological one.

It was first thought that the effect of insulin on protein synthesis was secondary to its effect on carbohydrate metabolism. It was felt that insulin stimulated glucose metabolism which in turn provided energy that could then be utilized for protein synthesis. More recent research, however, has made it appear highly unlikely that this is the case. Experiments carried out by Wool and Krahl (69) show that the effect of insulin on protein synthesis in the rat diaphragm *in vitro* is independent of the concentration of glucose in the medium for glucose concentrations between zero and 600 mg. %. In the same study it was shown that insulin stimulates the incorporation of labeled amino acids into proteins in diaphragms isolated from fed rats even when there is no extracellular glucose present in the diaphragm. This evidence strongly suggests a more direct effect of insulin on protein synthesis in this system.

It was found (70) that insulin stimulates the uptake of α-aminoisobutyric acid, a nonutilizable amino acid, by the isolated rat diaphragm. This led to the speculation that the effect of insulin on protein synthesis could be a result of its facilitating the transport of amino acids into cells. Further studies by Wool and Krahl (71) have essentially ruled out the possibility that this is the sole effect of insulin on protein synthesis. In these studies, the labeled amino acid was injected into intact rats, thus allowing the amino acid to accumulate in the diaphragm of the intact animals. The animals were then sacrificed, the diaphragms removed and incubated with or without insulin, and the rate of protein synthesis determined. The results showed that insulin stimulates the synthesis of proteins from labeled amino acids even when the amino acids are already present in the diaphragm, thus indicating a direct effect of insulin on protein synthesis.

The results of subsequent studies have led Wool to propose that the effect of insulin on protein synthesis is mediated by its effect on messenger RNA synthesis (72). He believes that the effect results from insulin stimulating the transfer of messenger RNA from the nucleus to the cytoplasm, a proposal which seems to agree with the existing facts.

POSSIBLE CAUSES OF DIABETES

The actual etiology of diabetes is not yet understood, although there are a number of factors which could account for the abnormal glucose tolerance exhibited by diabetics. The 3 most obvious possibilities are (*a*) a decreased production of insulin, (*b*) an increased rate of destruction of the hormone, and (*c*) an inability of tissue to respond to stimulation by insulin.

Juvenile diabetes is quite likely the result of the first. The pancreas loses the ability to produce and secrete insulin and the plasma insulin levels become very low, possibly even reaching zero (73).

As early as the 1940's, Mirsky (74) proposed that diabetes could be due to an increased destruction of insulin by an enzyme which he then referred to as "insulinase." Since that time numerous studies have been carried out to determine the nature of the enzyme responsible for the degradation of insulin. This led to the isolation of a purified preparation from acetone powders of livers which is capable of reducing insulin to its A and B chains (75–78). The reaction utilizes hydrogen from reduced glutathione, and the enzyme is therefore referred to as glutathione insulin transhydrogenase. There is no direct evidence for any increased activity of this enzyme nor any increased destruction of insulin in diabetic subjects, so its connection with the etiology of diabetes has not been established.

Neither of the first 2 factors seems to explain the impairment of glucose tolerance in cases of maturity-onset diabetes since individuals of this type may be characterized by normal or above normal insulin levels (79, 80). According to Lacy (81), the ultrastructure of the β granules in maturity-onset diabetics is similar to that of the normal human β cell. This suggests that the pancreas has not lost its ability to produce insulin, and another possible cause for the disease must be sought.

Maturity-onset diabetes could result from an inability of the peripheral tissue to respond to insulin. This decreased tissue response could very well be the result of the presence of substances which antagonize insulin. Numerous insulin antagonists have been described in some detail, and many of these are themselves hormones. It has been demonstrated (82) that growth hormone and the adrenal corticosteroids can inhibit the effect of insulin on glucose transport. Epinephrine, another insulin antagonist, increases blood sugar levels by increasing hepatic glucose output (83), and a similar effect on the liver is also produced by glucagon (83).

Insulin is a protein of sufficient molecular weight to produce antibodies (84) which are capable of neutralizing its action. This phenomenon may also be referred to as "insulin antagonism."

Bornstein and Hyde (85) have described a pituitary peptide with a molecular weight of about 4,500 which is able to inhibit the uptake of glucose by the isolated rat diaphragm. This substance is also capable of inhibiting the incorporation of acetate into fats by liver slices and the incorporation of amino acids into proteins in rat muscle (86). Its actual physiological significance has not been determined.

The studies of Samaan (87) with "typical" and "atypical" insulin and of Antoniades (88) with "bound" and "free" insulin indicate that binding of insulin to plasma proteins antagonizes its action in certain *in vitro* systems. Antoniades (88) has reported that bound insulin is biologically active in rat epididymal adipose tissue *in vitro* but is inactive in the isolated rat diaphragm, while free insulin is active in both systems. Recent studies (89) indicate that bound insulin increases the incorporation of labeled glucose into glycogen in muscle and adipose tissue and into fat in adipose tissue in intact rats. This suggests that the binding of insulin to plasma proteins does not antagonize its biological activity *in vivo*.

The insulin antagonists which are currently receiving the most attention are the synalbumin antagonist and plasma FFA, either or both of which could contribute significantly to the cause of diabetes.

The Synalbumin Insulin Antagonist.—It has been mentioned previously that insulin promotes the uptake of glucose by the rat diaphragm *in vitro*. This system has been used as a biological assay for insulin and under the proper conditions is an accurate method of determining the amount of insulin present in a given sample (3). The assay has also been used by Vallance-Owen (90) for the detection of insulin antagonism in the plasma of normal and diabetic subjects. His work on insulin antagonism has recently been summarized (91).

Vallance-Owen and Hurlock (92) found that the assay can be carried out using plasma from humans as the incubation medium. If insulin is added *in vitro* to the plasma of normal, fed subjects or obese, nonketotic diabetics, the insulin activity can be recovered almost quantitatively. In contrast to this, it was found that when plasma from uncontrolled or insulin-requiring diabetics is used as the incubation medium, the action of insulin is definitely antagonized.

It was subsequently shown by Vallance-Owen and his co-workers (93, 94) that the antagonist actually resides in the albumin fraction. The

albumin used in these studies was prepared by T.C.A.–ethanol fractionation, a procedure which has been shown by Schwert (95) to produce electrophoretically pure albumin.

Similar antagonism can be demonstrated in plasma-albumin from normal subjects, although this albumin is not nearly so active as that obtained from the plasma of diabetics. Vallance-Owen, Dennes, and Campbell (94) found that concentrations of 3.5–5% of diabetic plasma-albumin will completely antagonize the effect of 1,000 microunits of insulin in the rat hemidiaphragm assay and will also antagonize insulin *in vitro* at concentrations as low as 1.25%. Plasma-albumin from normal subjects will completely antagonize 1,000 microunits of insulin when used at concentrations of 3.5–5%, will show some antagonism at 2.5%, but exhibits no insulin antagonism at a concentration of 1.25%. These observations have recently been confirmed by Alp and Recant (96), who performed similar studies using a wide variety of albumin preparations. These workers studied albumin preparations isolated by the Cohn procedure (97) as well as a series of preparations isolated by the Debro procedure (98). Insulin antagonism was observed in all of these preparations, although the effect was not so great as that demonstrated by Vallance-Owen and his group. All of this evidence suggests that excessive insulin antagonism could have some connection with the etiology of diabetes.

Subsequent work by Vallance-Owen and his group (99) indicates that the antagonist is dependent on the presence of a functioning pituitary gland. This research involved a study of the plasma-albumin from 3 hypophysectomized patients, and the albumin obtained from these patients was found to be nonantagonistic in concentrations as high as 4%. One of the patients had been studied prior to hypophysectomy and his albumin had been shown to be antagonistic at that time.

It has also been shown (100) that the antagonist is dependent on the adrenal steroids. In 2 patients with a bilateral adrenalectomy and with cortisone therapy discontinued for at least 50 hr. prior to testing, the plasma-albumin was found not to antagonize insulin *in vitro*. After 1 of these patients subsequently was sustained on cortisone therapy, his plasma-albumin was retested and was found to be antagonistic.

It has been demonstrated (99) that when antagonistic albumin is passed through a partially acetylated cellulose column, it is nonantagonistic when eluted. This was the first indication that it is not the albumin itself but rather something associated with the albumin which is responsible for the insulin antagonism. Because of this, the term *synalbumin* antagonist was coined.

The synalbumin antagonist can be dialyzed away from the albumin after heat coagulation (100), but this, however, results in some loss in activity of the antagonist. The antagonist cannot be extracted from albumin with ethanol, chloroform, or a mixture of *n*-octane and acetic acid, which indicates that the antagonist is not a lipid, fatty acid, or steroid. This suggests that it is not a simple case of albumin-bound fatty acids inhibiting the action of insulin as shown in the studies of Randle, Garland, Hales, and Newsholme (101). The evidence shows that the antagonist could be a relatively low molecular weight polypeptide which led to the postulation (91) that the synalbumin antagonist might be the B chain of insulin.

Ensinck and Vallance-Owen (102) have shown that when ^{131}I-labeled insulin is administered to humans, the molecule is cleaved enzymatically and some of the reduced ^{131}I B chain becomes bound to the plasma-albumin. The A chain, on the other hand, appears to associate with the α_2 globulin fraction. These workers (102) have pointed out several similarities between B chain and the synalbumin antagonist; for example, both have a molecular weight of less than 4,000, both are capable of binding to albumin, and both dissociate from the albumin at extremes in pH.

Ensinck, Mahler, and Vallance-Owen (103) have recently presented further evidence to support the theory that the synalbumin antagonist could be B chain. They performed a series of experiments using reduced B chain, *S*-sulpho-B chain, and oxidized B chain. Their work showed that *S*-sulpho-B chain and reduced B chain antagonize the action of insulin in the hemidiaphragm assay, while oxidized B chain does not. They found that their *S*-sulpho-B chain preparation contained 0.5–0.9 sulfhydryl groups per mole of the chain. This, they feel, is responsible for the antagonistic activity of the *S*-sulpho-B chain and for the lack of antagonism shown by the oxidized B chain which contains no free sulfhydryl groups. It is indicated that the free sulfhydryl groups are essential for antagonistic activity since the reduced B chain preparations were rendered nonantagonistic by prior incubation with alkylating agents such as iodoacetamide or *N*-ethylmaleimide. The antagonistic activity of the reduced and *S*-sulpho-B chains is enhanced greatly by prior incubation with albumin which is probably because B chain itself is not very soluble unless complexed with albumin. These in-

vestigators (103) further demonstrated that B chain could be dissociated from albumin by passing the complex through an acetylated cellulose column or a Dowex 50 column in the sodium phase. The synalbumin antagonist had previously been shown to behave in a similar fashion.

It is proposed (103) that the B chain could result from the cleavage of insulin by glutathione-insulin transhydrogenase as shown by Tomizawa (104) and by Katzen and Stetten (78). It could antagonize the action of insulin by competing for the insulin binding sites at the cell membrane. It should be emphasized that insulin B chain has never been isolated from human serum or any of its components. If this could be accomplished, it would certainly add credence to the hypothesis that the synalbumin antagonist could be B chain.

Vallance-Owen (105) has shown that the plasma-albumin from prediabetics is antagonistic *in vitro* at a concentration of 1.25%. The term "prediabetes" may be defined as the metabolic state of a person at a period before he or she exhibits definite symptoms of diabetes. The presence of excessive synalbumin antagonism could therefore develop into a method of identifying prediabetics before they become overtly diabetic.

It is generally agreed that diabetes mellitus is hereditary in some way, although the mode of inheritance seems to be open to debate. Vallance-Owen (105) has studied a number of families with regard to the inheritance of excessive synalbumin antagonism, which he feels may indicate "essential diabetes." His evidence indicates that the transmission of excessive synalbumin antagonism could be by a dominant mode of inheritance, although the evidence is far from conclusive.

Lowry, Blanchard, and Phear (106) have performed experiments to show that the synalbumin antagonist does not inhibit the effect of insulin on adipose tissue. This could point to a fallacy in the whole line of reasoning or it could simply mean that synalbumin is capable of antagonizing some, but not all, of the actions of insulin. Also, no one has yet been able to demonstrate antagonistic activity for synalbumin or for B chain *in vivo*. A number of technical problems must be overcome before this can be accomplished; *i.e.*, B chain, if it is the antagonist, is quite insoluble and probably could not be administered alone, which would necessitate complexing it with a rather large quantity of albumin before infusing it into animals.

There are a number of questions yet to be answered about the synalbumin antagonist. For example, if it actually is B chain, does it arise from the incomplete synthesis of insulin or from increased destruction of insulin by glutathione insulin transhydrogenase? Also, what factors regulate the level of antagonist present in the plasma of normal and diabetic subjects? Nevertheless, these proposals merit serious thought and could provide answers to several important questions. It would certainly be advantageous to have a biochemical marker for the identification of prediabetics. This could also conceivably lead to fundamental knowledge about the etiology of diabetes mellitus and to possible ways of treating the disease.

The Glucose Fatty Acid Cycle.—It has been mentioned previously that the diabetic exhibits abnormal lipid metabolism as well as abnormal carbohydrate metabolism. Until quite recently, it had been generally agreed that the disturbance in lipid metabolism was secondary to the abnormalities in carbohydrate metabolism. Recent studies (107) suggest that the over-all disturbance in metabolism could result from high levels of FFA which result from an increased metabolism of fats by the diabetic. The implication is that the relationship between carbohydrate and lipid metabolism is reciprocal, and good evidence has been put forth to help verify this (107).

Randle and his associates (107) propose that the metabolism of glucose by the tissues inhibits the release of FFA from glyceride stores and that conversely the release of FFA inhibits the metabolism of glucose. According to this line of thought, the diabetic is unable to carry on normal carbohydrate metabolism because of elevated FFA levels.

Newsholme and Randle (108) demonstrated that anoxia, salicylate, and 2,4-dinitrophenol increase the rate of phosphorylation of fructose-6-phosphate to form fructose-1,6-diphosphate, a reaction which is catalyzed by phosphofructokinase. The reaction is inhibited in hearts from diabetic or starved rats or in hearts from normal rats which are perfused with media containing fatty acids or ketone bodies.

The stimulation of the phosphofructokinase reaction caused by anoxia is attributed (108) to increased concentrations of AMP and inorganic phosphate, which stimulate the reaction, and decreased concentrations of ATP, which inhibits the reaction. Alloxan-diabetes, starvation, fatty acids, and ketone bodies have no consistent effect on cencentrations of AMP, ATP, and inorganic phosphate and must, therefore, inhibit the reaction by a different mechanism. It is proposed (108) that the inhibition is caused by the increased concentration of citrate which these factors have been shown (109) to produce.

Randle's work (110) shows that the membrane transport of D-arabinose and D-glucose is greater in fed rats than in starved rats, a phenomenon which might be due to the presence of higher insulin concentrations in the fed animals. Fatty acids and ketone bodies will inhibit transport in fed rats but not in starved rats (110). Inhibition of transport by fatty acids and ketone bodies was demonstrated in starved rats when low concentrations (0.5 milliunit/ml.) of insulin but not when high concentrations (0.1 unit/ml.) of insulin were added. These workers feel that the above facts are explained by the hypothesis that fatty acids and ketone bodies impair the sensitivity of the transport system to stimulation by insulin.

Randle and his co-workers (110) also found that the phosphorylation of glucose by the perfused rat heart is inhibited by fatty acids and ketone bodies. A similar inhibition of phosphorylation of glucose was demonstrated in hearts from diabetic or starved rats.

It was found (110) that the rate of glycolysis was decreased in isolated rat hemidiaphragms and perfused rat hearts by fatty acids, ketone bodies, or pyruvate. These workers feel that the decreased rate of glycolysis is a result of a decreased uptake of glucose. The fact that more $^{14}CO_2$ is produced from 1-^{14}C-glucose than from 6-^{14}C-glucose under these conditions is an indication that some glucose is being oxidized through the pentose phosphate pathway.

Garland and Randle (109) showed that fatty acids, ketone bodies, and alloxan-diabetes inhibit the oxidation of pyruvate in the perfused rat heart and isolated hemidiaphragm. It is proposed (109) that this inhibition of pyruvate oxidation is the result of an increased acetyl CoA/CoA ratio which is produced by these conditions.

Garland and Randle (111) have reported that the concentration of fatty acyl CoA was increased in the rat heart *in vitro* by alloxan-diabetes, starvation, and perfusion with fatty acids. This is presumably caused by the increase in concentration of FFA which is due to an increased lipolysis and a decreased rate of fat synthesis. It was further shown (111) that the increased production of fatty acids and fatty acyl CoA could result in an increase in the concentration of citrate which then can inhibit the phosphofructokinase reaction. The concentration of citrate in diabetic rat hearts is lowered by insulin which might account for some of the increased carbohydrate metabolism brought about by insulin.

Garland *et al.* (112) illustrated that the oxidation of glucose in perfused rat hearts is inhibited when the perfusion media contains fatty acids or ketone bodies. They also found, however, that there is no decrease in oxygen consumption under these conditions. This was taken to mean that in the presence of fatty acids and ketone bodies, there is an increased oxidation of fatty acids to compensate for the decrease in glucose oxidation. This is further evidence for the reciprocal relationship between carbohydrate and fat metabolism.

From this evidence it is postulated (111) that the increased release of fatty acids from glyceride stores in diabetic rats results in increased levels of FFA, fatty acyl CoA, and ketone bodies. This in some way causes an increase in citrate concentration, and the over-all result is an inhibition of glucose transport, glucose phosphorylation, the phosphofructokinase reaction, and the oxidation of pyruvate.

INSULIN THERAPY

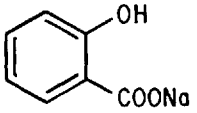
Insulin has been the mainstay of diabetes therapy ever since its discovery by Banting and Best. There are at least 7 different insulin preparations currently available in this country, the principal difference between these being in their duration of action. Crystalline insulin is effective but has an extremely short duration of action (6–12 hr.) (113). Longer acting preparations have been obtained by combining insulin with higher molecular weight proteins such as protamines and globins. Further work in this area resulted in the discovery of the lente insulins (114) in which duration of action can be controlled by a selective precipitation scheme. All of these preparations are insulin and exhibit the biological effects of insulin; they differ only in duration of action.

Although insulin offers many advantages in the treatment of diabetes, it also has a number of disavantages. It is a protein and is capable of producing allergic reactions. This can be further complicated when insulin is combined with a higher molecular weight, more highly antigenic protein, to increase its duration of action. Insulin is not effective orally and must therefore be administered parenterally which is an inconvenience at best. Insulin is a potent hormone and can produce dangerous hypoglycemia when doses are not regulated to correspond with conditions of diet and exercise. Good control of diabetes with insulin does not always prevent the occurrence of complications of diabetes such as diabetic neuropathy, glomerulosclerosis, diabetic retinopathy, and vascular disorders (115). Also, some patients are resistant to insulin (116) and require well over 200 units of insulin a day. This indicates a significant degree of resistance since it has been estimated (117) that a normal daily secretion of

insulin is about 55 units. Because of these numerous disadvantages, a great deal of research has been carried out to produce an insulin substitute which does not have these drawbacks.

ORAL HYPOGLYCEMICS

The Salicylates.—Long before the discovery of insulin it was known that sodium salicylate (I) is capable of lowering blood sugar in diabetics. Ebstein in 1876 (118) and Muller in 1877 (119)

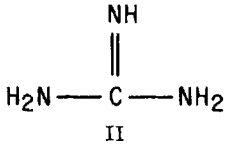


I

published reports on the ability of sodium salicylate to lower blood sugar. Not all salicylates exert this property. Acetylsalicylic acid, for example, has hypoglycemic acitivity when given in suitably high doses, while sodium gentisate which is chemically similar has no hypoglycemic acitivity.

The hypoglycemic salicylates cause an increased oxygen consumption in experimental animals (120) by the uncoupling of oxidative phosphorylation as shown by Brody (121). This uncoupling action increases aerobic glycolysis and increases glucose utilization, but the energy derived from the process is relatively little. The dosage of salicylates necessary to bring about a hypoglycemic effect is very high. It has been reported (122) that a daily dose of 4.8 Gm. is required to produce an adequate hypoglycemic effect. At this dosage level numerous side effects such as nausea, vomiting, hearing difficulties, and tinnitus appear quite frequently and thus the salicylates are not used clinically as hypoglycemic agents.

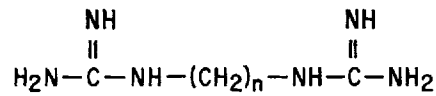
Guanidine.—The first reports on the ability of guanidine (II) to lower blood sugar were in 1918



II

(123). Hollunger (124) has shown that it inhibits respiration which leads to an increased glucose uptake by muscle, an increased glycolysis, and a decrease in blood glucose levels. Because of the relatively high toxicity of guanidine it was never widely used, although it did serve as a pattern for newer compounds which have been used as oral hypoglycemics.

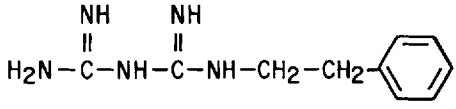
The Diguanidines.—These compounds (III), may be visualized as 2 guanidine molecules joined by a series of methylene groups. For the



III

most part, these compounds are more potent and less toxic than guanidine. Hypoglycemic activity increases with an increase in the number of methylene groups and apparently reaches a maximum in the decamethylene derivative which is 150 times more active than guanidine (125). This substance, known as synthalin A, was introduced into diabetes therapy in the 1920's The dodecamethylene diguanidine (synthalin B) is somewhat less active than synthalin A. Neither of these compounds compares favorably with insulin for effectiveness, and both also have a rather high tendency to cause kidney and liver damage (126), so their use as hypoglycemics was discontinued.

The Biguanides.—Further attempts to produce useful compounds resulted in 1929 in a series of biguanides which exhibited hypoglycemic activity (127). This discovery received little attention at the time, and little work was done in the area until 1957 when Unger and his associates (128) reported on the hypoglycemic activity of phenformin (IV) a newly synthesized biguanide.



IV

As a result of these studies, hundreds of new biguanides were prepared and tested for hypoglycemic activity, but none has been found to be superior to phenformin.

A great deal of information on the effects of phenformin and other biguanides on various biological systems is available, but the exact mechanism by which these compounds lower blood sugar is not understood. Phenformin was shown to increase the glucose uptake of the isolated rat diaphragm (129), but this effect was produced by a higher dose of the drug than is normally found in a physiological system. It has been found that glucose uptake by the diaphragm is accompanied by a decreased oxygen consumption and a decreased glycogen content of the diaphragm (130) which is opposite from the effect of insulin in this system. The increased glucose uptake of the diaphragm in the presence of phenformin appears to be a result of increased anaerobic glycolysis in the muscle (130). This is consistent with the findings of Steiner and Williams (131), who demonstrated that phen-

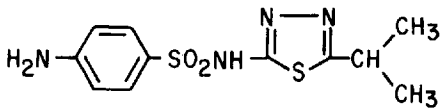
formin produces elevated levels of lactic acid, the end product of anaerobic glycolysis in muscle.

It has been shown (132) that phenformin increases the uptake of glucose by the rat epididymal fat pad *in vitro*. Daweke and Bach (133) used Buformin (N^1-*n*-butylbiguanide) to show an increased oxidation of glucose-1-^{14}C by rat adipose tissue at relatively low concentrations, but on the other hand, Wick and his co-workers (134) found that higher doses of phenformin inhibit glucose oxidation by adipose tissue.

Steiner and Williams (135) have proposed that the biguanides might act by inhibiting certain enzyme systems of the mitochondria which are concerned with Krebs cycle oxidations. This would lead to an indirect stimulation of the anaerobic glycolysis of glucose and in turn would cause an increased uptake of glucose. This theory accounts for the increased lactic acid production associated with biguanide treatment and is also consistent with the observations of Randle and Smith (136), who demonstrated that anaerobiosis can increase the glucose uptake of the isolated rat diaphragm.

The biguanides serve a useful function in diabetes therapy in that they are useful in treating certain cases which are unresponsive to other oralhypoglycemics. They affect carbohydrate metabolism directly but are not used in the absence of some supply of insulin since acidosis develops in such cases. Side effects such as anorexia, nausea, vomiting, weight loss, and muscular weakness occur quite frequently in phenformin treated subjects (137), although this compound does not exhibit the liver toxicity of the parent synthalins (138).

The Sulfonamides.—Savagone (139) was the first to publish information indicating that certain sulfonamides lower blood sugar in man. This led to the investigation of a number of sulfonamides as potential oral hypoglycemic agents. The work of Loubatieres (140) showed that one of the sulfonamides, 2-(*p*-aminobenzenesulfonamide)-5-isopropylthiodiazole (IPTD) (V), lowers



V

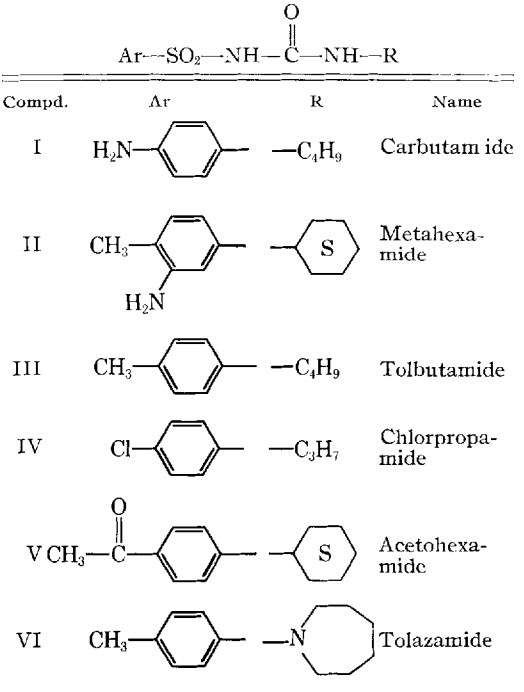
blood sugar levels, apparently by stimulating the β cells to secrete insulin.

Relatively little research was done on the sulfonamides as hypoglycemics until the advent of the sulfonylureas in the 1950's. At that time IPTD was studied clinically in human diabetics (141) and was found to lower blood sugar in some patients. It was found effective in the same general type of cases as the sulfonylureas; but since it is somewhat less potent than most sulfonylureas and has no obvious advantages over the sulfonylureas it never has been marketed.

The Sulfonylureas.—From a practical standpoint the sulfonylureas are currently the most important oral hypoglycemics. Their ability to lower blood sugar was first discovered by a group of German workers in 1951. Carbutamide (BZ 55) was the first of the sulfonylureas to be studied extensively from a clinical standpoint (142–144). The discovery that this compound is capable of controlling the effects of diabetes in some patients led to the synthesis and testing of literally hundreds of analogs and derivatives of carbutamide. It is beyond the scope of this review to consider the chemistry and pharmacology of large numbers of individual sulfonylureas, particularly since there are already numerous excellent reviews on the subject (145–148). This discussion will be concerned primarily with the pharmacological action of the sulfonylureas as a group.

Some of the more familiar sulfonylurea hypoglycemics are listed in Table I. Carbutamide, the first of the series to be studied thoroughly, was found to be an effective hypoglycemic but also exhibits liver toxicity which in some cases proved to be fatal (149). Because of this, the compound was never marketed in the U. S., although it is still in use in some European countries. Metahexamide, a somewhat newer compound, is an

TABLE I. – SULFONYLUREA HYPOGLYCEMICS



effective hypoglycemic (150) but also exhibits liver toxicity and has produced cases of obstructive jaundice (151) so is no longer being used clinically. Tolbutamide, chlorpropamide, and acetohexamide are all effective oral hypoglycemics which are currently in use in this country. Tolazamide, which was first synthesized in 1962 (152), is one of the newer sulfonylureas. It has been tested in animals and humans and shows promise of being a useful oral hypoglycemic (153).

All of the sulfonylurea hypoglycemics appear to act by the same mechanism, produce the same effects, and are effective in the same general types of cases. They differ mainly in potency, duration of action, and the frequency with which side effects occur. Of the many hundreds of sulfonylureas which have been synthesized, only a relatively few have a substantial amount of hypoglycemic activity and many of those are too toxic for clinical use.

The relationship between chemical structure and pharmacological activity for this class of compounds has been summarized by Mahler (147). The benzene ring should contain at least one substituent; best results are obtained when it is in the 4-position. Active compounds have been prepared where this substituent is methyl, amino, acetyl, chlorine, methylthio as well as several other groups or atoms. If a carboxyl, hydroxyl, nitro, or hydroxymethyl group is placed in the 4-position the resulting compound is inactive.

The group attached to the terminal nitrogen of the urea nucleus should be a certain size and should impart lipophilic properties to the molecule. Simple aliphatic groups are quite frequently placed in this position. The methyl derivatives are usually inactive, the ethyl derivatives exhibit some activity, and maximum activity is reached when the substituent contains 3–6 carbons. Activity is lost altogether if the substituent contains as many as 12 carbons. A number of active compounds have been prepared where this substituent is an alicyclic ring (154) with optimal activity usually being attained when this is a 5, 6, or 7 membered ring.

Most of the sulfonylureas are poorly soluble in water at a neutral pH but because of their weakly acidic character are more soluble at a slightly alkaline pH. This facilitates dissolution in the intestinal juices and absorption from the intestine. The absorption of tolbutamide from this site is nearly complete in most species (155). Most of the sulfonylureas are absorbed quite rapidly; Ridolfo and Kirtley (156) showed that carbutamide can be detected in the blood within 30 min. after oral administration.

The mode of metabolism of the sulfonylureas is quite variable and is dependent on the structure of the compound as well as the species of the animal. The mode and rate of metabolism of these drugs is important since many are converted to inactive metabolites, thus governing their potency and duration of action.

Carbutamide, which is structurally similar to the sulfa drugs, is metabolized by acetylation of the 4-nitrogen to produce an inactive metabolite (157). The 4-methyl group of tolbutamide is oxidized in the body to a carboxyl group (158) and the metabolite is without hypoglycemic activity. Chlorpropamide, which has a chlorine in the 4-position, is not metabolized in humans (159) and is excreted in the urine as the free drug. Acetohexamide is metabolized by the reduction of the ketone to a secondary alcohol (160) but the metabolite has hypoglycemic activity (161).

The biological half-life of these drugs is one of the best measures of their duration of action. The term "biological half-life" has been defined as (161) "the time required for the blood levels of the drug to decrease by 50 per cent during the postabsorptive, concentration-dependent disappearance phase." The biological half-life of a drug such as chlorpropamide, which is not metabolized, is dependent only on its rate of excretion. Johnson and his associates (159) determined the biological half-life of this drug to be 35 hr. The biological half-life of a drug which is metabolized in the body is dependent on the rate of metabolism as well as the rate of excretion of the unchanged drug. Baird and Duncan (162) estimated the biological half-life of carbutamide to be between 30 and 60 hr. The half-life of tolbutamide has been reported by a number of authors (163–165), and the average value is in the neighborhood of 5 hr. Recent studies by Smith, Vecchio, and Forist (161) indicate that the half-life of acetohexamide is 1.3 hr., indicating that the drug is metabolized very rapidly. Since the metabolite of this drug is an active hypoglycemic, the half-life of the metabolite should also be taken into consideration in estimating the duration of action of acetohexamide. The half-life for the metabolite is 4.6 hr. (161), which indicates that acetohexamide has a duration of action comparable to that of tolbutamide.

It should be emphasized that, although the duration of action of these compounds is proportional to their biological half-life, the 2 values are not equal to each other. Tolbutamide, for example, has a biological half-life of about 5 hr., but effective blood levels of the active drug are maintained for 12 to 18 hr. after oral administration (161).

Since all of these compounds are metabolized by different routes and at different rates it would be expected that the blood levels of drug would vary from compound to compound. This would quite logically have an effect on the relative hypoglycemic potencies of the compounds. McMahon and his co-workers (148) have determined the relative clinical potencies of a number of the common sulfonylurea hypoglycemics. The clinical potency was arrived at from considering the acute potency as well as the biological half-life of the various compounds. The clinical potencies of some of the more common sulfonylureas are: tolbutamide, 1; carbutamide, 1–3; chlorpropamide, 4–7; metahexamide, 10; acetohexamide, 1–4; and tolazamide, 5–15.

Numerous investigations have been carried out in recent years to determine the exact mechanism of action of the sulfonylurea hypoglycemics. A great deal of information has been accumulated and a number of facts have been well established. Some of the more pertinent observations are mentioned here.

Mirsky and Gitelson (166) have reported results which indicate that these compounds might decrease the activity of the insulin degrading enzyme in the rat liver. The decreased enzymatic activity which was observed was, however, very slight and it required an extremely high concentration of the sulfonylurea to bring it about. On the other hand, it has been found (167) that tolbutamide does not prolong the half-life of injected ^{131}I insulin. Because of this, it is rather doubtful that this effect is very important to the action of these compounds as hypoglycemics.

Some workers (168) have shown that sulfonylureas produce a slight increase in the glucose uptake of the rat diaphragm *in vitro*. This effect was not accompanied by an increased glycogen synthesis by the diaphragm as is the case when glucose uptake is stimulated by insulin. Most workers, however, have been unable to show an increased glucose uptake by the rat diaphragm in the presence of sulfonylurea hypoglycemics (169–171) which is in accord with the theory that sulfonylureas have no direct effect on carbohydrate metabolism.

The effect of sulfonylureas on the oxidation of labeled glucose by the rat epididymal fat pad *in vitro* appears to be somewhat confusing. Renold and his associates have reported that tolbutamide and chlorpropamide have a slight stimulatory effect on the oxidation of glucose by this tissue (172). Marques and Correa (173), on the other hand, found that acetohexamide, carbutamide, and tolbutamide did not increase glucose oxidation by isolated rat epididymal adipose tissue. They found that high concentrations (50 mcg./ml.) of chlorpropamide or tolazamide increased glucose oxidation slightly, but the effect was not nearly so great as that produced by insulin at a concentration of 20 microunits/ml. It is interesting to note that even in Renold's studies there was found to be no stimulation of fatty acid synthesis as is the case when insulin is added to the medium. It should also be pointed out that a number of substances other than hypoglycemics are able to stimulate the oxidation of glucose by adipose tissue *in vitro*. Some of these agents are epinephrine (174), ACTH (175), glucagon (176), serotonin (177), growth hormone (178), and prolactin (179). These observations suggest that even if the sulfonylureas do stimulate glucose oxidation in adipose tissue the effect is not necessarily important to their action as hypoglycemics.

It has been shown (180) that sulfonylureas are able to decrease the glucose output of the liver. This might contribute to their action as hypoglycemics, but it is unlikely to be their major action since Dulin and Johnston (181) have shown that sulfonylureas act as hypoglycemics in hepatectomized animals. It has been shown (182) that sulfonylureas are capable of inhibiting glucose-6-phosphatase activity in liver slices, but the concentration of drug required to bring about this response is well above the concentration normally present in blood. This makes it seem highly improbable that inhibition of glucose-6-phosphatase activity is important to the action of these compounds. It is also interesting that a similar concentration of sulfanilamide can inhibit glucose-6-phosphatase activity (182), but this compound does not lower blood sugar in intact animals.

It is generally agreed that the sulfonylureas are incapable of lowering blood sugar in totally pancreatectomized animals (183, 184, 157). They also have no hypoglycemic effect in alloxan-diabetic animals providing there is complete β cell destruction (181). This indicates that a functioning pancreas is essential to the action of these compounds.

It has been observed that pretreatment of animals with growth hormone or prolonged fasting of the animals decreases the response of these animals to sulfonylureas (185). Both of these conditions are known to decrease the insulin content of the pancreas. This further suggests that the action of the sulfonylureas is dependent on the presence of some insulin.

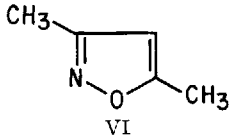
The storage and secretion of insulin by the pancreas has recently been reviewed by Lacy (81), who points out that insulin is stored in the β cells

in the form of β granules which are visible with the electron microscope. The process by which insulin is secreted is referred to as emiocytosis and involves the migration of the β granules to the cell membrane where they are liberated into the intercellular spaces. Williamson, Lacy, and Grisham (186) have demonstrated that tolbutamide stimulates emiocytosis in the β cells of the rat. Other workers (187) have shown a similar effect in the guinea pig. Vallance-Owen and his associates (188) have shown that a single oral dose of tolbutamide will increase plasma insulin-like activity by a factor of 2 within 2.5 hr. Yalow and Berson (189) made similar observations by using an immunoassay to measure plasma insulin levels.

The above observations indicate that the sulfonylureas have no direct effect on the metabolism of carbohydrates. The current feeling is that the primary action of the sulfonylureas is a stimulation of the pancreas to secrete insulin, but it should be emphasized that the mechanism by which they promote this release of insulin is not understood. This theory points out why it is essential to have a functioning pancreas for these compounds to be effective and illustrates why they cannot be used successfully to treat juvenile diabetics.

The sulfonylureas are an important class of therapeutic agents and can be used effectively in a great number of cases, but proper patient selection is an important factor in effective sulfonylurea therapy. In a recent report involving 9,214 diabetics studied over a 6-year period (190) it was concluded that the subject most likely to be controlled on these compounds is one who contracted diabetes after age 40, is overweight, has a daily insulin requirement of less than 30 units, and has had the disease for less than 5 years.

The Pyrazoles and Isoxazoles.—Dulin and Gerritsen were the first to report that 3,5-dimethylisoxazole (VI) is a potent oral hypo-



VI

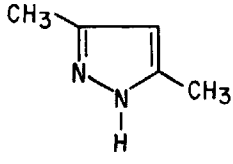
glycemic agent (191) in fasted, intact rats which are injected with 100 mg. of glucose at the time of treatment. The compound is effective at a dose as low as 10 mcg./150 Gm. rat which indicates that it is about 188 times more potent than tolbutamide and is the most potent oral hypoglycemic reported. These observations have opened a whole new area of research in oral hypoglycemics and have led to a great deal of information concerning this compound as well as a number of similar compounds.

This study (191) demonstrated that 3,5-dimethylisoxazole increases the oxidation of labeled glucose by fasted, intact rats, an effect which is shared by insulin but not the biguanides (129). The action of 3,5-dimethylisoxazole is different from that of the sulfonylureas as it is effective in alloxan-diabetic rats and the sulfonylureas are not. The compound was found to be ineffective in lowering blood sugar in spinal transectomized, eviscerated rats, which suggests that the action of the compound is dependent on the liver and/or the intestine.

In a subsequent study by Dulin, Lund, and Gerritsen (192) it was demonstrated that this isoxazole derivative also has a profound effect on fat metabolism as it is capable of lowering plasma FFA levels within 30 min. after oral administration to fasted, glucose-primed, intact rats. The compound had no effect on the rate of oxidation of labeled palmitate which was continuously infused into the animals, but there was an increase in the specific activity of plasma FFA during the infusion of the labeled palmitate. This led the authors to suggest that the depression in plasma FFA was probably due to an inhibition of lipolysis rather than to a stimulation of fatty acid oxidation.

It is interesting to note that the FFA levels are depressed within 30 min., while it requires about 2 hr. for blood sugar to be depressed significantly. This could mean that the effect on blood sugar is secondary to the effect on plasma FFA levels, although there is no proof that the two effects are directly related.

The work with 3,5-dimethylisoxazole has led to subsequent publications dealing with related compounds derived from pyrazole (193, 194). 3,5-Dimethylpyrazole (VII) was found to pro-



VII

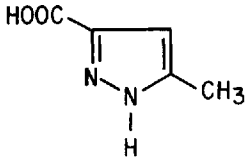
duce biological effects similar to those reported earlier for the analagous isoxazole and was shown to be 50 times as potent as tolbutamide in lowering blood sugar in glucose-primed, fasted, intact rats. It was also found to have a significant effect on fat metabolism (193).

It was shown (193) that 3,5-dimethylpyrazole increases the rate of oxidation of labeled glucose in fasted, intact rats and was also found to be effective in alloxan-diabetic rats. It has no effect

on the rate of oxidation of labeled glucose by rat epididymal adipose tissue *in vitro* and also has no effect on the liberation of FFA from this tissue *in vitro*.

As was found to be the case with 3,5-dimethylisoxazole, the pyrazole was found to lower plasma FFA levels in glucose-primed, fasted, intact rats. The administration of the pyrazole to rats continuously infused with labeled palmitate again resulted in no increased oxidation of the palmitate but did produce an increase in the specific activity of the plasma FFA. This again is suggestive that the FFA depression is probably a result of a decreased mobilization of fatty acids from glycerides in fat depots. The depression of plasma FFA occurred within 15 min., while it required 2 hr. to depress glucose levels significantly, which further suggests that the effect of these compounds on blood glucose levels could be secondary to their effect on fat metabolism.

Since 3,5-dimethylpyrazole depresses fat mobilization *in vivo* but not *in vitro*, since it depresses blood glucose levels in fasted, glucose-primed, intact rats but not in eviscerated animals, and since its action on blood sugar is antagonized by SKF 525-A, an inhibitor of drug metabolism, it was suggested that the action of the compound could be due to a metabolite rather than to the compound itself. A study carried out by Smith, Forist, and Dulin (195) has shown that 3,5-dimethylpyrazole is metabolized to 5-methylpyrazole-3-carboxylic acid (VIII) in the rat. Further work by Gerritsen and Dulin (194) shows that this metabolite is a potent hypoglycemic and is probably responsible for many of the effects which are produced when 3,5-dimethylpyrazole is administered to animals.



VIII

5-Methylpyrazole-3-carboxylic acid is 116 times more potent than tolbutamide in intact, fasted, glucose-primed rats (194). Like the dimethyl derivative, it also increases the rate of oxidation of labeled glucose in fasted, intact rats, is effective in alloxan-diabetic rats, and rapidly depresses plasma FFA. It is readily apparent from Fig. 1 that the depression in plasma FFA occurs much more rapidly than the depression in blood sugar.

This study also points out a number of differences between 3,5-dimethylpyrazole and 5-methylpyrazole-3-carboxylic acid (194). The carboxy compound has hypoglycemic activity in spinal transectomized, eviscerated rats, a property which is not shared by the dimethyl derivative. This indicates that the dimethyl derivative must be oxidized to the acid before it is able to lower blood sugar. The evidence indicates that this oxidation probably occurs in the liver and apparently occurs quite rapidly since blood sugar is depressed within 2 hr. in animals treated with the dimethyl compound.

Another difference between the two pyrazole derivatives is that 5-methylpyrazole-3-carboxylic acid increases glucose oxidation by rat epididymal adipose tissue *in vitro* and also inhibits the release of FFA by the same tissue *in vitro* (194). Neither of these effects is exhibited by 3,5-dimethylpyrazole which further suggests that it is the metabolite which is the active form of the drug. Like other pyrazoles and isoxazoles, but unlike insulin, the carboxy pyrazole derivative does not increase glucose uptake by the isolated rat diaphragm (194).

There is not sufficient information available to understand completely the mechanism of action of these compounds, although a number of points are quite clear. They affect both carbohydrate and fat metabolism in a variety of biological systems; but whether these two effects are related or independent of each other has not been established. If the two effects are related, the effect on fat metabolism is almost certainly the primary effect since this effect is manifested so much more rapidly than the blood sugar depression, a point which is made abundantly clear by Fig. 1. Further evidence for the primary effect on fat metabolism is that there have been no instances reported where these compounds lowered blood sugar without first having lowered plasma FFA levels.

There is good evidence to support the theory

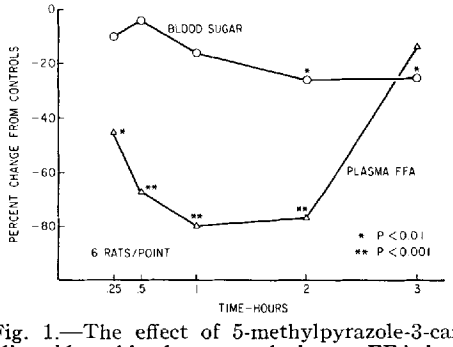


Fig. 1.—The effect of 5-methylpyrazole-3-carboxylic acid on blood sugar and plasma FFA levels in fasted, intact, glucose-primed rats (194). Reprinted with the permission of the *Journal of Pharmacology and Experimental Therapeutics*.

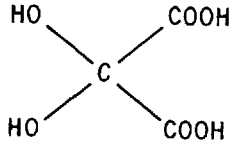
that a depression of plasma FFA levels could produce a depression in blood sugar. At low FFA levels the oxidation of fatty acids is minimized thus allowing for the oxidation of relatively large quantities of glucose for energy production which would result in a lowering of blood sugar levels. This idea is also in agreement with the work of Randle and his associates (107) which shows that high levels of FFA inhibit the utilization of glucose. The exact mechanism of action of these compounds is, however, probably much more complex than this.

One wonders why certain barbiturate anesthetics are able to inhibit the effect of these compounds on carbohydrate metabolism, but not on fat metabolism as shown by Gerritsen and Dulin (193). One possibility is that the barbiturate antagonizes the secondary action of the compounds on carbohydrate metabolism, but not the primary action on fat metabolism. Another question which must be answered is why the effect of 3,5-dimethylpyrazole on carbohydrate metabolism is blocked by evisceration, but the effect on FFA depression is not (193). There appears to be no ready answer to this question at this time.

A recent publication by Wright, Dulin, and Markillie (196) describes the preparation of 41 pyrazole derivatives which were tested for hypoglycemic activity. A number of the compounds having methyl groups in the 3 and 5 positions were found to be active oral hypoglycemics, 1 of them being about 100 times more potent than tolbutamide. It seems likely that these compounds act by the same mechanism as the original isoxazoles and pyrazoles, so little if any difference would be expected.

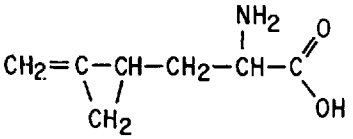
In summary, the pyrazoles and isoxazoles are among the most interesting hypoglycemics to be studied. Further study of this group of compounds could result in the discovery of important information concerning the causes, effects, and treatment of diabetes. It is also conceivable that an effective and useful antidiabetic drug could evolve from the series.

Miscellaneous Hypoglycemics.—Mesoxalic acid (IX) has been reported to decrease blood sugar levels in laboratory animals (197). Relatively little information has been published on the compound, but it appears to have an action similar to the sulfonylureas. It is effective in the presence of some endogenous insulin, but exerts no hypoglycemic effect in totally pancreatectomized animals (197). This led to the speculation (198) that the compound exerts its action by stimulating the β cells to secrete insulin. The calcium salt of this acid is on the Japanese market but has not been studied extensively in this country.



IX

Two compounds, hypoglycin A (X) and hypoglycin B, that have been isolated from the unripe fruit of a tropical tree (*Blighia sapida*) have been found to possess hypoglycemic activity (199). Chen and his associates (200) found hypoglycin A to be more effective than hypoglycin B, but found both compounds to be highly toxic.



X

Patrick (201) has studied the effects of hypoglycin A on various biological systems. He found that it is effective in alloxan-diabetic rats, but its action is different from that of insulin. This compound does not increase oxygen consumption in intact rats nor does it increase muscle glycogen. It does not increase the glucose uptake of the isolated rat diaphragm and does not stimulate the oxidation of glucose by rat epididymal adipose tissue *in vitro*. It does not appear to increase carbohydrate utilization greatly by any biological system. Patrick (201) has proposed that the liver is the primary site of action of the compound.

L-Leucine has been reported to lower blood sugar in some children with idiopathic hypoglycemia (202) and in some patients with islet cell tumors (203). Yalow and Berson (204) have shown that L-leucine produces elevated plasma insulin levels in these patients which probably accounts for its hypoglycemic effect. Fajans and his associates (205) have reported that L-leucine can also reduce blood sugar in normal healthy subjects who have been treated with insulin or sulfonylureas to increase plasma insulin levels. They have also shown that a modest but significant hypoglycemic effect is produced when L-leucine is administered to healthy subjects without sulfonylurea or insulin pretreatment. Fajans (206) has recently summarized his findings which indicate that L-leucine-induced hypoglycemia results from a decreased hepatic glucose output and an increased peripheral utilization of glucose. The stimulation of insulin release is proposed as the primary mechanism producing these effects.

TABLE II.—THE BIOLOGICAL EFFECTS OF VARIOUS HYPOGLYCEMIC AGENTS ON VARIOUS ANIMAL SYSTEMS

Effect	Insulin	Sulfonyl-ureas	Biguanides	3,5-Dimethyl-pyrazole	5-Methyl-pyrazole-3-carboxylic Acid
Effective in alloxan-diabetic animals	+	−	+	+	+
Effective in eviscerate animals	+	−	+	−	+
Increase glucose oxidation in intact animals	+	+	−	+	+
Increase glucose uptake by rat diaphragm *in vitro*	+	−	+	−	−
Increase glycogen content of isolated rat diaphragm	+	−	−	−	−
Increase glucose oxidation by rat epididymal adipose tissue *in vitro*	+	−	−	−	+
Lower plasma FFA levels	+	+	−	+	+

A large number of other substances have been found to exhibit hypoglycemic activity, but most of these show no real advantages over compounds which are in current use. 4-Dimethylamino-*N*-methyl-2,2-diphenylvaleramide was shown to be 4 times more potent than tolbutamide in fasted intact rats (207); it is proposed that it acts by decreasing the glucose output of the liver. Goldner and Jauregui (208) have reported that certain antihistaminic drugs possess hypoglycemic activity. Hultquist (209) has shown that iodoacetic acid and iodoacetamide exhibit hypoglycemic activity in rats, seemingly by the stimulation of the β cells to secrete insulin. Tris-(hydroxymethyl) aminomethane has also been reported to possess hypoglycemic properties (210).

This is only a partial list of substances which have been reported to have significant hypoglycemic activity. A complete list would be far too extensive for the purposes of this review.

Summary.—It may be seen from Table II that, although many different classes of compounds are capable of lowering blood sugar and counteracting certain other effects of diabetes, they do not all act by the same mechanism. It points out that the sulfonylureas, which are dependent on a functioning pancreas for their action, are not effective in alloxan-diabetic or eviscerate animals (181, 184). The biguanides, on the other hand, have been found to lower blood sugar in alloxan-diabetic (129) and eviscerate (138) animals. The biguanides do not increase glucose oxidation in intact animals, whereas insulin and the pyrazoles do (193). The biguanides are the only compounds in the table other than insulin which are generally agreed to stimulate glucose uptake by the isolated rat diaphragm (129), but even these compounds do not increase the glycogen content of the diaphragms as does insulin (211). The pyrazoles (193) and the sulfonylureas (212) as well as insulin are capable of lowering plasma FFA levels in intact animals. The table also illustrates that 5-methylpyrazole-3-carboxylic acid can be differentiated from 3,5-dimethylpyrazole by virtue of the effectiveness of the acid in eviscerate rats as well as by its effect on glucose oxidation in rat epididymal adipose tissue *in vitro* (194). It is obvious by now that none of these compounds qualifies as an oral insulin since none exhibits all of the biological effects of insulin. They are properly classified as "oral hypoglycemics" or "oral insulin substitutes."

CONCLUSIONS

The above observations lead to the realization that although a substantial amount of information about the metabolic effects of diabetes has been accumulated, a great deal more must be learned before certain important conclusions can be reached. The etiology of diabetes is not well understood, nor are its hereditary aspects. The effects of insulin on various biological systems are quite well documented, but the mechanisms by which it produces these effects are not clearly understood. Treatment of diabetes with oral hypoglycemics must be termed only partially successful at this time, and more research in the area is required to produce new compounds which are more similar to insulin in their biological action and which are effective in treating a greater number of diabetic patients. Long-range goals should be aimed at learning more about the causes of diabetes so that steps can be taken to prevent the disease rather than simply treating the symptoms. The cause is a worthy one and merits the vast amount of research being carried out in the area.

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Spectra–Structure Correlations of Phenothiazines by Infrared, Ultraviolet, and Nuclear Magnetic Resonance Spectroscopy

By R. J. WARREN, I. B. EISDORFER, W. E. THOMPSON, and J. E. ZAREMBO

The infrared, ultraviolet, and nuclear magnetic resonance spectra of 23 phenothiazines of varying structure have been recorded and analyzed. Spectra–structure correlations and assignments of the absorption bands are presented and discussed. The collection of phenothiazines includes all of the commercially available analogs currently in use in the field of medicine and provides a comprehensive source for the identification and structure elucidation of samples from biological, forensic, and medical research.

ALTHOUGH the literature is replete with publications on phenothiazines, there has been to date no attempt to organize and correlate spectral data on this class of compounds. The purpose of this paper is to report the results of such a spectral study. Infrared, ultraviolet, and nuclear magnetic resonance spectra of 23 phenothiazine derivatives have been analyzed, and the correlations from these spectral data make possible a positive identification of all of the commercially available pharmaceutically useful phenothiazines.

EXPERIMENTAL

The infrared spectra were recorded between 4000–625 cm.$^{-1}$ with a Perkin-Elmer model 21 spectrometer with a sodium chloride prism. The phenothiazines were prepared as natural films.

The ultraviolet spectra were recorded between 200 and 400 mμ with a Cary model 14 recording spectrophotometer using matched fused silica cells with a 1-cm. light path.

The NMR spectra were recorded in $CDCl_3$ with tetramethylsilane as the internal standard on a Varian Associates model A-60 spectrometer.

All spectral data were obtained on the same samples. These were analytical standards with a purity of 98% or greater. (See Table I.)

DISCUSSION OF RESULTS

Infrared Spectra.—The infrared spectra of the phenothiazines are shown in Figs. 1–6. Seventeen of the 23 phenothiazines are substituted in the

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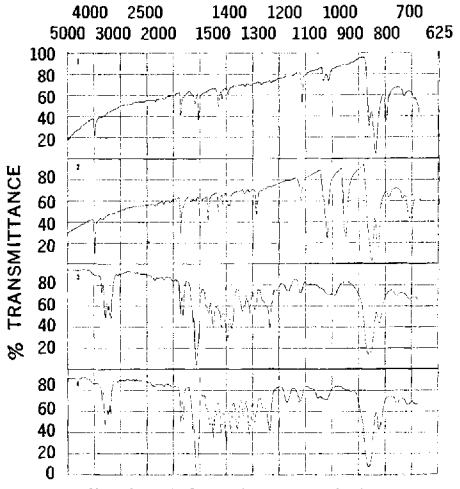


Fig. 1.—Infrared spectra 1–4.

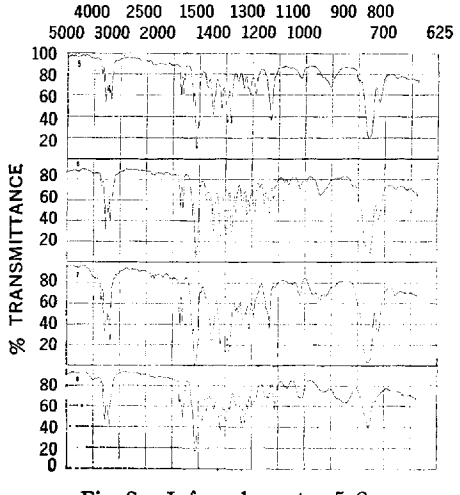
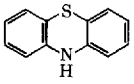
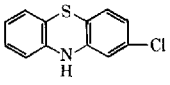
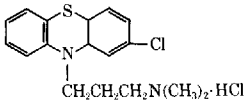
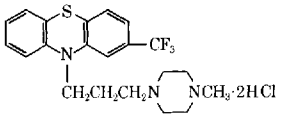
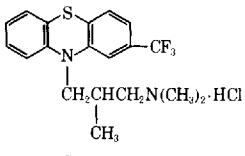
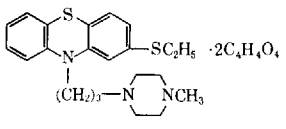
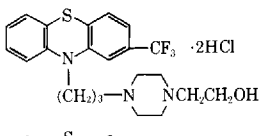
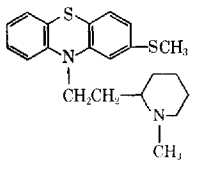
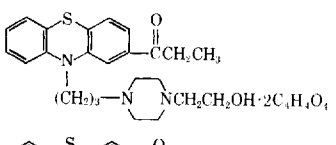
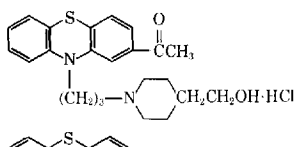
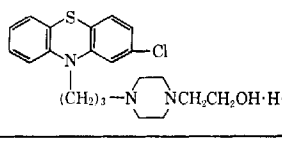


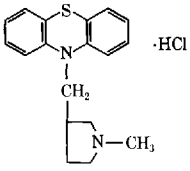
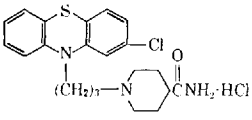
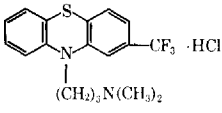
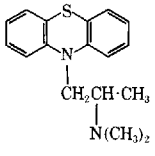
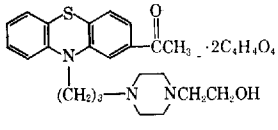
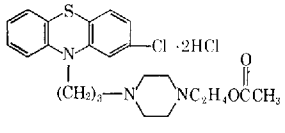
Fig. 2.—Infrared spectra 5–8.

TABLE I.—SPECTRAL DATA FOR PHENOTHIAZINES

Compd.	Structural Formula	U. V. Data[a] $\lambda_{max.}$ mμ	Log ε	$\lambda_{min.}$ mμ	Log ε	I.R. Spec. No. of Free Base	NMR Spec. No. of Free Base
Phenothiazine		253 320	4.64 3.64	280	3.00	1	1
2-Chlorophenothiazine		256 320	4.71 3.69	285	3.03	2	2
Chlorpromazine hydrochloride		256 310	4.54 3.60	280	3.16	21	3
Prochlorperazine dihydrochloride		256 309	4.54 3.62	280	3.11	20	17
Trifluoperazine dihydrochloride		258 307.5	4.50 3.50	280	3.08	16	13
Trifluoromeprazine hydrochloride		308 258 238sh	3.60 4.55 4.18	279 240 223	3.21 4.18 4.02	15	5
Thiethylperazine maleate		316 264	3.69 4.59	300 239	3.51 4.28	8	20
Vespazine hydrochloride		309 259	3.58 4.53	279 224	3.23 4.00	9	19
Thioridazine hydrochloride		314 263	3.66 4.58	288	3.72	10	9
Carphenazine maleate		278 243	4.39 4.46	256 230	4.25 4.36	14	15
Piperacetazine hydrochloride		280 244	4.35 4.40	256 220	4.15 4.23	12	12
Perphenazine hydrochloride		310 257	3.66 4.57	278 225	3.23 4.06	11	18

continued on next page

TABLE I.—(*Continued*)

Compd.	Structural Formula	U. V. Data[a] $\lambda_{max.}$ mμ	Log ε	$\lambda_{min.}$ mμ	Log ε	I.R. Spec. No. of Free Base	NMR Spec. No. of Free Base
Methdilazine hydrochloride		304 254	3.61 4.50	276 222	3.17 3.93	7	21
Pipamazine hydrochloride		312 257	3.65 4.57	280	3.22	19	10
Triflupromazine hydrochloride		308 258	3.57 4.53	280 224	3.21 4.01	17	6
Promethazine hydrochloride		302 252	3.58 4.49	274 220	3.21 3.97	4	7
Acetophenazine dimaleate		280 243	4.35 4.44	255 230	4.19 4.32	13	14
Thiopropazate hydrochloride		310 257	3.66 4.57	280 226	3.26 4.12	18	16

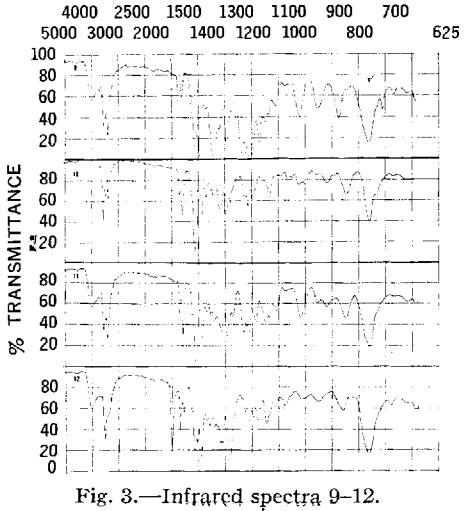


Fig. 3.—Infrared spectra 9–12.

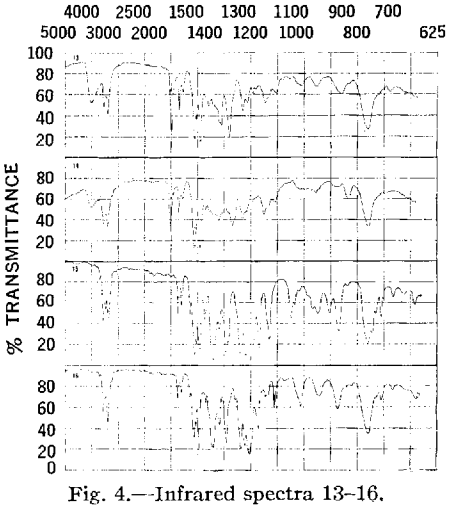
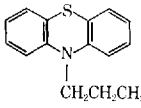
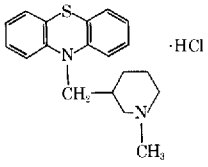
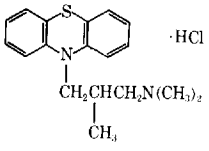
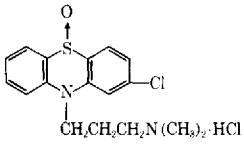
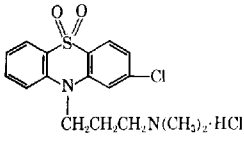


Fig. 4.—Infrared spectra 13–16.

TABLE I.—(*Continued*)

Compd.	Structural Formula	U. V. Data[a] $\lambda_{max.}$ mμ	Log ε	$\lambda_{min.}$ mμ	Log ε	I.R. Spec. No. of Free Base	NMR Spec. No. of Free Base
Promazine		306 254	3.64 4.53	277 222	3.20 3.95	3	8
Mepazine hydrochloride		304 254	3.59 4.48	276 221	3.15 3.96	6	11
Trimeprazine hydrochloride		308 255	3.67 4.53	278 222	3.22 3.95	5	4
Chlorpromazine sulfoxide hydrochloride		240 275 298 342.5	4.52 4.03 3.88 3.72			22	22
Chlorpromazine sulfone hydrochloride		233 271 294 332	4.54 4.16 3.89 3.76			23	23

[a] U.V. spectra run in 95% ethanol.

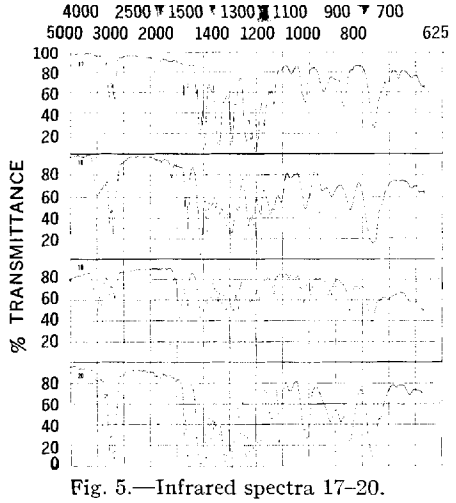


Fig. 5.—Infrared spectra 17–20.

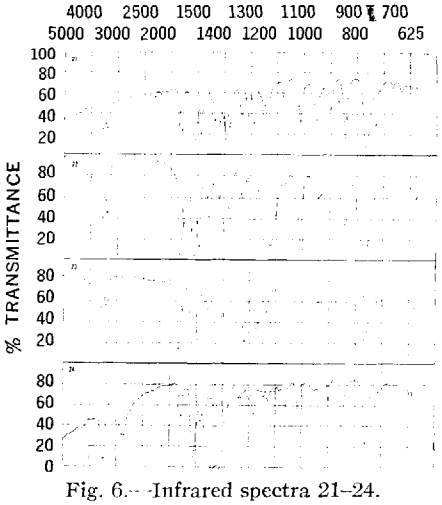


Fig. 6.—Infrared spectra 21–24.

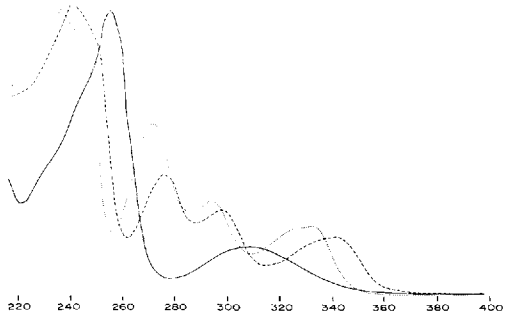


Fig. 7.—Ultraviolet spectra. Key: ——, phenothiazine; ---, sulfoxide; . . ., sulfone.

2,10-positions. These 2,10-disubstituted phenothiazines have a characteristic and unique over-all pattern in the infrared spectrum. There are 4 bands in the region 1000–700 cm.$^{-1}$ which are common to all. These bands occur at 915–928 cm.$^{-1}$, 840–870 cm.$^{-1}$, 785–800 cm.$^{-1}$, and 730–755 cm.$^{-1}$. The band at 785–800 cm.$^{-1}$ exhibits some splitting in certain cases, but it is steady in location. The bands listed are all strong and comprise a definite over-all pattern which dominates the fingerprint range of the spectrum. The bands are assignable to the phenothiazine ring system and the substitution in the 2-position. Bellamy (1) gives the range 770–735 cm.$^{-1}$ for the out-of-plane bending vibration of 4 adjacent hydrogens in an aromatic ring and the 900–800 cm.$^{-1}$ for 1,2,4-trisubstitution.

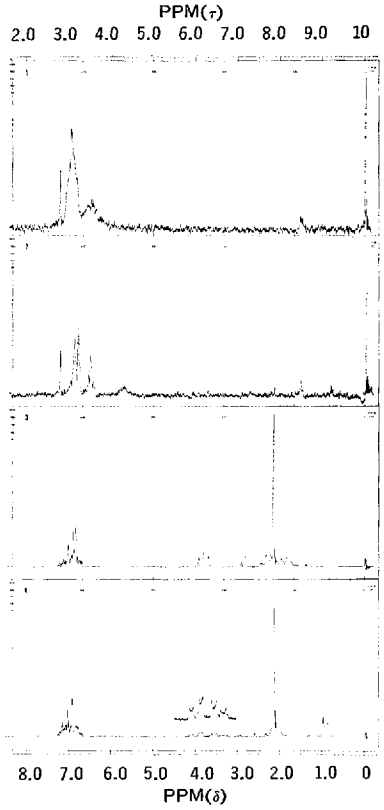


Fig. 8.—NMR spectra 1–4.

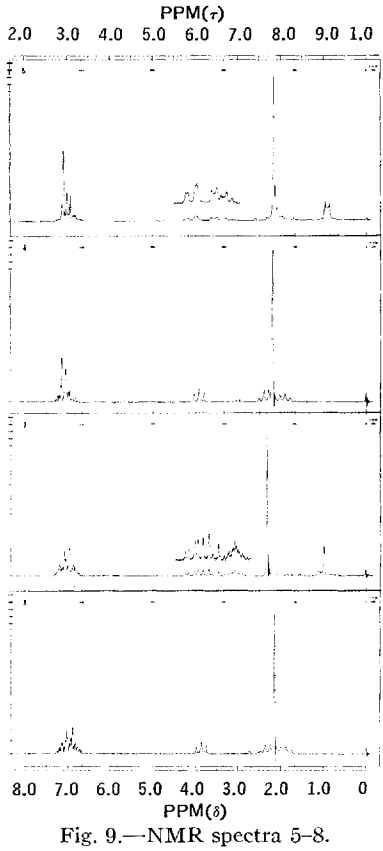


Fig. 9.—NMR spectra 5–8.

Phenothiazines with no substituent in the 2-position all show very strong absorption in the 720–770 cm.$^{-1}$ range which is assignable to the out-of-plane bending vibrations of the 4 adjacent hydrogens of the phenothiazine ring system (1).

Two other characteristic bands for the phenothiazine system are found at 1590 and 1560 cm.$^{-1}$. These bands are quite consistent in location, although they vary as to which is the more intense. They also may be assigned to the aromatic system.

In the case of phenothiazine amine salts (spectrum No. 24, chlorpromazine HCl) a strong, broad band centered between 2300 and 2500 cm.$^{-1}$ is characteristic of the R_3NH^+ ion combined with X^-. The relatively small negative ion X^- can approach the amine cation R_3NH^+ from only one direction, forming the ion pair $R_3NH^+X^-$ with the hydrogen atom bound strongly to the negative ion. Water of hydration tends to raise the frequency and lower the intensity of the absorption (2).

Two of the important oxidation products of phenothiazines are the sulfoxide and sulfone. The presence or absence of these may be determined by examining the spectra for S=O and SO_2 bands. The single S=O band occurs at 1000 cm.$^{-1}$ (±20). The —SO_2 asymmetric stretching band occurs at 1250–1300 cm.$^{-1}$ and the symmetric stretching band at 1140–1160 cm.$^{-1}$. All exhibit strong absorption bands.

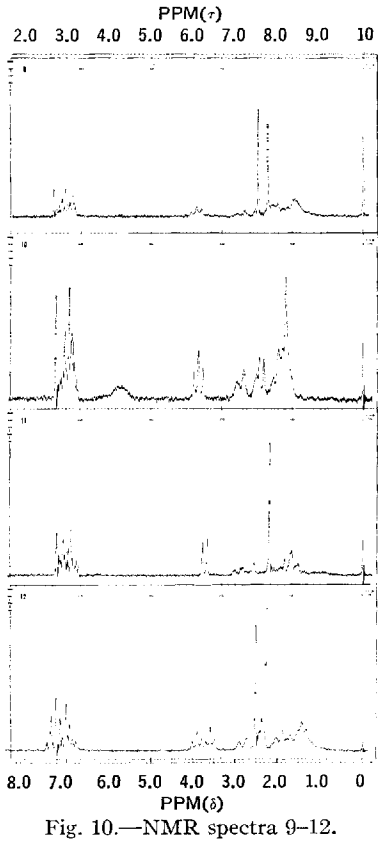


Fig. 10.—NMR spectra 9–12.

Ultraviolet Spectra.—The ultraviolet spectra of the phenothiazines are characteristic both in wavelength and in intensity. Two peaks are observed, the first and most intense in the range 250–265 mμ and the second in the range 300–325 mμ. The exact location of the peaks in both regions is dependent upon the nature of the group in the 2-position. Halogen substituents such as chloro- or trifluoromethyl appear to exert a slight influence in the form of small bathochromic shifts of 2–4 mμ on the more intense peak in the 250–265-mμ region. The trifluoro effects a slightly stronger shift than the chloro analog. It has been further noted that the alkyl side chain containing an amine group causes slight shifts in the peak locations, and the amount of shift is related to the length of the side chain, which is to say the proximity of the amine group to phenothiazine nucleus. The amine group has been found to exert a slight influence even when located at the end of a 4-carbon chain.

Phenothiazines with a carbonyl group in the 2-position are exceptions to the general rule regarding location of the 3 ultraviolet peaks. This type of phenothiazine exhibits strong absorption peaks in the range 240–245 mμ and 275–285 mμ.

Because of the distinctive ultraviolet spectrum and the intense absorption detectable at low concentrations (0.015 mg./ml.), it is possible to make a qualitative determination quickly and with a minimum amount of sample.

The presence or absence of the sulfone and sulfoxide decomposition products is easily ascertained by examining the ultraviolet spectrum. The spectrum of sulfone or sulfoxide is quite different and easily detected (Fig. 7).

Nuclear Magnetic Resonance.—For NMR study (Figs. 8–13) and correlations the phenothiazine spectra can be separated into 5 groups: 4 groups according to the type of side chain on the nitrogen in the 10-position, a fifth group consisting of the oxidation products (sulfone and sulfoxide). The groups to be considered are: (*A*) those having an aliphatic side chain with no heterocyclic ring; (*B*) those having a side chain containing a piperazine ring; (*C*) those having a side chain containing a piperidine ring; (*D*) those containing a pyrrolidine ring; and (*E*) oxidized phenothiazines.

Group A.—All of the compounds belonging to group *A* have features in common. All contain an *N,N*-dimethyl group, all have a CH_2 group attached to the 10-position of the phenothiazine ring, and, of course, all contain aromatic protons from the phenothiazine nucleus itself. It is, therefore, not surprising that their NMR spectra are similar in many respects, and have characteristic absorbance bands unique to their general group. The *N,N*-dimethyl group is found at 131 c.p.s. for the compounds with straight chain amines and at 138 c.p.s. in the case of branched chain amines. The position of the branched chain amine is influenced by its proximity to the ring N. The CH_2 group on

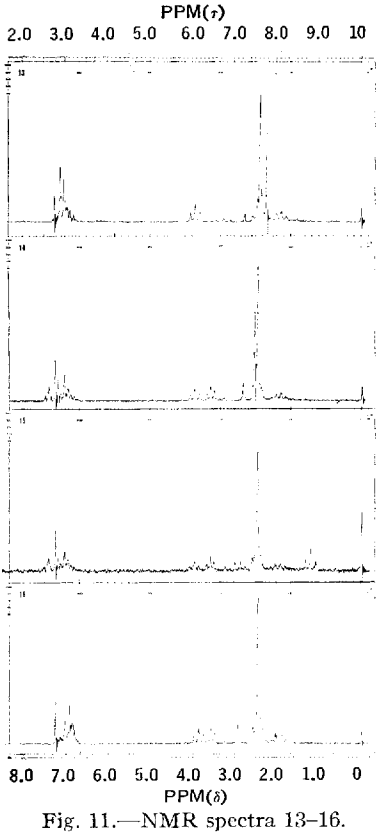


Fig. 11.—NMR spectra 13–16.

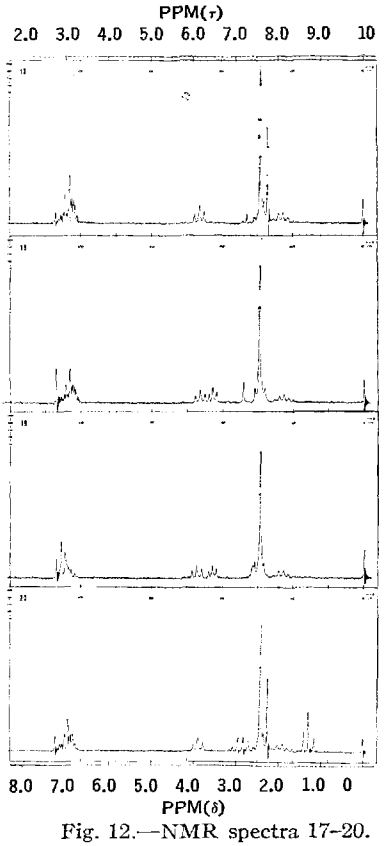


Fig. 12.—NMR spectra 17–20.

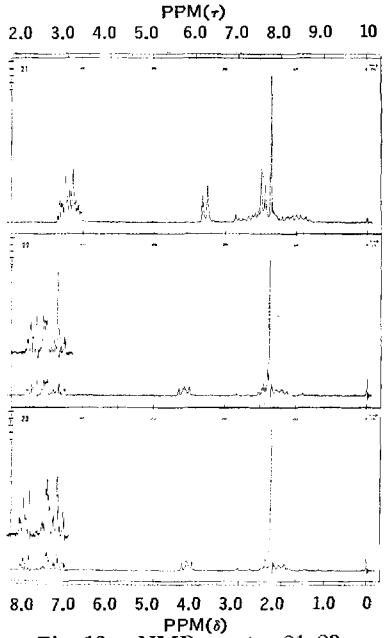


Fig. 13.—NMR spectra 21–23.

the nitrogen atom in the 10-position is found to be a triplet at 232 ± 3 c.p.s. for straight chain amines and in the same area but with more splitting in the case of branched chain amines due to the increased number of adjacent protons. All of the phenothiazine spectra show a complex pattern for the aromatic protons in the area 400–440 c.p.s. (3).

Group B.—Phenothiazines having a piperazine ring in their side chain have common features attributable to their specific group which distinguish them from other phenothiazines. The 8 protons in the piperazine ring being equivalent are found as a single peak at 146 ± 2 c.p.s. This is the strongest peak and the dominant feature of a phenothiazine in this group. The CH_2 attached to the N in the 10-position is found at 234 ± 4 c.p.s. In those piperazine derivatives where the piperazine ring is attached directly to a CH_2 in the chain (6 of the 9 shown) the CH_2 adjacent to the piperazine is located at 215 c.p.s. The complex aromatic pattern is located at 400–450 c.p.s.

Group C.—The phenothiazines containing a piperidine group in the side chain have chemical shifts due to the piperidine ring in the range 90–130 c.p.s., depending on the position and nature of the substitutent. The CH_2 attached to the N at the 10-position is again found at 235 c.p.s. with 1 exception, where the CH_2 is the only link between the N and the piperidine ring. In this case the CH_2 is a doublet located at 220 and 227 c.p.s. The aromatic pattern is found at 400–450 c.p.s.

Group D.—The group containing a pyrrolidine ring shows a chemical shift in the range of 100–180 c.p.s. due to the substitution on the ring. The CH_2 which links the N at 10-position and the pyrrolidine ring is a doublet at 224 and 231 c.p.s.

Group E.—This group contains the sulfoxide and sulfone of chlorpromazine. The effect of the introduction of oxygen into the system is an increase in chemical shift as compared with chlorpromazine. The $N(CH_3)_2$ is shifted 4–6 c.p.s. to 135–137 c.p.s. The CH_2 in position 10 is shifted to 254–256 c.p.s. and the aromatic protons show additional splitting and shift to 490 c.p.s.

SUMMARY

Infrared, ultraviolet, and nuclear magnetic resonance spectral data of phenothiazine compounds have been presented and correlated. On the basis of the information presented here, it is possible to make a rapid identification with a minimum amount of sample of any of the commercially available phenothiazines used in pharmaceutical preparations.

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Drug Absorption from the Rectum II

Rates of Absorption and Elimination from the Blood for Various Suppository Bases

By JOSEPH F. BORZELLECA and WERNER LOWENTHAL

To facilitate the calculations involved in Teorell's equation No. 25, a program in Fortran with Format was used in the IBM 1410 digital computer system. Using plasma salicylate levels, the rates of drug absorption and elimination from the blood and the specific apparent body volumes were determined for salicylic acid and sodium salicylate administered in 4 different suppository bases. The rate of absorption for sodium salicylate was found to be greater than that for salicylic acid except for polyoxyethylene sorbitan monostearate base. The rates of elimination from the blood for the 2 drug forms do not vary significantly. The specific apparent volumes for salicylic acid were less than that for the sodium salt, suggesting a lesser degree of storage for the acid form. Since Teorell's equation assumes apparent first-order rates of absorption and elimination from the blood, and this equation describes the data presented, this suggests that salicylic acid and its sodium salt are absorbed into the blood from the 4 bases tested and eliminated from the blood by apparent first-order kinetics. Two sets of data from the literature were also analyzed, and constants for 2 other sets could not be computed because of limitations on the computer.

GARRETT *et al.* (1) used an analog computer to solve a four-factor linear homogeneous equation of exponentials to explain the disappearance of ^{47}Ca from the blood. These authors (2) then programmed a distributive model on the analog computer to determine rate constants.

Riegelman and Crowell (3) using a mathematical solution for radial diffusion, derived equations to explain the absorption of radioactive compounds from the rectum. From the data collected on rats using solutions of ^{131}I compounds (NaI, CHI_3, and triiodophenol), the authors derived an equation which in its simplified form is:

$$\log N - N_f/N_0 - N_f = -kt \qquad \text{(Eq. 1)}$$

where

N = the dose detected at time t,
N_f = the dose detected at the end of the experiment,
N_0 = the total dose administered, and k was characterized as a pseudo first-order constant which included the diffusion coefficient, absorption constant, and any differences due to formulation.

No rate constants to characterize absorption through the rectal membrane, drug disappearance rate from the blood, or rates of elimination were determined.

Teorell (4) originally derived a general equation (equation No. 25) on a theoretical basis to describe the absorption and elimination of drugs administered by routes other than the intravenous. This equation involves 5 variables, 2 of which can be determined experimentally, and 3 are unknown. Because of the difficulty in solving this equation, it has been used only rarely. This could explain why much of the recent work concerned with the kinetics of drug absorption, distribution, and excretion has resulted in derivation of specific equations with limited application. However, the availability of computers makes solving the equation of Teorell a less formidable task.

Recently, Wiegand and Sanders (5), using blood levels determined for 2 drugs given orally as solutions to dogs, or aspirin tablets administered to humans, solved Teorell's equation with a digital computer. A program in Fortran without Format was written to solve the equation for absorption rate constant, drug elimination rate constant from the blood, and specific apparent body volume.

It is the purpose of this paper to apply the equation and a modification of the program presented by Wiegand and Sanders (5) to suppository dosage form, and to show that Teorell's equation combined with computer technology has wide application.

RESULTS AND DISCUSSION

In the work reported here, the program in Fortran without Format was modified to Fortran with Format for use in the IBM 1410 digital computer system. The explanation of the various steps were discussed by Wiegand and Sanders (5). By

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Previous paper: Lowenthal, W., and Borzelleca, J. F., *J. Pharm. Sci.*, **54**, 1790(1965).

TABLE I.—COMPUTER CALCULATED CONSTANTS FOR DATA PRESENTED BY LOWENTHAL AND BORZELLECA (6)

Suppository Base and Drug[a]	$k_a \pm$ (S.E.)[b]	$k_d \pm$ (S.E.)	$V_d \pm$ (S.E.)
Cocoa butter SA	1.275 (0.487)	0.0293 (0.0531)	0.542 (0.108)
Cocoa butter NaS	2.199 (0.377)	−0.0368 (0.0115)	0.814 (0.052)
PEG SA	1.045 (0.105)	−0.0080 (0.0132)	0.697 (0.038)
PEG NaS	1.970 (0.376)	0.0340 (0.0172)	3.342 (0.269)
S-55 SA	0.812 (0.166)	0.0564 (0.0341)	0.359 (0.047)
S-55 NaS	1.245 (0.195)	0.0318 (0.0160)	0.851 (0.067)
PSMS SA	0.927 (0.324)	0.0607 (0.0585)	0.484 (0.103)
PSMS NaS	0.352 (0.530)	0.210 (0.351)	1.211 (1.653)

[a] PEG is polyethylene glycol mixture; S-55 is a synthetic mixture of glycerides; PSMS is polyoxyethylene sorbitan monostearate. SA is salicylic acid and NaS is sodium salicylate. *Reference 6* gives a complete description of the bases. [b] S.E., standard error.

use of the computer program, plasma, serum, or blood drug levels can be used to determine absorption rate constants, drug disappearance rate constants from the blood, and specific apparent body volumes.

The equation to be fitted to the plasma concentration data is:

$$C = \frac{k_a}{V_d(k_a - k_d)} a_0 (e^{-k_d t} - e^{-k_a t}) \quad \text{(Eq. 2)}$$

where

a_0 = the drug dose in mg./Kg.,
C = the plasma, serum, or blood drug concentration in mcg./ml.,
t = time in hours,
k_a = the apparent first-order absorption rate constant in hr.$^{-1}$,
k_d = the apparent first-order drug disappearance rate constant from the blood in hr.$^{-1}$, and
V_d = the specific apparent volume of drug distribution in L./Kg.

The plasma salicylate levels presented in our previous communication (6) were averaged and used to determine k_a, k_d, and V_d. The use of averages has been shown to be an acceptable procedure (5). The initial estimates for k_a, k_d, and V_d, required as input data, were those determined by Wiegand and Sanders (5) for aspirin tablets, and were 1.55, 0.209, and 0.144, respectively. For sodium salicylate in PSMS base, using the data averaged for the 6 dogs, constants could not be calculated even after several attempts using various estimates of k_a, k_d, and V_d. The plasma levels of

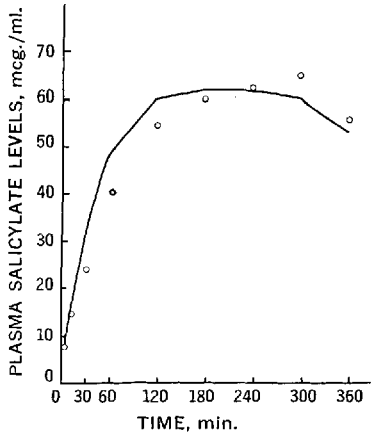

Fig. 1.—Plasma salicylate levels from salicylic acid in cocoa butter suppository base. Key: O, experimental points; —, theoretical curve.

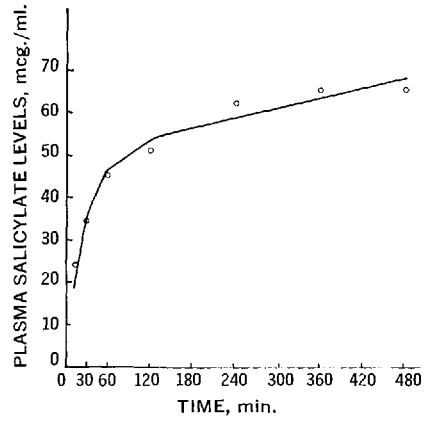

Fig. 2.—Plasma salicylate levels from sodium salicylate in cocoa butter suppository base. Key: O, experimental points; —, theoretical curve.

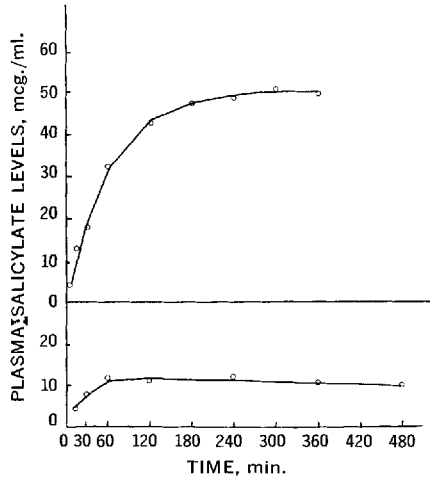

Fig. 3.—Plasma salicylate levels from sodium salicylate in PEG suppository base (bottom) and from salicylic acid in PEG suppository base (top). Key: O, experimental points; —, theoretical curves.

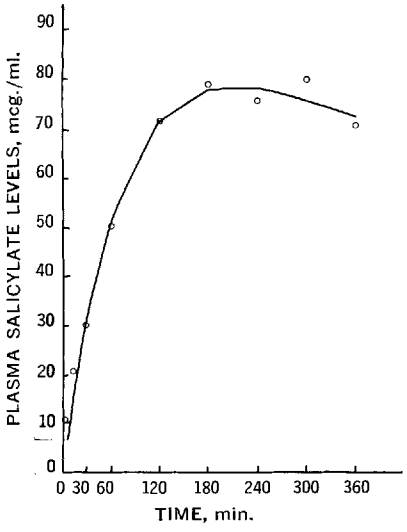

Fig. 4.—Plasma salicylate levels from salicylic acid in S-55 suppository base. Key: O, experimental points; —, theoretical curve.

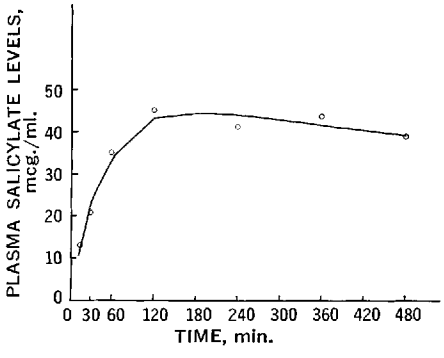

Fig. 5.—Plasma salicylate levels from sodium salicylate in S-55 suppository base. Key: O, experimental points; —, theoretical curve.

1 of the dogs were abnormally high when compared to the other 5 dogs. Constants were readily obtained using the averages obtained from 5 dogs. The calculated constants are given in Table I. Figures 1–6 show the curves for the calculated equation and the experimental data used.

Very few communications give 6 or more blood level concentrations with time data, so that demonstration of the usefulness of this equation and program is made more difficult. Blume and Nohara

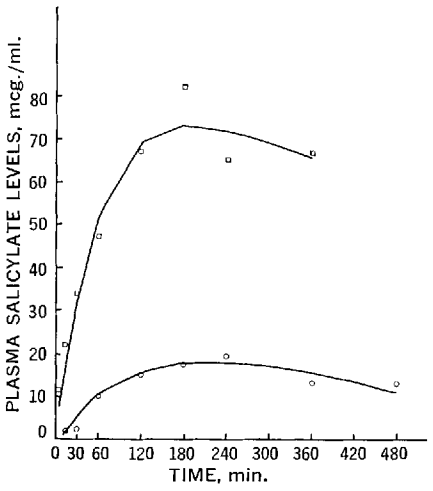

Fig. 6.—Plasma salicylate levels from salicylic acid (□) and sodium salicylate (O) in PSMS suppository base. Key: —, theoretical curves.

(7) gave sodium salicylate solutions orally and rectally to rabbits and determined plasma salicylate levels. From their data the constants were calculated and are shown in Table II; equation and data are plotted in Fig. 7. The same estimates for k_a, k_d, and V_d as listed above were used. Fincher *et al.* (8) administered orally to dogs capsules containing 3 different particle size distributions of sulfisoxazole. The drug in experiment *A* had an average diameter of 1.7 μ; in experiment *B*, 37 μ, and experiment *C*, 95 μ. The constants calculated from these data are shown in Table II and the equations and data plotted in Fig. 8. The estimates of k_a, k_d, and V_d used were 2.30, 0.254, and 3.74, respectively. The data from Swintosky *et al.* (9), and Walkenstein *et al.* (10) had sufficient number of observations and appear to follow Eq. 2. The computer was not able to calculate the constants because it was unable to handle power functions greater than 99. In the power function e^{-kt}, when t becomes large, k does not have to be very large for e to be raised to the 99th power.

In the previous communication (6), it was reported that salicylic acid resulted in higher plasma levels than the sodium salt, but it can be seen from the constants listed in Table I that the salicylic acid is absorbed at a slower rate, except in the case of PSMS base. The rates of elimination from the blood for the 2 forms do not vary significantly. The specific apparent volume for salicylic acid was less than that for the sodium salt, suggesting a

Table II.—Computer Calculated Constants for Data Presented in the Literature

Source and Ref.	$k_a \pm$ (S.E.)[a]	$k_d \pm$ (S.E.)	$V_d \pm$ (S.E.)
(7), Oral	5.930 (2.371)	0.0965 (0.0307)	134.004 (10.972)
(7), Rectal	2.767 (0.741)	0.179 (0.044)	101.643 (10.234)
(8), Expt. *A*	0.709 (0.143)	0.262 (0.048)	0.598 (0.086)
(8), Expt. *B*	0.686 (0.209)	0.165 (0.046)	0.879 (0.172)
(8), Expt. *C*	0.673 (0.203)	0.0493 (0.0249)	1.483 (0.250)

[a] S.E., standard error.

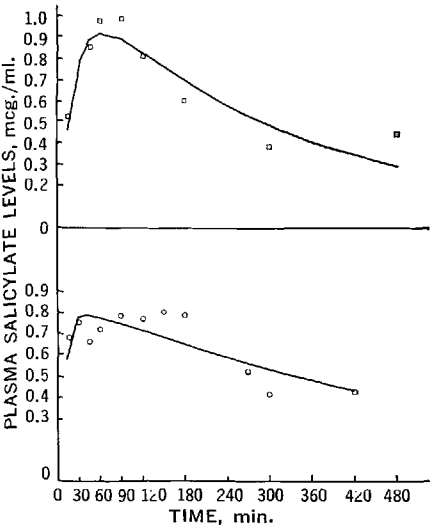

Fig. 7.—Plasma salicylate levels from sodium salicylate solutions (7). Key: ○, oral administration; □, rectal administration; —, theoretical curve.

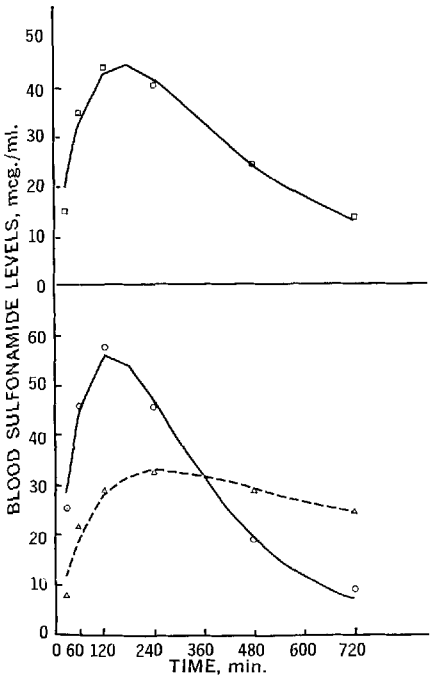

Fig. 8.—Plasma sulfonamide levels from oral administration of sulfisoxazole capsules (8). Key: ○, capsule *A*; □, capsule *B*; △, capsule *C*; — and - - -, theoretical curves.

lesser degree of storage for the acid form. This may be due to the slower rate of absorption resulting in less time for storage before excretion. The negative elimination rates obtained with sodium salicylate in cocoa butter and salicylic acid in PEG base indicate that the plasma levels were still rising at the time the experiment was stopped.

Equation 2 assumes apparent first-order rates of drug absorption and elimination from the blood. This equation describes the data presented, suggesting that salicylic acid and the sodium salt are absorbed into the blood from the 4 bases tested and eliminated from the blood by apparent first-order kinetics.

The constants presented in Table II indicate that sodium salicylate was absorbed at a faster rate orally than rectally, and eliminated from the blood at a slower rate. The large V_d indicates a large amount of the drug is stored in tissue. The constants calculated from the data of Fincher *et al.* (8) show that as average particle diameter increases there is a slight and probably nonsignificant decrease in rate of absorption, a decrease in rate of elimination from the blood, and an increase in specific apparent body volume.

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Gas Chromatographic Determination of Codeine in Serum and Urine

By EMANUEL SCHMERZLER, WILLIAM YU, M. I. HEWITT, and I. J. GREENBLATT

A method for gas chromatographic detection of codeine in serum is described. An application to a clinical drug evaluation study is given. A method for simultaneous detection of free and total codeine and its metabolites, norcodeine and morphine, in urine is also presented.

USE OF GAS-LIQUID chromatography as a means of comparing serum levels in subjects receiving oral codeine dosages has been investigated. Although an efficient SE30 column enabled detection of nanogram quantities, codeine was not resolved from norcodeine on this nonpolar phase, as previously reported by Anders and Mannering (1). Elliot *et al.* (2) used the acetates formed inside the column (1) to detect higher levels present in urine of narcotic addicts. The authors found small interfering peaks present in blanks which precluded the use of codeine acetate for reliable quantification of low levels present in serum. However, there is a great difference in reaction rate between codeine and norcodeine under mild acetylation conditions. This was used to remove norcodeine before injection on SE30 columns. The specificity and accuracy of the results were checked, using an XE60 column on which free codeine and norcodeine are well resolved.

A method for the estimation of codeine and its metabolites in urine is also described.

METHODS

Codeine in Serum or Urine.—To 2.5 ml. of serum or urine in a 15-ml. glass-stoppered conical centrifuge tube is added 9 ml. of 10% butanol in freshly distilled chloroform (3). The tube is hand shaken 5 sec. Then 0.12 ml. of 16 *N* KOH is added (0.05 ml. for urine). Again mix by hand 5 sec., then mechanically shake in 10 min. After centrifuging, the aqueous serum and jell layers are aspirated. If jell formation is excessive, the aqueous layer is aspirated and the jell broken by vigorous reshaking before recentrifuging. A 7-ml. aliquot of the organic layer is transferred to a clean centrifuge tube. Care must be taken not to transfer any of the strongly basic aqueous layer. A quantity of 1.5 ml. of 0.01 *N* H_2SO_4 is added. Shake 10 min. Mechanical shaking for 3 min. is followed by centrifuging and transfer of the acid layer to a new 15-ml. tube. Quantities of 0.04 ml. of 16 *N* KOH and 8 ml. of distilled chloroform are added. Shake 10 min. After centrifuging, remove the aqueous layer. Dry the chloroform extract with anhydrous sodium sulfate. Evaporate to dryness with a stream of nitrogen at 60° in 2-ml. conical centrifuge tubes. Twenty microliters (50 μl. for urine) of an internal standard solution, 0.1 mg./ml. of cholesterol acetate in ethyl acetate, is added just prior to injection of 2 μl. of this mixture.

On SE 30, codeine and norcodeine are not resolved and peaks observed represent any contributions from either compound. To determine codeine alone, a trace amount of acetic anhydride is added

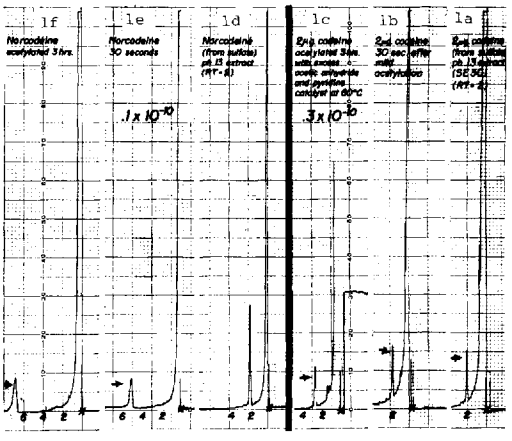

Fig. 1.—Norcodeine removal technique (SE30). Key: (1a) codeine; (1b) codeine injected 30 sec. after mild acetylation; (1c) codeine after acetylating 3 hr. at 60° with pyridine catalyst; (1d) norcodeine; (1e) norcodeine after 30 sec. acetylation; and (1f) norcodeine after 3 hr. acetylation. Note the retention time of codeine = norcodeine = 2 min.

TABLE I.—RETENTION OF RESINATED AND NONRESINATED PREPARATIONS

Retention Times	XE60 (240°) min.	SE30 (240°) min.
Codeine	2.2	2.0
Norcodeine	2.8	2.0
Codeine acetate	2.8	2.6
Morphine diacetate	5.1	3.4
Norcodeine-*N*-acetamide	16.0	5.0
Norcodeine-*N*-acetamide-6-acetate	19.0	6.8
Cholesterol acetate	6.7	16.0
Cholestane	1.6	5.6

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TABLE II.—TOTAL CODEINE AND NORCODEINE, RELATIVE UNITS (SE30)

Subject and Age	15 mg. Resinated Codeine Time, hr. 2	5	7	9	11	15 mg. Nonresinated Codeine Time, hr. 2	5	7	9	11
A, 37	3.6	1.6	0.5	0.3	0.1	1.4	1.6	0.5	0.4	0.2
B, 21	6.4	3.6	2.4	0.4	0.1	6.4	4.4	3.6	2.0	0.4
C, 23	0.8	5.0	1.2	0.6	0.4	3.2	2.0	1.6	0.4	0.1
D, 24	4.8	1.6	0.5	0.5	0.3	5.6	1.2	0.5	0.2	0.1
E, 46	5.2	2.7	0.9	0.6	0.1	4.8	4.0	1.9	1.0	0.6
Av.	4.2	2.9	1.1	0.5	0.2	4.3	2.6	1.6	0.9	0.3

TABLE III.—SERUM CODEINE (mcg./100 ml.) AFTER NORCODEINE REMOVAL ON SE30

		Codeine				Codeine	
		Time, hr.				Time, hr.	
Subject	Medication	2	5	Subject	Medication	2	5
B	Resinated	2.8	1.2	*B*	Nonresinated	3.3	2.2
E	Resinated	3.6	1.2	*E*	Nonresinated	2.6	1.3

TABLE IV.—SERUM CODEINE (mcg./100 ml.) ON XE60

		Codeine				Codeine		
		Time, hr.				Time, hr.		
Subject	Medication	2	5	9	Subject	Medication	2	5	9
B	Resinated	3.6	1.2	0.1	*B*	Nonresinated	3.6	2.8	0.4

(1 μl. of a 1/50 solution of acetic anhydride in ethyl acetate for serum or 1/10 for urine). The mixture is stirred and 2 μl. injected into the chromatograph within 30 sec. (Fig. 1). Norcodeine may be estimated by difference if an injection before acetylation is made first. Suitable standards and blanks are included with each analysis.

On XE60 the 2 alkaloids are resolved as free compounds, and acetylation is unnecessary.

Codeine and Metabolites in Urine.—Three milliliters of urine is brought to pH 9 with 0.3 Gm. of sodium bicarbonate and 0.3 ml. of 10 *N* sodium hydroxide. Nine milliliters of 10% butanol in chloroform is added and, at this point, the procedure continues as described above through the extraction at pH 2 into dilute sulfuric acid. The acid layer is adjusted to pH 13 with 0.05 ml. of 10 *N* sodium hydroxide. Codeine and norcodeine are extracted into 8 ml. of distilled chloroform. The aqueous layer containing morphine sulfate is completely transferred to another tube, adjusted to pH 9 with 0.2 Gm. of sodium bicarbonate and extracted 10 min. with 8 ml. of chloroform containing 1% ethanol. After centrifuging, the aqueous layer is discarded. Both organic layers are dried with anhydrous sodium sulfate and evaporated to dryness under nitrogen at 60° in 2-ml. centrifuge tubes. The codeine and norcodeine may be chromatographed by dissolving the extract in 50 μl. of internal standard solution and injecting 2 μl. on the XE60 column.

Free morphine tails excessively on XE60 or SE30 and must be acetylated completely to chromatograph well. Acetylation of the pH 9 extract containing free morphine is carried out by adding 0.1 ml. of a mixture of acetic anhydride, pyridine, and ethyl acetate (1:1:1) and warming about 2 hr. at 60°. The solution is evaporated to dryness and dissolved in 50 μl. of internal standard solution.

Total codeine, norcodeine, and morphine are obtained by first hydrolyzing with acid. Concentrated HCl (10% by volume) is added to 3 ml. of urine and the sample placed in a boiling water bath for 1 hr. (4). As a check, or if the presence of occasional interfering compounds compel it, the codeine and norcodeine in the pH 13 extract may be completely acetylated as for morphine.

Gas chromatographic data were obtained on a Jarrel-Ash instrument model 28-710.

SE30 column: 6-ft., 4-mm. i.d., silanized glass column with 5% (w/w) SE30 on acid washed 80/100 mesh Gas-Chrom P. Temperatures: injector, 260°; detector, 260°; column, 240°. Nitrogen carrier flow rate = 70 ml./min. measured at room temperature. Theoretical plates = 1600.

XE60 column: 4 ft., 4-mm. i.d., silanized glass column with 4% (w/w) XE60 on acid washed 90/100 mesh Anakrom A. Temperatures: injector, 260°; detector, 260°; column, 240°. Nitrogen flow = 70 ml./min. measured at room temperature. Theoretical plates = 1500.

The liquid phases were applied by filtration technique (5).

RESULTS

The objective was to determine if a 15-mg. resinated oral codeine preparation[1] would show either slower absorption or last longer in human blood when compared with a nonresinated codeine preparation of the same composition except for absence of resin complexing. Five healthy male volunteers were tested. After 10 days, those individuals who received the resinated material then received the nonresinated material in the same dosage and vice versa. (Table I.)

All 10 blood series were examined for total (unresolved) codeine and norcodeine values on an SE30 column. The peaks were calculated, arbitrarily, using codeine standards in micrograms per 100 ml. of serum. In Table II, these results are recorded

[1] Strional which contains 15 mg. of codeine base as cation exchange resin complex, 10 mg. of methaqualone as cation exchange resin complex, 162 mg. of acetylsalicylic acid U.S.P. Strional was supplied by the Strasenburgh Laboratories, Rochester, N. Y.

TABLE V.— URINARY CODEINE, NORCODEINE, AND MORPHINE ON XE60 (SUBJECT *I*)

Time, hr.	Vol.	pH	Codeine		Norcodeine		Morphine	
			Free	Total	Free	Total	Free	Total
			mcg./100 ml.		mcg./100 ml.		mcg./100 ml.	
4	35	7	40	70	70	170	5	40
11	320	7.5	30	90	75	330	20	100

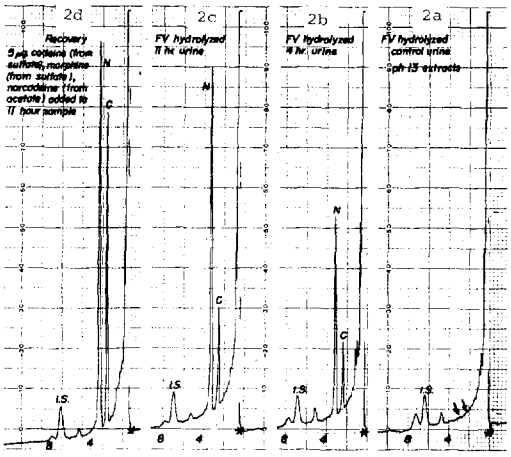

Fig. 2.—Shows hydrolyzed (FV, subject *I*) urine extracted at pH 13 for total codeine (*C*) and norcodeine (*N*). Cholesterol acetate = internal standard (*I. S.*). Key: (2a) control; (2b) 4 hr. after ingestion of resinated codeine; (2c) 11 hr.; (2d) recovery. (XE60.)

in relative units. The averages obtained using resinated or nonresinated preparations are about the same. The specific determination of codeine at key time periods was made in 2 of the individuals (*B* and *E*) by the technique of norcodeine removal on SE30 (Table III). In one case values for free codeine were also checked on an XE60 column (Table IV). Codeine levels for these individuals show the same essential hourly pattern as for the combined alkaloids. In all methods, results were read from standard curves, since these were not quite linear at serum levels. Recoveries of added codeine ranged from 70–140% and averaged 105%.

Codeine Metabolites in Urine.—The method for codeine and metabolites was used to study the urine of a female patient (Table V). After 11 hr., the conjugated forms of all 3 alkaloids are present in greater concentrations than at 4 hr. Figure 2 shows chromatograph for total codeine and norcodeine in this individual.

The urines of 4 patients receiving resinated codeine and 1 patient receiving free oral codeine preparation were examined after 3.5 hr. by norcodeine removal technique on SE30. The data presented in Table VI indicate wide variations in the ratio of codeine to norcodeine.

TABLE VI.—URINARY CODEINE AND NORCODEINE BY NORCODEINE REMOVAL TECHNIQUE ON SE30

Subject	Medication	Codeine, mcg./100 ml.	Norcodeine,[a] mcg./100 ml.
F	Resinated	570	70
G	Resinated	160	90
H	Resinated	220	70
I	Resinated	30	65
J	Nonresinated	260	100

[a] Calculated using standards made by dissolving authentic norcodeine in chloroform. Results based on norcodeine salts carried through extraction procedure give results about 10 times greater.

SUMMARY

Methods are presented for gas chromatographic analysis of codeine in serum and its metabolites in urine. In a comparison of 2 oral codeine preparations, no significant difference in serum levels between resinated and nonresinated codeine was found. Over-all precision of the methods average 100 ± 20% in serum with better precision at the higher levels in urine.

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Toxicology of a Series of Phthalate Esters

By DAVID CALLEY, JOHN AUTIAN, and WALLACE L. GUESS

Certain types of plastic materials require the addition of appreciable quantities of plasticizers to impart specific physical-chemical properties to the final item. Presently, many vinyl devices used with drug products may contain plasticizers of the phthalate type. For this reason, a series of phthalic acid esters were evaluated for parenteral toxicity including LD_{50} values and hexobarbital narcosis. Experiments utilized in the study also included i.p. injections in mice for acute toxicity profiles, i.v. administration in rabbits for blood pressure and respiration effects, and intradermal injections into rabbits for irritation effects. A further study was conducted to note what effects repeated i.p. doses of the phthalates would have on mice over a period of time, examining the effects on organs, weight gain, and the blood. Finally, tissue culture experiments were conducted to attempt to correlate certain of the toxicity manifestations. The most salient feature of the toxicity of these compounds was the central depression on the animals and the rather low order of toxicity by the parenteral route of administration.

CERTAIN plastic materials (*e.g.*, polyvinyl chloride) require the introduction of an appreciable quantity of plasticizers to impart specific physicochemical and mechanical properties. In medical practice, these materials are used as bags, tubings, vehicles for administration, and collection or storage devices. A high degree of safety must be established and maintained in order to prevent any adverse effect when and if an ingredient from the plastic device migrates into a parenterally administered product. Previous reports from this laboratory have indicated that certain plastic devices used medically can release one or more ingredients into tissue or solvent systems (1–3). Furthermore, these "migrated" ingredients, when in sufficient concentrations, can elicit definite pharmacological responses. One study by Guess, Autian, and Meyers (4) demonstrated that a group of citric acid esters used as plasticizers for vinyl formulations produced definite toxicological effects when administered by parenteral routes. These same compounds, however, were extremely safe even in large quantities when administered orally to animals.

Presently, one of the most commonly used groups of plasticizers is the phthalic acid esters. These and related compounds generally have low volatility (5), low water solubility (6), some degree of absorption through the skin, and toxicity when taken into the body by the inhalation of vapors or by the oral route (7–9). Little information, however, is available as to the degree of toxicity or modes of actions of the phthalic acid esters when they are introduced parenterally (10, 11). This paper reports a series of biological experiments in which 8 of the esters were evaluated as to parenteral toxicity to mice and rabbits and *in vitro* toxicity to cell cultures.

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EXPERIMENTAL

Materials

Dimethyl phthalate, diethyl phthalate, dibutyl phthalate, di-isobutyl phthalate, di-(methoxyethyl) phthalate (Eastman Chemical Products, Inc.), butyl benzyl phthalate (Monsanto Chemical Co.), di-(2-ethylhexyl) phthalate (Eastman Chemical Products, Inc.), and dicapryl phthalate (Harchem Division, Wallace & Tiernan, Inc.).

All of the compounds were commercial products. Thin-layer chromatography and infrared analysis demonstrated that each of the samples had a relatively high degree of purity.

Methods

Acute Toxicity.—*LD_{50} Determinations.*—These were conducted according to the method and tables described by Thompson and Weil (12) and Weil (13). Each phthalate was administered intraperitoneally in Swiss Webster white mice of uniform weight and age in 4 dosage levels ranging from 0.5 Gm./Kg. to 16 Gm./Kg. Dosage levels were spaced in geometric progression, increasing by a factor of 2.

Effect of Phthalate on Hexobarbital Narcosis.—This study was conducted to measure acute CNS stimulation or depression following sublethal intraperitoneal administration of emulsified phthalates. Groups of 10 white mice weighing 14 to 20 Gm. were administered 500-mg./Kg. doses of the phthalate esters and, after an interval of 30 min., injected i.p. with 60 mg./Kg. of sodium hexobarbital. A control group of 10 mice received an equivalent volume of 3% acacia in place of the phthalate.

Rabbit Intradermal Irritation Tests.—Phthalate emulsions in concentration of 100 mg./ml. were injected 0.2 ml. intradermally into the cleanly shaven backs of rabbits. Inflammatory response at the injection site was measured by injection of 1 ml./Kg. of 1% trypan blue into the marginal ear vein after an interval of 15 min. (14, 15).

Acute Intravenous Toxicity.—Effects of phthalate emulsions on rabbit blood pressure, respiration rate, electrocardiogram pattern, and electroencephalogram pattern were recorded on the Grass Polygraph. Rabbits were anesthetized with approxi-

mately 33 mg./Kg. of sodium pentobarbital. Phthalates were emulsified in buffered 3% acacia solution and administered in repeated doses of 50 mg./Kg. through the cannulated external jugular vein directly into the heart. Blood pressure changes were recorded *via* the cannulated common carotid artery with the Statham transducer. Respiratory changes were determined from the cannulated trachea connected to a low pressure transducer (model PT 5A) through the Grass recorder. The EEG was taken using occipital leads, and the EKG taken from leads inserted subdermally in the limbs and chest. Two rabbits receiving only 3% acacia in equivalent volumes served as controls for record comparison.

Tissue Culture.—Toxicity of the phthalates to strain L 929 mouse fibroblast cells and to chick embryo cells was tested according to a method developed in our laboratory (16). Porous pads were wet with 0.05 ml. of a 50 mg./ml. emulsion of phthalates. The pads were placed on the surface of the agar and the cultures observed over a 48-hr. period for appearance of cell mortality.

Subacute Toxicity Studies.—These studies were conducted using 4 phthalic acid esters: diethyl, di-(methoxyethyl), butyl benzyl, and di-(2-ethylhexyl). From 20 to 30 white mice of uniform initial weight and age were injected daily with an emulsion of each phthalate for a period of 6 weeks. Dosage levels ranged from 125 mg./Kg. to 500 mg./Kg., depending on the toxicity or tolerance level for each. Weight gains were recorded weekly. Organ-to-body weight ratios for liver, kidney, lungs, heart, spleen, and testes were calculated at the end of the study and compared with normal values of a control group receiving only 3% acacia suspension. Pathology studies were made on certain of the organs using the standard formalin fixation and hematoxylin-eosin stains. Blood studies were made before injections began and again at the termination of the project before sacrifice. These studies included white and red cell counts, white cell differential counts, hematocrit, and hemoglobin levels.

RESULTS AND DISCUSSION

Acute Toxicity Studies

LD_{50} Determination.—The results of this study indicated that acute lethal i.p. toxicity of the phthalic acid esters studied was generally of a low order. LD_{50} values ranged from 1.58 Gm./Kg. for dimethyl phthalate to 14.19 Gm./Kg. for di-(2-ethylhexyl) and dicapryl phthalate (Table I). Although water solubility of all the compounds was extremely low, ranging from 1.5 Gm./100 Gm. to insolubility, a correlation appeared to exist between this factor and degree of toxicity indicated by LD_{50} values. The 3 esters with greatest solubility also exhibited greatest toxicity. As would be expected, an inverse relationship existed between toxicity and molecular weight of the compounds.

Effect of Phthalates on Hexobarbital Narcosis.—Sleeping time of the control group was 46 min. (Table II). Only 2 compounds gave indications of CNS stimulation as represented by significantly shortened sleeping time. These were di-(2-ethylhexyl) and dicapryl phthalate, each with a sleeping time of 36 min. These averages were significantly different ($p > 0.05$) from the control average. All other phthalates appeared to demonstrate CNS depression. Notable were diethyl, di-isobutyl, and butyl benzyl phthalates, with sleeping times of 88, 72, and 62 min. These 3 values were also significantly different ($p > 0.05$) from the control group.

TABLE I.—ESTIMATED LD_{50} VALUES FOR A SERIES OF PHTHALATE ESTERS

Phthalate Ester	i.p. LD_{50} in Mice, Gm./Kg.	95% Confidence Limits	Mol. Wt.	Solubility in Water, Gm./100 Gm.
Dimethyl phthalate	1.58	0.98 to 1.99	194	0.45
Diethyl phthalate	2.83	2.42 to 3.29	222	0.1
Dibutyl phthalate	4.00	2.94 to 5.45	278	Insol.
Di-isobutyl phthalate	4.50	3.36 to 6.02	278	0.01
Di-(methoxyethyl) phthalate	2.51	1.82 to 3.45	282	0.85
Butyl benzyl phthalate	3.16	2.51 to 3.98	312	Insol.
Di-(2-ethylhexyl) phthalate	14.19	12.62 to 15.76	390	0.01
Dicapryl phthalate	14.19	11.21 to 15.87	390	0.03

TABLE II.—HEXOBARBITAL SLEEPING TIME IN MICE

	Mice in Group, No.	Sleeping Time ± S.E., min.	CNS Effect: Depression	CNS Effect: Stimulation
Control group (0.5 ml. 3% acacia)	10	46 ± 1.66		...
Dimethyl phthalate	10	52 ± 1.15	+	...
Diethyl phthalate	10	88 ± 2.94[a]	+	...
Dibutyl phthalate	10	55 ± 2.18	+	...
Di-isobutyl phthalate	10	72 ± 2.83[a]	+	...
Di-(methoxyethyl) phthalate	10	50 ± 1.71	+	...
Butyl benzyl phthalate	10	62 ± 2.45[b]	+	...
Di-(2-ethylhexyl) phthalate	10	36 ± 1.14[b]	...	+
Dicapryl phthalate	10	36 ± 1.14[b]	...	+

[a] Indicates significant difference from control value ($p > 0.01$). [b] Indicates significant difference from control value ($p > 0.05$).

No satisfactory explanation for the evidence of CNS stimulation found for di-(2-ethylhexyl) and dicapryl phthalate could be elicited from these experiments, particularly in view of the fact that both of these showed a tendency to lower blood pressure at higher dose levels. The only supporting evidence of stimulation was seen in the EEG patterns after i.v. administration of dicapryl phthalate (see below). The order of the potentiation of narcosis followed the water solubility of the compounds. In view of the extremely lipid nature of the CNS, this order of narcosis potentiation was surprising. However, these compounds were administered as emulsions by the i.p. route, and from this route, the degree of absorption and consequently evidence of narcosis potentiation could have been due to a concentration at the site of action effect.

Rabbit Intradermal Irritation Tests.—Three phthalates—dimethyl, diethyl, and di-(2-ethylhexyl)—caused rapid and intense concentration of extravasated trypan blue at the injected sites (Table III), indicating marked inflammatory response.

TABLE III.—IRRITATIVE RESPONSE IN RABBITS TO INTRADERMAL INJECTIONS OF PHTHALATE ESTERS

Phthalate Ester	Degree of Extravasation[a] 10 min.	15 min.	26 min.
Dimethyl phthalate	++	+++	+++
Diethyl phthalate	+++	+++	+++
Dibutyl phthalate	+	+	++
Di-isobutyl phthalate	−	−	++
Di-(methoxyethyl) phthalate	++	++	++
Butyl benzyl phthalate	−	+	++
Di-(2-ethylhexyl) phthalate	+++	+++	+++
Dicapryl phthalate	−	+	+
0.85% NaCl (negative control)	−	−	−
20.00% EtOH (positive control)	+++	+++	+++

[a] Inflammatory response indicated by degree of dye extravasation; −, no color, negative reaction; +, mild; ++, moderate; +++, marked.

Others exhibited mild to moderate inflammatory response as indicated by low color intensities with greater lapses in time before appearance of the dye at the injection site. Dicapryl phthalate was the least irritating according to this test, showing only mild tissue response during the observation period. It should be noted that with the exception of di-(2-ethylhexyl) phthalate, the activity of these compounds as irritants was related to molecular weight.

Acute Intravenous Toxicity.—*Blood Pressure.*—With the exception of diethyl phthalate, none of the intravenously administered phthalates had dramatic or significant effect on the anesthetized rabbit blood pressure until a minimum total dose level of 350 mg./Kg. had been given. At this point, 4 of the phthalate compounds [diethyl, dimethyl, di-(2-ethylhexyl), and dicapryl] showed some depression of the blood pressure, indicating a vascular response to toxicity. In no case did the electrocardiograms or vector analysis of these results indicate direct cardiac toxicity. Therefore, the available evidence suggests an indirect cardiovascular toxicity at the higher i.v. dose levels. In the case of diethyl phthalate, each dose of 50 mg./Kg. administered i.v. caused a transient (about 3 min. duration) fall in blood pressure of about 20 mm. Hg, or approximately a 22% decrease. The blood pressure then gradually returned to the predose level, and no subsequent change ensued until administration of the next dose. A total dose of 650 mg./Kg. was given i.v. without death or other significant change in the animal, indicating a low order of toxicity. Administration of 5 doses of 3.0 ml. each of the vehicle (3% acacia) in control rabbits elicited no changes in blood pressure.

Respiration.—All of the phthalates administered i.v. caused an increase in the respiratory rate of the anesthetized rabbit. Since all were administered in a buffered (pH 7.0) emulsion over a period of about 2-3 min., the stimulation of respiration could not be attributed to a pH effect on the chemoreceptors. In 3 cases (dimethyl, diethyl, and diisobutyl phthalate), there were significant increases in respiratory rate after the administration of a total dose of 100 mg./Kg. The per cent increase for these 3 were 66.6, 71.2, and 114.3, respectively. The rate gradually returned toward normal over a period of about 5 min. It is of interest to note that these 3 compounds exhibited the highest order of toxicity in the LD_{50} study.

Electroencephalograms.—The electroencephalogram tracings obtained from occipital leads showed varying patterns after the administration of different phthalates intravenously. Figure 1 depicts several of the patterns seen both pre- and post-phthalate administration. The CNS depression pattern, obtained after administration of di-(methoxyethyl) phthalate, shows decreased frequency. The stimulation pattern, obtained after administration of dicapryl phthalate shows increased frequency, which appears to be a confirmation of the CNS stimulation exhibited in the hexobarbital narcosis study. It is also recognized that other influences such as anoxia, etc., may complicate EEG tracings, but no indication of these contingencies were noted in our experiments.

Tissue Culture Toxicity.—None of the phthalate emulsions demonstrated toxicity to chick embryo cells in the amounts used (0.05 ml. of a 50 mg./ml. emulsion). Three of the phthalates did show toxicity to mouse fibroblast cells (L-cells) in these same amounts. Microscopic examination of the dead cell zones revealed that the cells were intact and indicated absence of corrosive activity. Other results have shown that mouse cells are more sensitive to liquid toxicants than chick cells. Table IV shows the results of each phthalate on each system used. It should be noted that only the

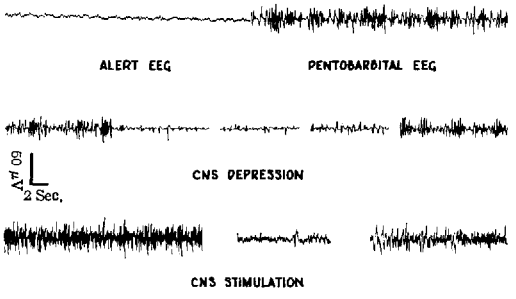

Fig. 1.—Occipital EEG patterns on rabbit. Key: ordinate and abscissa of insert (L) enlarged 2× for legibility of axis captions.

TABLE IV.—TOXICITY OF PHTHALATE ESTERS TO CULTURED CELLS

Phthalate Ester	Chick Embryo Cells, 50 mg./ml.[a]	Mouse Fibroblasts, 50 mg./ml.[a]
Dimethyl phthalate	−	+
Diethyl phthalate	−	+
Dibutyl phthalate	−	−
Di-isobutyl phthalate	−	−
Di-(methoxyethyl) phthalate	−	+
Butyl benzyl phthalate	−	−
Di-(2-ethylhexyl) phthalate	−	−
Dicapryl phthalate	−	−

[a] −, no cell toxicity; +, cell death.

compounds most soluble in water demonstrated toxicity to the cultured cells. This is not too surprising since the cell system is almost entirely aqueous. Two of the 3 exhibiting cell toxicity were also most irritating in the rabbit intradermal tests.

Subacute Toxicity Studies (Mice)

Body Weight Gains.—After 6 weeks of mouse intraperitoneal injections of 4 phthalates—diethyl, di-(methoxyethyl), butyl benzyl, and di-(2-ethylhexyl)—body weight in all groups, including controls, was approximately equal. The control group, however, reached maximum weight by the end of the third week, while in comparison, all phthalate-injected mice exhibited some degree of weight gain retardation, notably those receiving di-(2-ethylhexyl) phthalate, and, to a lesser extent, butyl benzyl phthalate. The diethyl phthalate group showed weekly weight gains most similar to those of the control group, and the di-(methoxyethyl) group exhibited an intermediate pattern. These differences in weight gain patterns may indicate a decreased food intake due to peritoneal cavity irritation caused by the repeated injections. It was observed on autopsy of each group of mice that some degree of peritonitis had been induced by the chemicals. The extent of peritonitis in the group of mice receiving the di-(2-ethylhexyl) phthalate was extreme. In nearly all of these cases, there was extreme adhesion formation, liver abscess, and adhesions to the diaphragm, and even testicle abscesses. These mice writhed and stretched after an injection, further supporting the observation of irritation of the entire peritoneal cavity.

Organ Weight–Body Weight Ratios.—Examination of organ-body weight ratios (Table V) indicated that liver weights in the di-(2-ethylhexyl) group were significantly greater and testis weights in the di-(2-ethylhexyl) and in the di-methoxyethyl) group were significantly less than the control group (in both groups $p > 0.01$). In regard to liver weights, however, it is doubtful that a genuine difference existed. Gross and pathological examination of these organs, particularly in the di-(2-ethylhexyl) group, indicated that organ weights were likely distorted by the effects of peritonitis. Adhesions, lesions, and other anomalies tended to prevent precise excision and cleaning of the organ before weighing. In addition, abscesses in the tissue changed the normal weight ratio. Testis weights indicated atrophy of this organ in the 2 above groups.

Hematology.—The hematological pattern of the several groups of mice receiving daily injections of the phthalate emulsions over a 6-week period indicated no significant deviations from the control group receiving only 3% acacia injections, with the possible exception of di-(2-ethylhexyl) phthalate. In this case, there was a slight decrease from original values in the hematocrit and hemoglobin and a slight increase in the red blood cell count. In all cases including controls there was a slight increase in white cell count, but this is to be expected after 6 weeks of daily injections.

Pathology.—In all of the test mice used in this study, there was some degree of peritonitis. Organs showing evidence of gross abnormalities were submitted for histopathological evaluation. These evaluations confirmed the original observations indicating acute peritonitis. Some organs from the phthalate injected groups were far more severely damaged than organs from the control group. In the case of di-(2-ethylhexyl) phthalate, nearly all organs showed presence of cloudy sedimentation accompanied by adhesions of the diaphragm, liver, and intestines and by abscess formation in the livers of a few. Liver and spleen of mice injected daily with di-(methoxyethyl) phthalate were found to have acute peritonitis and peri-portal hepatitis in the liver and extramedullary hematopoiesis in both the liver and spleen. The same pattern was also observed in the mice receiving the butyl benzyl phthalate, and in 1 testis there was an abscess of unknown etiology. In view of the irritation to rabbit tissue caused by intradermal injection, these results were to be anticipated. In these cases, a fairly strong irritant, comparable to the irritating action of 20% solution of ethanol, was injected daily and this was apparently enough to cause adhesions, peritonitis, and even some abscess

TABLE V.—EFFECT OF PHTHALATE ESTERS ON MOUSE TISSUE WEIGHTS

Group	Dosage, mg./Kg.	Organ Wt.–Body Wt. Ratio × 10^3					
		Liver	Heart	Lungs	Kidney	Spleen	Testes
Control		65.00 ± 1.83	4.72 ± 0.15	7.50 ± 0.36	13.29 ± 0.87	6.97 ± 0.85	6.94 ± 0.53
Diethyl phthalate	125	65.55 ± 3.45	4.51 ± 0.17	8.71 ± 3.78	13.27 ± 0.83	7.02 ± 0.80	7.07 ± 0.41
Di-(methoxyethyl) phthalate	250	65.12 ± 3.84	4.26 ± 0.25	7.56 ± 0.56	11.75 ± 0.75	8.04 ± 0.73	5.65 ± 0.34[a]
Di-(2-ethylhexyl) phthalate	250	76.02 ± 2.80[a]	4.30 ± 0.21	7.97 ± 0.39	13.16 ± 0.36	7.54 ± 0.73	5.48 ± 0.19[a]
Butyl benzyl phthalate	500	65.86 ± 2.73	4.53 ± 0.11	8.25 ± 0.40	13.20 ± 0.45	7.62 ± 0.54	6.17 ± 0.28

[a] Significantly different from control group averages ($p > 0.05$).

formation. The control animals in this study showed no gross or microscopic pathology.

SUMMARY

A group of phthalic acid esters were studied for both acute and subacute toxicity in animals. The acute toxicity experiments included the evaluation of LD_{50}, hexobarbital sleeping time effects, rabbit intradermal tests, acute intravenous toxicity studies, and tissue culture effects. Subacute toxicity dealt with effect on body weight gain, organ–body weight ratios, effect on tissue of various organs, and effect on the hematopoietic system. Results of the study indicated that the group of phthalate esters reported in this paper had a low degree of toxicity when administered parenterally and that their degree of toxicity was parallel to their water solubility (greater solubility, greater activity) and to their molecular weight (lower molecular weight, greater activity). This low order of toxicity to parenteral administration appears to indicate that their use in applications implicated in this study are probably warranted and safe.

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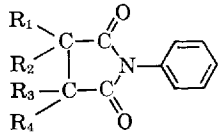
The mechanisms and the relative rates of alkaline hydrolysis of succinanil and methyl-substituted succinanils have been investigated partly to help to elucidate the mechanism of carboxyl facilitated formation and hydrolysis of amides observed in these laboratories and also because a number of important hypnotics possess similar structures. As may be expected, the observed rates for the several imides roughly parallel those found for the corresponding anhydrides, succinanil being more reactive than the methyl-substituted compounds. The relative rates were in the order: unsubstituted > monomethyl > mesodimethyl > 2.2 dimethyl > racemic dimethyl > trimethyl > tetramethyl; the first member of the series reacting 83 times faster than the last. The tetramethyl anil was found to be sufficiently stable to co-exist as the major species in equilibrium with its cleaved product at pH of 8.

ALTHOUGH a number of cyclic imides, including glutethimide, methsuximide, phensuximide, etc., are widely used as drugs, relatively little has appeared in the literature concerning the rate of hydrolysis of such imides. Results of studies on the effect of structure on some aspects of this hydrolytic reaction are presented at this time. In particular, the investigation has been concerned with the influence of alkyl substitution on the rate of hydrolysis of *N*-phenyl succinimides (succinanils) (I). These reactions are of interest



I

R_1, R_2, R_3, R_4, = H or alkyl

not only because of the close relationship of these compounds to pharmaceuticals but also because

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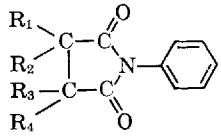

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they shed light on possible mechanisms of biochemical reactions.

It has been long recognized that methyl substitution generally increases ring stability. Thorp and Ingold and their students spent 15 years collecting evidence to substantiate this effect (1). Whether methyl substitution leads to greater thermodynamic stability for cyclic compounds than for their open-chain counterparts has not, however, been established with certainty. There is, nevertheless, evidence that methyl substitution aids in stabilization of the cyclic structure by accelerating the rate of ring closure (2). It had not been definitely ascertained, however, that methyl groups also stabilize the cyclic forms by inhibiting the rate of ring opening. Studies comparing anhydrides of dicarboxylic acids with their methyl-substituted analogs indicate that this may be the case (3). Whether this phenomenon is due to a steric hindrance to the attacking nucleophile or a steric hindrance to the linear separation of the 2 carboxylate groups is not yet clear.

Imide hydrolysis in general has not been the subject of great interest in recent literature, and hydrolysis of cyclic imides has received even less attention. Edward and Terry (4) have investigated the hydrolysis of succinimide in basic solutions and have shown that the rate of hydrolysis is first order in hydroxyl ion and first order in undissociated succinimide. Zerner and Bender (5) have shown that the hydroxyl-ion concentration is between pH 3.5 and 6.5.

EXPERIMENTAL

Materials.—The succinanils used in this investigation were prepared according to the method described by Fieser (6) for succinanil and were recrystallized from distilled water. Melting points of the products were compared with values given in the literature. Since the preparative method was straightforward, a good correspondence with melting points given in the literature was assumed as sufficient evidence for the existence of the compounds.

The dioxane used in this investigation was purified and made anhydrous by the treatment of technical grade dioxane as described by Vogel (7). Barium hydroxide solutions were prepared from carbon dioxide-free distilled water and sealed under nitrogen in glass ampuls. These samples were standardized against standard hydrochloric acid solutions. Corrections for appropriate ionic strength and temperature as affecting the partial dissociation of $Ba(OH)^+$ were estimated from the data of Gimblett and Monk (8).

The nitrogen which was used in the kinetic runs on the pH stat was passed over an Ascarite plug and bubbled through aqueous potassium hydroxide solution to remove carbon dioxide and prehumidified in a saturator at the reaction temperature. All the water used in these kinetic studies was freshly boiled and carbon dioxide free.

All other chemicals used in the preparation of buffers, kinetic solutions, and analytical reagents were analytical or reagent grade.

Kinetic Procedures for Hydrolysis of Succinanil and Methyl Succinanil.—The hydrolytic rate constants for these 2 compounds as a function of pH and temperature were determined in phosphate buffers of ionic strength 1.0 in a thermostated oil bath. Since it was found that the phosphate buffer catalyzed the hydrolysis of these compounds, each experiment involved determination of the hydrolysis rate at 5 buffer concentrations at the same pH and extrapolation to zero buffer concentration. The reaction solutions consisted of 99 ml. of the appropriate concentration of phosphate buffer and 1 ml. of a 1.2% solution of the anil in dioxane. The dioxane solution was added after the buffer solution had reached the required temperature in the oil bath. Five-milliliter samples were withdrawn periodically and quenched by diluting to 25 ml. with 0.25 *M* phosphate buffer at pH 6.0. These samples were analyzed spectrophotometrically at 240 mμ which is $\lambda_{max.}$ for the anilic acids. The appearance of the anilic acid was followed in this manner, and plots of $\log (A_\infty - A)$ *versus* time yielded straight lines from which k_{obs} could be calculated. Figure 1 shows plots of k_{obs} *versus* buffer concentration for succinanil at several pH values.

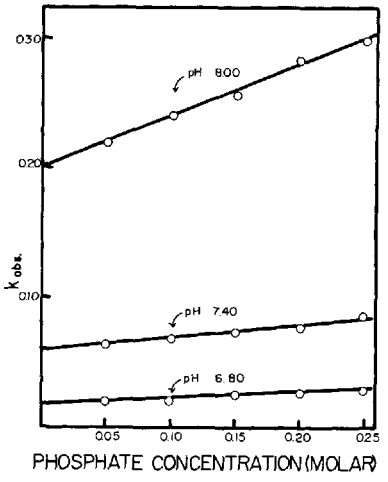


Fig. 1.—Plots of k_{obs} as a function of buffer concentration for hydrolysis of succinanil at 40°.

Kinetic Procedures for Hydrolysis of Methyl; meso-2,3-Dimethyl; 2,2-Dimethyl; Racemic 2,3-Dimethyl; and Trimethyl Succinanils.—The hydrolytic rate constants for this group of compounds were determined by use of a Radiometer TTT 1 automatic titrator with a SBR2 type Titrigraph and a TTA 3 titration assembly. Twenty-five milliliters of carbon dioxide-free water containing 1.45 Gm. of potassium chloride was placed in a water-jacketed beaker attached to a constant-temperature water bath. When the temperature had equilibrated, 1 ml. of a 1.2% solution of the anil in dioxane was added by means of a syringe and needle. The pH of the solution was maintained by the automatic addition of 0.025 *N* sodium hydroxide solution. Since the ml. of base added is directly proportional to the

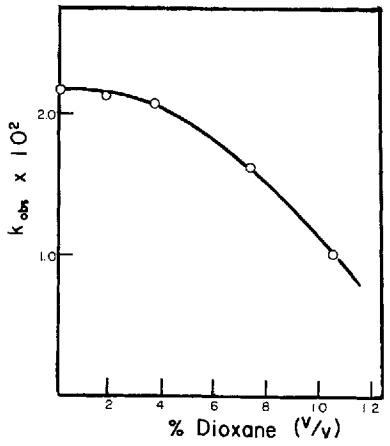


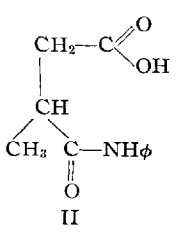
Fig. 2.—Plot showing dependence of k_{obs} for hydrolysis of methyl succinanil on dioxane concentration at 40° and pH 8.85.

amount of anilic acid being produced, plots of log (ml.$_\infty$ − ml.) *versus* time yielded straight lines from which k_{obs} could be calculated. Spectrophotometric analysis indicated that the reaction had essentially gone to completion.

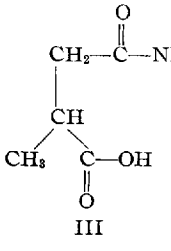
Since dioxane was used in these kinetic runs, the influence of dioxane concentration on k_{obs} was evaluated. Figure 2 shows values of k_{obs} for methyl succinanil as a function of dioxane concentration. At dioxane concentrations used in these experiments (3.84%), k_{obs} differed only slightly from k_{obs} at zero dioxane concentration.

Kinetic Procedure for Hydrolysis of Tetramethyl Succinanil.—Because of its slow rate of hydrolysis at lower pH values and because of the limitations of the pH stat method at higher pH values, the hydrolytic rate constants for tetramethyl succinanil were obtained in barium hydroxide solutions and were carried out directly in the sample cell of a thermostated Cary model 11 spectrophotometer. Twelve microliters of a 1.5% solution of the anil in dioxane was used for each run, along with 3 ml. of the barium hydroxide solution. The formation of tetramethyl succinanilic acid was followed by the change in absorption at 240 mμ. Plots of log (A_∞ − A) *versus* time yielded straight lines from which k_{obs} was calculated. Spectrophotometric analysis at the end of each run indicated that the anil had essentially hydrolyzed completely to the anilic acid.

Identification of Reaction Products.—All reaction products were identified spectrophotometrically. However, it must be mentioned at this time that in the hydrolytic cleavage of certain of the anils studied in this series, 2 different products are possible. For example, hydrolytic cleavage of methyl succinanil can yield the 2 isomers, II and III.



II



III

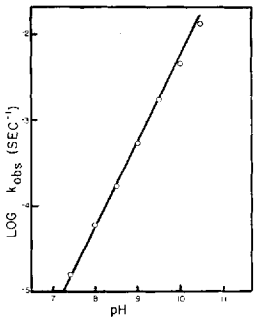


Fig. 3.—pH rate profile for hydrolysis of methyl succinanil at 40°. The solid line has been drawn with a slope of 1.00.

Probably a mixture of II and III is actually produced. Although one may expect purely on steric and electronic considerations that compound II would be the more prominent species, in this investigation no attempt was made to isolate and identify the products. The same was also true for 2,2-dimethyl and trimethyl succinanil.

RESULTS AND OBSERVATIONS

The second-order rate constants for a series of methyl-substituted succinanils were determined at temperatures ranging from 25 to 55°, and the kinetic parameters E_a and $\Delta S^\ddagger$ were calculated. From past work it might be expected that the hydrolysis would be independent of pH at low values (5), but at the pH values employed in this investigation, the reaction has been shown to be first order with respect to hydroxyl-ion concentration. It would appear, therefore, that the rate constants which are to be compared all stem from an identical mechanism. Figure 3 shows a pH-rate profile for the hydrolysis of methyl succinanil at 40°.

Table I lists all the anils studied in this investigation and their second-order rate constants calculated at 25°. The apparent E_a and $\Delta S^\ddagger$ values are shown also. The $\Delta S^\ddagger$ values were all calculated at 25°. The E_a values listed in this table for the first 6 compounds are 12.6 Kcal. less than those values calculated from the slopes of Arrhenius-type plots for runs made at constant pH. This accounts for the change in K_w with temperature.

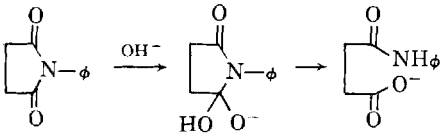
DISCUSSION

The observed markedly decreased rate of hydrolysis of the succinanils induced by methyl substitution is not altogether unexpected. Although the nature of the activated states involved in the cleavage of these imides differs significantly from those responsible for formation of hydrolysis of the amic acids, there are some similarities. Considering the

TABLE I.—RATE CONSTANTS FOR OH$^-$-CATALYZED HYDROLYSIS OF ANILS AT 25°

Compd.	k (1/m/sec.)	E_a, Kcal./mole	$\Delta S^\ddagger$, (E.U.)
Succinanil	5.45	12.3	−15.9
Methyl-	5.20	12.8	−15.9
meso-2,3-Dimethyl-	4.98	10.0	−19.5
2,2-Dimethyl-	3.57	10.6	−22.2
Racemic 2,3-dimethyl-	2.92	11.2	−20.7
Trimethyl-	0.815	11.7	−22.0
Tetramethyl-	0.0669	9.4	−34.3

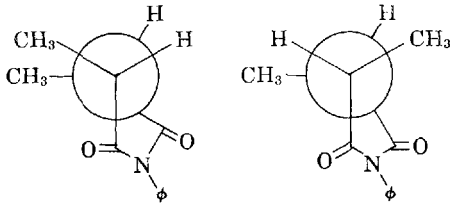
second-order character of the reaction, one can write Scheme I.



Scheme I

From this postulated mechanism, it is obvious that there are 3 general phenomena which could explain the decrease in hydrolytic rate with increased methyl substitution: (*a*) polar contribution of the methyl groups which would tend to reduce the $\delta+$ charge on the carbonyl carbon, thus reducing the rate of hydroxyl attack; (*b*) steric inhibition by the substituted methyl groups to the attacking nucleophile; and (*c*) steric hindrance to ring opening due to the presence of the methyl groups.

This last phenomenon has been pointed out by Bordwell and co-workers (9) in their studies on sultones. These workers show large decreases in rates of hydrolysis with increasing methyl substitution; they attribute this effect largely to steric hindrance of ring opening. They argue that in sultone hydrolysis, the ring opening must occur by a rotation about the bonds holding the severed groups, and that methyl substitution restricts this rotation. This line of reasoning may be applied in the present investigation to explain the difference in hydrolytic rates for the *meso* and racemic isomers of 2,3-dimethyl succinanil. Examination of Newman projections of these compounds indicate that in the *meso* isomer (IV) the methyl groups are already in an



meso IV

Racemic V

eclipsed position, and a rotation on cleavage would reduce this methyl–methyl interaction. On the other hand, a rotation in the racemic isomer (V) would increase methyl–methyl interactions. Since the polar effects and steric inhibition to nucleophilic attack in these two isomers would be expected to be similar, the steric inhibition to ring opening seems to be a reasonable approach for explaining the difference in hydrolytic rates of these 2 compounds (Table I).

Bruice and Pandit (3) have investigated the solvolysis of certain cyclic anhydrides, as well as the solvolysis of mono esters of these anhydrides and concluded that although a restriction of rotation of reacting groups away from each other markedly increases the rate of anhydride formation, it does not decrease the rate of anhydride solvolysis. They point out that although certain mono-esters of maleic acid form the anhydride about 50 times faster than the corresponding succinic monoesters, maleic anhydride solvolysis proceeds about 8 times faster than that of succinic anhydride. Their findings do not actually refute the suggestions of Bordwell *et al.*, since maleic anhydride hydrolysis would not involve a rotation but would probably relieve a strain imposed by the cyclic structure.

From the previous discussion and from the data presented in this investigation, it becomes apparent that no definite conclusions can be drawn concerning the differences in the hydrolytic rates of methyl-substituted succinanils at this time. This, of course, is due to the fact that the 3 possible phenomena which may be responsible for the change in rates cannot be separated. It can be suggested, however, that polar effects and steric inhibition to nucleophilic attack are mostly responsible for the differences in rates among the lower methyl-substituted compounds in this series and that a "steric hindrance to ring opening" effect becomes more pronounced as methyl substitution is increased. This is based on the fact that the relative rates change only slightly in the lower methyl-substituted members, while there is a greater rate change in the tri- and tetramethyl-substituted members of this succinanil series.

SUMMARY

Although hydrolytic cleavage of succinanils is not strictly related to anhydride formation occurring during the formation and hydrolysis of the succinanilic acids in dilute aqueous solutions, certain similarities are thought to exist. The hydrolytic rate constants were determined in aqueous buffered solutions for succinanil and methyl succinanil; in barium hydroxide solutions for tetramethyl succinanil; and by use of a pH stat for methyl, *meso*-2,3-dimethyl, 2,2-dimethyl, racemic 2,3-dimethyl, and trimethyl succinanil.

The fact that increased methyl substitution decreases the rates of hydrolysis in this series was not unexpected in light of previously compiled data. The interpretation of these results appears to be rather complicated since there were probably several factors, such as polar effects, steric inhibition to nucleophilic attack, and steric hindrance to ring opening, which would tend to decrease the hydrolytic rate. Although it was not possible to separate these effects, it is suggested that the last effect became more prominent as methyl substitution was increased.

The findings of this investigation seem to indicate that not only does methyl substitution markedly enhance ring formation, but it is also responsible for increased ring stability.

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Interaction of Aqueous Polyethylene Glycol Solutions with Iodine

By C. F. HISKEY and F. F. CANTWELL

An experimental study of the interaction of iodine with the ether linkage of some polyethylene glycols was made. The conditions for producing 1 : 1 complexes of $-CH_2$ \ O : I_2 / $-CH_2$ were determined. Some qualitative observations are presented on the effects of polymer weight, polymer–iodine ratios in solution, and on the behavior of these complexes in analytical reactions.

SINCE the turn of the century (1) it has been known that iodine solutions in pure solvents are either violet or brown. Violet solutions are formed in such solvents as carbon disulfide, carbon tetrachloride, and simple aliphatic hydrocarbons. In these solvents the iodine has an absorption spectrum similar to that which it has in the gaseous state with an absorption maximum in the vicinity of 518 to 520 mμ, and with a molar absorptivity of about 1000 (2). Brown solutions are formed when the iodine is dissolved in alcohols, ethers, ketones, organic acids, and basic nitrogenous solvents. Hildebrand and Glascock (3) showed that, at least in alcohols and ethers, 1:1 complexes were formed, and later Mulliken (4) showed by means of molecular orbital argumentation, that iodine would bind to the oxygen of an ether at right angles to the plane of the molecule. These solutions have an absorption maximum around 450 to 460 mμ with a molar absorptivity a little bit less than that in the violet solution.

The blue form of iodine which is observed in the starch reaction or in the reaction of iodine with the cyclodextrins, benzonitrile, and coumarin complexes appears, from the X-ray diffraction studies of Cramer (5) to be due, not to the normal diatomic iodine form, but instead to a polymeric blue modification kept inside the channels within these inclusion compounds. Hence, these compounds are quite different from the ether–iodine complexes discussed here.

It is a well known phenomenon that the brown solutions tend to become violet when heated, reverting to their original color on cooling. Also it has been observed that in the case of the violet color of the carbon disulfide solution this may be shifted to the brown color as the solution is cooled in a dry ice–ether bath. Beckman (6) by means of precise molecular weight determinations in both the violet and the brown solutions established that the iodine is present in the diatomic state in both instances. Thus, the current view is that when the iodine forms a weak coordination complex the brown color is observed, but these complexes may be easily dissociated merely by heating.

In this paper it is our intention to report some phenomena which were observed when we attempted to do iodometric titrations in the presence of aqueous solutions of some polyethylene glycol polymers[1] (PEG). Their structures may be written as $HOCH_2 \cdot\cdot (CH_2OCH_2)_n \cdot\cdot CH_2OH$, where the average n varies from 7.2 to 8.1 in the case of PEG 400, from 20.1 to 22.3 for PEG 1000, and from 67.0 to 83.0 in the case of PEG 4000. The authors have written the formula this way instead of the usual way, *viz.*, $H(OCH_2CH_2)_{n+1}OH$, because we wish to stress the polyether character of these molecules. From the work of a group of investigators and inventors (8), it is well known that iodine forms complexes with such polymeric materials, but it seemed to us that a qualitative description of what we observed plus some of the quantitative measurements which were made would provide the analyst with an understanding of what occurs in the above-mentioned solutions.

EXPERIMENTAL, RESULTS, AND DISCUSSION

To study the quantitative aspects of the interaction of polyethylene glycols with iodine, it was decided to work with the higher molecular weight PEG 4000 which had qualitatively been observed to yield solid, filterable precipitates with KI_3 solutions. It was anticipated that a PEG which yields a precipitate with iodine in aqueous solutions would permit an evaluation of the limiting molar ratio I_2–PEG in the complex. To make this evaluation it would be necessary to form the precipitates in solutions with large excesses of KI_3 relative to the

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[1] Marketed as Carbowax by the Union Carbide and Chemical Co., New York, N. Y.

PEG. For this purpose the concentration of PEG 4000 is expressed as *molarity of* $—CH_2OCH_2—$ (mol. wt. = 44), and the contribution of the end $—CH_2OH$ groups to the molecular weight is neglected.

In the experiment, 10.0 ml. or less of an appropriately diluted stock solution of PEG 4000 was pipeted into ground-glass-stoppered flasks, enough water was added to make 10.0 ml., and then 10.0 ml. of 0.0554 *M* KI_3 solution was added. The mixtures were swirled and set aside in a dark place for from 3 to 6.5 hr., after which time the suspension was vacuum filtered through a membrane filter. An aliquot of each filtrate was pipeted into a flask, about 5 ml. of 1 *N* H_2SO_4 and 10 ml. of H_2O were added, and the solution was titrated to a colorless end point. The amount of unprecipitated iodine in the 20.0-ml. of reaction mixture thus is determined, and by difference the amount of complexed and precipitated iodine is known.

The data obtained are plotted in Fig. 1, with the ordinate representing the number of mmoles

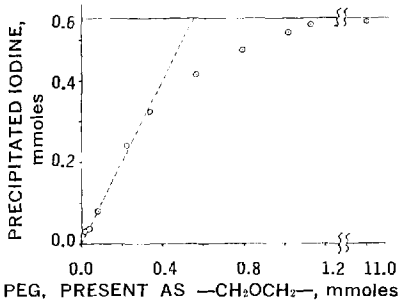

Fig. 1.—Precipitation of I_2 by PEG 4000.

of iodine precipitated and the abscissa representing the mmoles of $—CH_2OCH_2—$ added. It will be observed that at high ratios of iodine to ether, *i.e.*, where very small quantities of $—CH_2OCH_2—$ were added, each mole of $—CH_2OCH_2—$ added precipitated 1 mole of I_2. Thus, initially the points fall on the straight dotted line with a slope of unity. In the light of Hildebrand and Glascock's experimental observations and Mulliken's calculations, cited previously, this is exactly what one would expect.

As the amount of PEG added increases so that the iodine remaining in solution is depleted, a deviation from the straight line is observed, and the experimental points form a curve which asymptotically approaches the limiting value of 0.0554. This can be understood in terms of the competing equilibria in these solutions, the iodine being complexed by both the ether and the iodide ion present in solution. If the iodine–ether complexation constant was very much larger than that of the triple iodide ion, the experimental points would remain on the straight line right up to the limiting value of 0.0554. The fact that they do not merely indicates that they are approximately of the same order of magnitude. The value for the reaction ($I^- + I_2 \rightleftharpoons I_3^-$) is about 725 (9) at 25°.

In the course of the above experiment some interesting qualitative observations were made. These observations concern the color and character of the precipitates. In those solutions where the residual iodine concentration was high, the precipitates were dense and dark black with a submetallic lustre resembling that of iodine itself. On the other hand, where the mole ratio of ether to iodine was large, *i.e.*, 10 and higher, the precipitates were very much more voluminous and were light reddish-brown in color, becoming lighter as the ratio was increased.

Thus, it is apparent that when every ether oxygen is coordinated with iodine, a precipitate resembling iodine crystals in color and lustre is observed, but when the coordination is made to every tenth oxygen or less, the color of the precipitate appears more like that of a dilute ether solution of iodine. It was also evident that only a small fraction of the ether linkages had to be coordinated before the solubility of the PEG was reduced so that precipitation occurred.

Another interesting phenomenon was observed when precipitates with low iodine:$—CH_2OCH_2—$ ratios were washed with distilled water. These light reddish-brown precipitates immediately became much darker in color, and their appearance changed to that of the precipitates with the I_2:$—CH_2OCH_2—$ ratios of approximately 1. This occurred whether they were washed by decantation or directly on filter paper. In addition it was observed that there was a substantial reduction in the volume of the precipitate. Furthermore, the filtrate or the supernatant from such a washing has a light but definite yellow color. A possible explanation of this phenomenon follows.

The PEG 4000 precipitate can be assumed to have the iodine randomly distributed among its ether oxygens, but weighting each individual molecule sufficiently so that it is made insoluble. However, not every oxygen position is coordinated with iodine as evidenced by the light color of the precipitate. On washing, however, disproportionation occurs with iodine from some of the molecules being transferred to others, producing complexes that have a higher I_2:$—CH_2OCH_2—$ ratio and ones which have a much lower ratio. The former of these which approach the 1:1 complex are dark and insoluble, whereas the latter having a relatively small number of ether oxygens complexed with iodine, are light colored and water soluble. The effect is indeed a very striking one to observe.

Additional Qualitative Observations.—In light of the phenomena which have been described above, it was thought appropriate to make a qualitative comparison between the interaction of the KI_3 solutions with PEGs of various molecular weights. Those available were PEG 400, 1000, and 4000. The purpose of this study was to determine the relative ease of precipitating the lighter and heavier PEGs, using minimal amounts of iodine to accomplish that objective. The actual experiment performed was to dissolve 6.0 Gm. of each of the PEGs in 10.0 ml. of water. This makes a solution which is 13.6 *M* in ether linkages. Again the terminal $—CH_2OH$ groups are ignored. These 10.0-ml. portions of PEG solutions were titrated dropwise with an 0.0554 *M* KI_3 solution with continuous agitation. In all 3 cases, as the first drop and each successive drop of KI_3 was added, a reddish-brown precipitate formed in the area where the drop initially contacted the solution. However, it dissolved with agitation to give a yellow solution

TABLE I.—ASSAY RESULTS WITH POLYETHYLENE GLYCOLS

PEG	ml. KI_3 to Achieve Precipitation	mole Ratio, $-CH_2OCH_2-$ / KI_3	I_2, % Easily Titratable
400	8.5	290	98
1000	4.2	585	43
4000	3.5	713	...

which became more and more wine-red in color as more KI_3 was added. Finally, as the titration continued, there came a point at which the reddish-brown turbidity persisted, and further addition of KI_3 only increased the amount of turbidity. The second column of Table I lists the ml. of titrant required just to reach the point of permanent turbidity for each of the PEGs.

It will be seen that as the molecular weight of the PEG increases, the amount of KI_3 needed decreases. In the third column of Table I this is further illustrated by presenting the mole ratio of ether linkages to the KI_3 at that point. After the point of permanent turbidity had been reached, enough additional KI_3 was added to bring the volume of that reagent to 10.0 ml. The precipitates obtained were watched for the next 1.5 hr. to observe alterations in their characteristics. In the case of the PEG 400, the solution initially had a reddish-brown suspension in it, but with time and occasional swirling of the flask, this precipitate coalesced to give a few drops of a dark black liquid. When the supernatant liquid was decanted, these globules collapsed and appeared to wet the glass. The thin films formed in this way were dark red by transmitted light.

In the case of PEG 1000, as the precipitate aged, a dark black portion of liquid material similar to that observed with the PEG 400 and some light reddish-brown wax-like solids were observed. In the case of the PEG 4000, the changes which occurred were not very striking, except that the very light brown precipitates that formed initially appeared to get darker with time and to diminish to some extent in volume. After about 15 min. this precipitate had settled out, leaving a very lightly straw-colored solution above it.

The situation obtained in these solutions after the rapid addition of the additional KI_3 to make the final 10.0 ml. is one where the mole ratio of ether oxygens to iodine is still quite high, being about 246. It is evident for precipitation to occur under such circumstances, that the iodine be concentrated on some molecules, rendering them insoluble, while the bulk of the remaining molecules have so few iodine atoms on them that they remain in solution. This is the final situation which prevails after a period of time sufficiently long to bring the system to equilibrium.

When the lower molecular weight PEG is used, the iodine concentrates on the precipitated molecules to give them an iodine–ether ratio close to unity as evidenced by their dark color. With the higher molecular weight polymers a lower ratio leads to precipitation.

The changes that occurred with time are those associated with bringing the system to this disproportionate equilibrium and to coalescence of the liquid-like product from the lower polymers, and perhaps some aging of the higher polymer precipitates.

Several interesting additional observations were made. The first is concerned with the relative ease of dissociating these precipitated complexes. This was studied by titrating the iodine with standard $Na_2S_2O_3$, employing moderate agitation of the solution during the titration. The titrations were performed rapidly, *i.e.*, in a matter of several minutes, to see how quickly the iodine became available. The titrant was added until the supernatant was just colorless. The per cent recovery of the iodine is given in the fourth column of Table I. In the case of the PEG 400, nearly all of the iodine was recovered immediately, and with vigorous and prolonged agitation, the small residual amount of dark material could be brought into solution with all of the iodine being recovered. With PEG 1000, however, this is not so easily accomplished. The iodine is tenaciously retained by the precipitate and only 43% could be readily recovered. With the PEG 4000, recovery was essentially zero or very little, and also the precipitate interfered with the end point detection.

A second observation, relating to the dissociation of these complexes was made when a solvent like propylene glycol was added. If it was added to the extent of about 50 vol. %, precipitation of the iodine–PEG complexes was avoided completely. If the propylene glycol was added with heating after precipitation had been effected, the precipitates redissolved, and quantitative recovery of the iodine was achieved. The explanation here, of course, is that an additional coordinating reagent has been added which is competing nearly as strongly as the PEG itself for the iodine. Consequently, the PEG coordinates only a small fraction of the iodine, insufficient in amount to achieve precipitation. Furthermore, this alcohol has a greater solvent effect than water, for both the PEG and the PEG–iodine precipitate.

Two additional observations of interest were the following: aqueous solutions of the PEGs can extract, to a degree, the iodine present in the violet-colored chloroform and carbon tetrachloride solutions, and the vapor pressure of iodine over well-dried PEG–iodine precipitates was significantly lower than that of iodine itself. If the PEG 1000–iodine precipitate was heated on a spatula, the iodine could be driven off, but the reaction was slow and the temperature had to be in excess of 100°.

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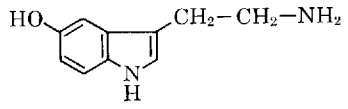
Preparation and Biological Evaluation of Some *N*-Amino Acid Substituted Derivatives of Serotonin

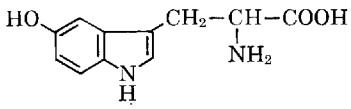
By ALBERT J. FORLANO and ROBERT L. S. AMAI*

A series of *N*-amino acid substituted derivatives of serotonin was prepared. The method entailed condensing the α-halo analogs of alanine, isoleucine, phenylalanine, and valine with 5-benzyloxy-serotonin using a method adapted from Fisher's peptide synthesis. The activity of these compounds was compared with 5-hydroxytryptophan in an Actophotometer to determine their effect on generalized activity in mice. The results are presented along with some possible explanations for the biological observations.

HIGH cerebral levels of serotonin are known to produce a significant increase in central stimulation (1–3). Woolley (4) stated that hallucinations, agitation, and other signs of excitation are found in individuals with elevated cerebral levels of serotonin and that decreased levels can lead to depressive states. There is also an implication that an abnormality in serotonin metabolism may be responsible for schizophrenia (4). The biosynthesis of serotonin (I) in animals commences with the natural amino acid tryptophan which is obtained from dietary sources (5). 5-Hydroxytryptophan (II) is produced by hydroxylation of the amino acid in the 5-position of the indole ring by an enzyme system having NADP as one of the coenzymes. The latter



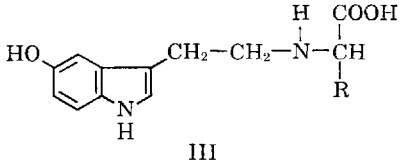
I



II

compound (II) is easily decarboxylated to the hormone serotonin (I) by 5-hydroxytryptophan-decarboxylase, which contains pyridoxal phosphate as a coenzyme. Serotonin is not produced in animals by the direct 5-hydroxylation of tryptamine (5).

Shaw and Woolley reported that exogenous serotonin does not pass from the blood into the brain very readily (6–8). Instead, the parenteral administration of serotonin produces powerful intestinal contractions, increased tissue permeability, and a rise in blood pressure. Therefore, if one wanted to observe the central effects of serotonin, a substance like 5-hydroxytryptophan (II) would be necessary. This compound can pass through the blood brain barrier where it is subsequently converted to serotonin (2, 9). Since serotonin (I) cannot pass from the blood into the brain, it appears likely that the carboxyl group of 5-hydroxytryptophan may be necessary for its transport into the brain. Evered and Randall (10) have shown that it is possible to transport cytoactive drug molecules into cells by linking them to actively transported natural amino acids as carriers. Considering these factors, it was believed that a series of compounds with unique properties could be produced if serotonin was linked to some natural amino acids. These compounds would: (*a*) have the free carboxyl group that appears to be necessary for transport across the blood brain barrier and (*b*) have natural amino acids for anchoring purposes to carrier and metabolic receptor sites. The proposed series of compounds are derivatives of structure III, where R represents the tail portion of the various amino acids used.



III

The synthesis of these compounds was initiated by treating 5-benzyloxyindole (IV) with oxalyl chloride to produce 5-benzyloxy-3-indoleglyoxalyl chloride (V). The acid chloride was converted to the amide (VI) by treatment with concentrated aqueous NH_3 and was subsequently reduced to 5-benzyloxytryptamine (VII) with $LiAlH_4$. The 5-benzyloxy derivatives of III were produced by condensing the appropriate α-

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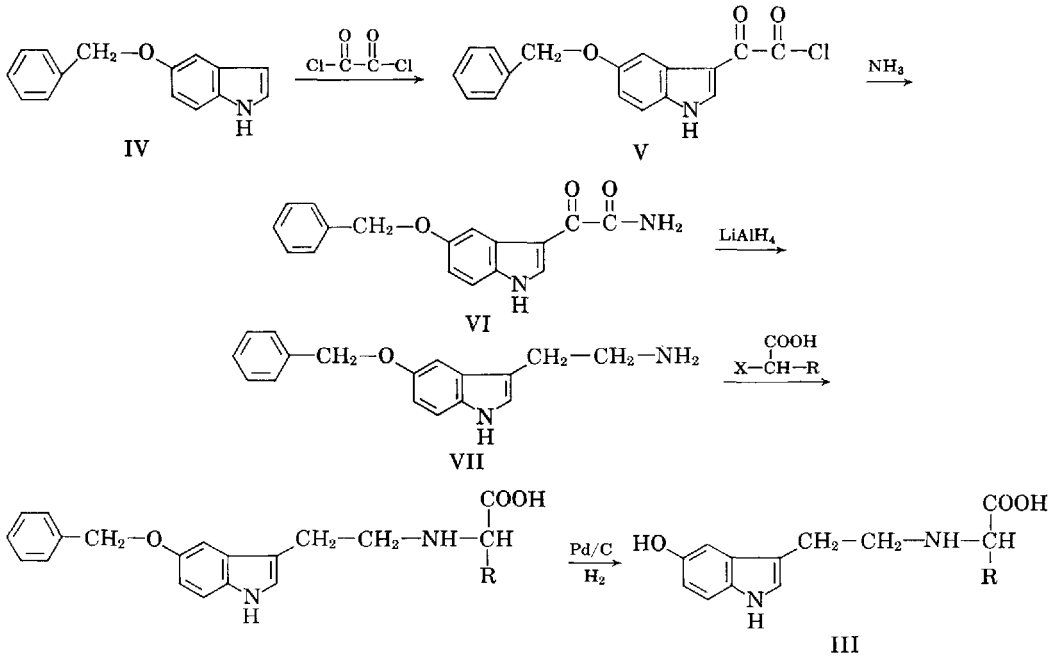
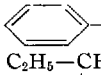


III*a*, R = CH_3—(alanine derivative)
III*b*, R = $(CH_3)_2$—CH—(valine derivative)
III*c*, R =  —CH_2—(phenylalanine derivative)
III*d*, R = C_2H_5—CH(—CH_3)—(isoleucine derivative)

Scheme I

halo analogs of the amino acids with the amine (VII). These were debenzylated by catalytic hydrogenation using Pd/C catalyst. The complete reaction is presented in Scheme I.

EXPERIMENTAL[1]

The first part of this section deals with the preparation of 5-benzyloxytryptamine (VII) which was used as the common intermediate for condensation with the α-halo analogs of the amino acids.

5-Benzyloxytryptamine (VII).—This compound was produced in 41% yield based on the starting material using the method of Lipp *et al.* (11). 5-Benzyloxytryptamine HCl (12) was converted to the base by treatment with aqueous KOH and extraction with diethyl ether.

N - (5 - Hydroxy - 3 - indole - ethyl) Alanine (IIIa).—Compounds (III*a–d*) were prepared by a modification of the method suggested by Fischer and Otto (13, 14) for the synthesis of peptides. Seventeen grams (0.064 mole) of 5-benzyloxytryptamine was dissolved in 50 ml. of recently boiled and cooled *N,N*-dimethylformamide (DMF). In a separate vessel 10.85 Gm. (0.10 mole) of α-chloropropionic acid (Eastman Red Label) was mixed with 20 ml. of DMF and enough 1.0 *N* KOH to neutralize the acid. The 2 solutions were transferred to a reflux flask and water was slowly added until the mixture became homogeneous. An additional 0.01 mole of KOH was added, and the solution was refluxed for 12 hr. During this time more base was added if necessary to maintain the pH slightly basic; however, large excesses were avoided because of the possible hydrolysis of DMF. The reaction mixture was diluted with twice its volume of 1 *N* aqueous KOH and any unreacted amine which precipitated was removed by extraction with ethyl ether. The solution was adjusted to pH 4.0 with glacial acetic acid and placed in a refrigerator for 24 hr. The product *N*-(5-benzyloxy-3-indolethyl) alanine was removed by filtration and washed with hot isopropyl alcohol. It was then suspended in 150 ml. of ethanol and water mixture (1:1) which contained 2.0 Gm. of 10% Pd/C (Matheson, Coleman and Bell). The product was debenzylated in a Parr hydrogenator at a pressure of 50 psig for 1 hr. The catalyst was removed by filtration and the solvent was removed under vacuum. The residue was crystallized from an isopropanol–water mixture and dried yielding 6 Gm. (23% yield) of *N*-(5-hydroxy-3-indole-ethyl) alanine·$1H_2O$, m.p. 238–243° dec.

Anal.—Calcd. for $C_{13}H_{18}N_2O_4$: C, 58.70; H, 6.79; N, 10.55. Found: C, 59.00; H, 6.93; N, 10.30.

N - (5 - Hydroxy - 3 - indole - ethyl) Valine (IIIb).—This compound was prepared in a similar manner to the alanine derivative (III*a*). Seventeen grams (0.064 mole) of 5-benzyloxytryptamine was reacted with 18.1 Gm. (0.10 mole) of α-bromo-

[1] All melting points were taken on a Fisher Johns apparatus and are uncorrected. Microanalyses were performed by Organic Microanalysis, Montreal, Ontario, Canada.

β-methylbutyric acid (Eastman Red Label). The *N*-(5-benzyloxy-3-indole-ethyl) valine derivative had a m.p. of 225–230° after crystallization from an ethanol–water mixture. After debenzylation and crystallization from the isopropanol–water mixture, 7.0 Gm. (25% yield) of the title compound was produced, m.p. 240–245° dec.

Anal.—Calcd. for $C_{15}H_{20}N_2O_3$: C, 65.20; H, 7.30; N, 10.14. Found: C, 64.90; H, 7.19; N, 10.35.

N - (5 - Hydroxy - 3 - indole - ethyl) Phenylalanine (IIIc).—This compound was prepared in a similar manner to the alanine derivative (III*a*). Sixteen grams (0.06 mole) of 5-benzyloxytryptamine was refluxed with 20 Gm. (0.086 mole) of α-bromo-β-phenyl propionic acid (K & K Laboratories, New York, N. Y.) for 3 hr. The benzyloxy derivative had a m.p. of 200–205°. The debenzylated product was crystallized from an isopropanol–water mixture, and 6.5 Gm. (20% yield) of the title compound was produced, m.p. 235–238° dec.

Anal.—Calcd. for $C_{19}H_{20}N_2O_3$: C, 70.35; H, 6.21; N, 8.64. Found: C, 70.03; H, 6.54; N, 8.73.

N - (5 - Hydroxy - 3 - indole - ethyl) Isoleucine (IIId).—This compound was prepared in a similar manner to the alanine derivative (III*a*). Sixteen grams (0.06 mole) of 5-benzyloxytryptamine was refluxed with 20 Gm. (0.10 mole) of α-bromo-β-methyl valeric acid (K & K Laboratories, New York, N. Y.) for 3 hr. yielding the 5-benzyloxy derivative, m.p. 205–210° dec. The debenzylated product was crystallized from an isopropanol–water mixture and 5 Gm. (17.2% yield) of the title compound was produced, m.p. 212–215° dec.

Anal.—Calcd. for $C_{16}H_{22}N_2O_3$: C, 66.18; H, 7.64; N, 9.65. Found: C, 65.39; H, 7.52; N, 9.81.

BIOLOGICAL TESTING

The objective of the biological testing was to determine whether these compounds had any central stimulating activity. Woolley (4) stated that high serotonin levels in the brain caused hallucinations and other signs of excitation. Since cerebral serotonin levels cannot be raised by extracerebral injections of serotonin (2, 9) a precursor substance such as 5-hydroxytryptophan (II) was used as the standard for comparison of the serotonin activity of the new compounds. The test group consisted of four 25-Gm. white mice. All the activity measurements were conducted in an Actophotometer (Metro Industries, Long Island City, N. Y.) which is designed to measure the general activity of the mice. Subcutaneous doses of normal saline solution, 2 mg. of 5-hydroxytryptophan, or the molar equivalent of compounds III*a–d* (2.26, 2.40, 2.94, and 2.62 mg., respectively) were administered, and the activity of the mice was determined periodically during a period of 5 hr. It was necessary to add minimal amounts of 0.1 *N* NaOH to solubilize some of the compounds so that the total dose was contained in 0.2 ml. The second series consisted of the simultaneous injection of equimolar mixtures of 5-hydroxytryptophan (2 mg.) and the test compounds. The results are presented in Tables I and II.

DISCUSSION

While the synthesis of serotonin and 5-benzyloxytryptamine are not new, the preparation of these *N*-substituted amino acid derivatives does represent a new concept. It is well established that natural amino acids can readily pass through cell walls with the aid of active transport systems. Serotonin, however, cannot pass from the blood into the brain very readily (6–8); therefore, the joining of the hormone to an amino acid *via* a covalent bond to assist in transporting it into the brain proposes an interesting concept.

The biological testing of these compounds suggest some reasons for their lack of central activity. The data in Table I show that a 2.0-mg. dose of 5-hydroxytryptophan per mouse slightly lowered

TABLE I.—ACTIVITY OF MICE[a] FOLLOWING SUBCUTANEOUS INJECTIONS OF THE TEST COMPOUNDS

	Time, min.										
Compd.	10	15	30	45	60	90	120	150	180	240	300
Saline control	59	80	102	110	144	157	165	185	216	227	231
5-Hydroxytryptophan[b]	19	21	59	60	74	82	82	91	91	95	140
III*a* alanine der.[c]	50	75	99	102	131	145	155	171	194	214	214
III*b* valine der.[c]	57	71	118	118	119	129	129	145	145	157	...
III*c* phenylalanine der.[c]	70	80	85	85	85	89	94	99	111	112	...
III*d* isoleucine der.[c]	58	66	104	...	149	173	173	173	175	181	189

[a] In terms of Actophotometer readings. [b] At a level of 2 mg./mouse; 15 mg./mouse produced marked excitement, convulsions, and agitation (15). [c] At an equimolar dose level with 5-hydroxytryptophan.

TABLE II.—ACTIVITY OF MICE[a] FOLLOWING SUBCUTANEOUS INJECTIONS OF MIXTURES OF 5-HYDROXYTRYPTOPHAN AND TEST COMPOUNDS

	Time, min.										
Mixture	10	15	30	45	60	90	120	150	180	240	300
Saline	59	80	102	110	144	157	165	185	216	227	231
5-HTP[b] + III*a*[c]	28	37	57	57	60	74	75	75	95	104	141
5-HTP[b] + III*b*[c]	39	51	64	67	86	90	102	102	105	132	149
5-HTP[b] + III*c*[c]	42	61	99	102	102	140	140	142	147	166	166
5-HTP[b] + III*d*[c]	31	68	100	101	118	123	151	169	174	179	185

[a] In terms of Actophotometer readings. [b] 5-Hydroxytryptophan at a level of 2 mg./mouse. [c] Test compounds at a concentration equal to the molar equivalent of 5-hydroxytryptophan.

the generalized activity when compared to that of a saline control. This is substantiated by the work of Woolley (15), who also noted that 2.5-mg. doses of 5-hydroxytryptophan per mouse caused either normal or slightly subdued activity. A possible reason for the lack of activity of small doses of 5-hydroxytryptophan is suggested by Gal *et al.* (16), who observed that intracerebral injections of labelled L-tryptophan in rats and pigeons produced a significant increase in cerebral serotonin levels; D-tryptophan, however, did not increase the serotonin levels but in fact caused a decrease. They also noted that intraperitoneal injections of L-tryptophan, at a dose level 17 times greater than the intracerebral dose did not result in the formation of any labelled serotonin in the brain. While it is true that Gal used tryptophan and not 5-hydroxytryptophan, there may be still some analogy. In the present study racemic 5-hydroxytryptophan was injected subcutaneously. Possibly some of the D-5-hydroxytryptophan entered the brain where it acted as an antagonist to the decarboxylation of L-5-hydroxytryptophan resulting in a decreased cerebral serotonin level with generalized reduction of activity. Another factor to be considered is that other organs besides the brain such as the liver can decarboxylate 5-hydroxytryptophan and bind serotonin resulting from extracerebral administration. These factors may account for the lack of stimulation demonstrated by the 2-mg. dose of 5-hydroxytryptophan. Woolley (15) also stated that large doses of 5-hydroxytryptophan (15 mg./mouse) produced marked excitement, convulsions, and agitation. It appears thus that the smaller doses are metabolized outside the brain and produced the classical symptoms on the gastrointestinal tract and blood pressure. The administration of larger amounts may be partially available to the brain because the extracerebral metabolism sites become saturated and allow some to enter the brain, thus resulting in central stimulation.

The data in Tables I and II show that the derivatives do not produce greater stimulation than 5-hydroxytryptophan when used alone or in combination with the latter compound; in fact, there is a diminution of central activity when compared to the saline control. While it is possible that the use of larger doses of the derivatives may have caused some evidence of central activity, higher doses could not be used because of the pain and tissue necrosis at the injection site. Consequently, one would not have been able to determine if the increased activity was due to central stimulation or to pain of injection.

In conclusion, therefore, the reason for the lack of central stimulation is not readily apparent at this time.

SUMMARY AND CONCLUSIONS

1. The synthesis of a series of *N*-(5-hydroxy-3-indole-ethyl) amino acid derivatives containing alanine, valine, phenylalanine, and isoleucine is described.

2. These compounds were evaluated in mice to determine their effect on generalized activity.

3. The results indicated that these compounds when used either alone or in combination with 5-hydroxytryptophan did not produce any significant increase in activity over the 5-hydroxytryptophan standard.

4. The results suggest that these compounds either are unable to enter the brain or that they are functioning as metabolic antagonists of 5-hydroxytryptophan.

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Applications of Aqueous Thermometric Titration to Pharmaceutical Analysis

By ALBERT B. DELEO and MARVIN J. STERN*

Aqueous thermometric titration with standard HCl, NaOH, or $AgNO_3$ as titrant was used successfully for the analyses of aminophylline (ethylenediamine and theophylline), chlorpheniramine maleate, chlorpromazine hydrochloride, hydrochlorothiazide, and niacinamide. Most of the determinations were based on titration volumes. In cases where the curvature of the enthalpogram in the vicinity of the end point was severe, the quantitative result was derived from the temperature change due to the reaction. Exploratory studies of the applicability of simple aqueous thermometric titration, without prior separation of ingredients, to the analysis of solid and liquid dosage forms of the above compounds were conducted. With all but 1 of the solid dosage forms investigated, no interferences from inert ingredients were observed. In some of the liquid dosage form systems studied, the vehicles interfered with the titrations, while in others they did not.

THE POSSIBLE application of aqueous thermometric titration to pharmaceutical analysis was reported in a recent communication from this laboratory (1). The present paper describes the procedures and results of the aqueous thermometric titration of selected pure medicinal substances and of some preliminary studies concerning the application of thermometric titration to dosage form analysis.

The large number of systems to which thermometric titration may be applied has been the subject of several reviews.[1] The investigation described in this report was concerned primarily with simple acid-base and precipitation reactions of nitrogenous compounds. It is well known[1] that a thermometric titration will often produce a well-defined end point in a system where free energy methods such as potentiometric titration fail. Thus, in the first phase of this study, compounds whose official or other assay procedures require *nonaqueous* titration were chosen for testing the applicability of *aqueous* thermometric titration.

The second phase of this study was concerned with the assay of dosage forms. At present, "inert" binder, filler, and sealer materials, required to manufacture most solid dosage forms, often necessitate the use of separation procedures prior to the assay of the active ingredients. The problem also exists with liquid dosage forms where "inert" substances in the vehicles often interfere with the use of visual indicators and electrodes. Unfortunately, most separation procedures are tedious as well as time consuming.

While the enthalpogram for a thermometric titration of a single reacting substrate undergoing a single-step reaction is characterized (ideally) by a single "break" at the end point, the enthalpogram for a titration of 2 substrates (or a single substrate undergoing a 2-step reaction) may or may not be characterized by 2 breaks. First, the free energies of the 2 reactions must be sufficiently different so that the reactions do not occur simultaneously. Second, the enthalpies of the 2 reactions must also be sufficiently different so that the point where one reaction ceases and the second commences is discernible. One hopes then that the enthalpogram of a thermometric titration of a dosage form will be characterized by a single end point corresponding to the reaction of the active ingredient (*i.e.*, the other substances present do not react) or, if more than one substance reacts, that the thermodynamic factors are favorable, so that the enthalpogram exhibits a series of end points, the volumes between the end points corresponding to individual reactions occurring during the titration. If either hope is fulfilled, the need for prior separation of the active ingredient is eliminated.

EXPERIMENTAL

Materials.—Powdered samples of aminophylline U.S.P. (anhydrous), chlorpheniramine maleate U.S.P.,[2] chlorpromazine hydrochloride U.S.P.,[3] hydrochlorothiazide U.S.P.,[4] and niacinamide U.S.P.

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[1] See for example: Zenchelsky, S. T., *Anal. Chem.*, **32**, 289R(1960); Jordan, J., and Ewing, G. J., "Handbook of Analytical Chemistry," Meites, L., ed., McGraw-Hill Book Co., Inc., New York, N. Y., 1963, Sect. 8, p. 3ff.; Wendlandt, W. W., "Chemical Analysis," vol. 19, Elving, P. J., and Kolthoff, I. M., eds., Interscience Publishers, Inc., New York, N. Y., 1964, Chap. 8, p. 271ff.

[2] Supplied by Schering Corp., Bloomfield, N. J.
[3] Supplied by Smith Kline & French Laboratories, Philadelphia, Pa.
[4] Supplied by Ciba Pharmaceutical Co., Inc., Summit, N. J., and Merck Sharp & Dohme Research Laboratories, West Point, Pa.

were dried according to standard procedures but not purified further. Dosage forms were purchased on the open market. Standard HCl and NaOH solutions were purchased as certified volumetric solutions. Standard $AgNO_3$ solutions were prepared from reagent grade $AgNO_3$. Only recently boiled water was used throughout. The acetone used in the hydrochlorothiazide titrations was reagent grade.

Apparatus.—The titration cell assembly, which consisted basically of a small stoppered standard Dewar flask, a 2000-ohm bead-in-glass-probe thermistor, a right-angled titrant delivery tip, and a glass stirrer with 4 quadrantal blades, is shown in Fig. 1. The thermistor and delivery tip extended to ~3 mm. above the stirrer blades. The delivery tip was positioned so that the flow of titrant was counter to the flow of the solution in the flask. The stirrer was powered with a Synchro-Tork stirring motor (General Laboratory Supply Co., Paterson, N. J.). Titrant was delivered with a 0.6 ml./min., constant-flow Menisco-matic buret (American Instrument Co., Inc., Silver Spring, Md.).

The titration cell, stirring motor, and buret were enclosed in a constant-temperature air bath, maintained at 25.0 ± 0.1°. The air bath was a closed system in which air was recirculated. Cooling was achieved with copper cooling coils, in and around the air duct, through which cold water was circulated. A 200-w. (at 115 v.a.c.) heating coil in the air duct was set, with an auto-transformer, to maintain the air bath at approximately 24.5°. A 100-w. (at 115 v.a.c.) heating coil, also in the air duct, was controlled with a Micro-Set (mercury-column) thermoregulator (Precision Scientific Co., Chicago, Ill.) set at 25.0°. The voltage to the 100-w. heater was adjusted with a power rheostat to give minimum "overshoot." The exhaust fan and air entry portals were diagonally opposite each other and were equipped with deflector vents to cause turbulent flow of air in the bath. The temperature could be maintained at the desired level over extended periods. The bath was also equipped with a safety

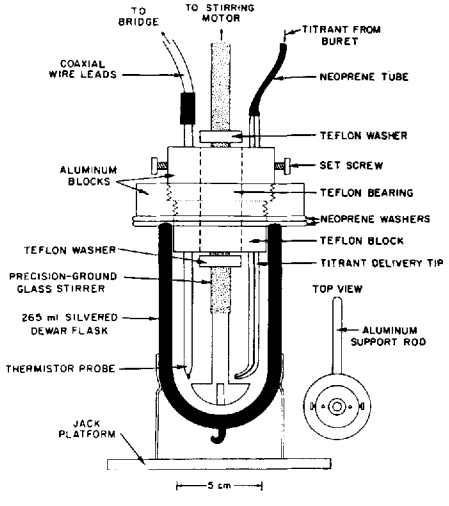

Fig. 1.—Titration cell assembly.

circuit breaker which automatically shut off the 200-w. heater in case the temperature of the bath exceeded 35°. Switches on the air bath permitted the operation of the stirrer and buret from the outside.

The thermistor was actually 1 arm of a simple bridge circuit, only slightly modified from the one described by Jordan and Alleman (2). The imbalance potential of the bridge was recorded with a 5-mv. Dynamaster recorder (The Bristol Co., Waterbury, Conn.). In cases where the magnitude of the heat change was low, the bridge signal was amplified by a factor of 5 with a solid-state variable recorder amplifier (Instruments and Communications, Inc., Wilton, Conn.).

Titration Procedure

Pure Substances.—One-liter volumetric solutions were prepared and allowed to reach thermal equilibrium, along with the standard titrants, for several hours in the air bath. The slow and low solubility of hydrochlorothiazide in water necessitated dissolving the compound in 20 ml. of acetone and slowly dispersing the acetone solution in water while stirring. The small amount of acetone (2%) appeared to have negligible effect on the titrations.

Exactly 100 ml. of solution was placed in the Dewar flask which was then positioned on the stationary titration cell head with a laboratory jack. The stirrer and bridge were turned on and the system allowed to attain thermal equilibrium. Equilibration generally required 5–10 min. and was indicated by a linear base line on the recorder. The buret was then turned on and the titration allowed to proceed until well after the end point(s) was reached.

Dosage Forms.—All dosage forms were titrated without prior separation procedures. A suitable quantity of the dosage form was dispersed, in the Dewar flask, in enough thermally equilibrated (25.0 ± 0.1°) water to make ~100 ml. In the case of hydrochlorothiazide tablets, a slurry of the powdered tablet in 5 ml. of acetone was prepared and then diluted to ~100 ml. with water. Titrations were then performed in the same manner as described for pure substances.

U.S.P. Assays

The pure substances were also analyzed by the procedures (usually nonaqueous titration) given in the U.S.P. (3).

Treatment of Data

An idealized enthalpogram for the thermometric titration of a single substrate undergoing a single reaction is shown in Fig. 2. The factors contributing to the various portions of the curve have been discussed. (See *Footnote 1.*) The section of the curve between the start of the titration and the end point is called the titration branch. The extrapolation method for obtaining the titration volume, ΔV, and the temperature rise *due to the reaction* (in arbitrary units), ΔT, is indicated on the curve.[5] For 1 titration system, chlorpromazine hydrochloride *versus* NaOH, ΔV's were also determined with

[5] Enthalpograms are usually recorded with a temperature rise reflected as an *increased* voltage. In the present investigation, this convention was used.

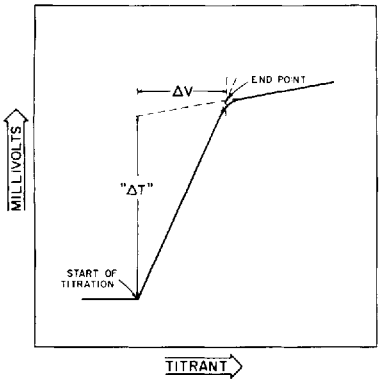

Fig. 2.—Idealized enthalpogram.

an automatic end point determination device. The procedure used has been described (4).

Pure Compounds.—Most of the analyses of the pure compounds were made by calculating the amount of substrate from the measured ΔV and the known concentration of the titrant. In 2 cases, theophylline in aminophylline *versus* NaOH and hydrochlorothiazide *versus* NaOH, the curvature in the vicinity of the end point was too severe to allow reliable extrapolation for measurement of ΔV. In these cases, quantitative determinations were made from the ΔT measurements. ΔT values from titrations of various concentrations (always 100 ml.) of the compound under consideration and of hydrochloric acid *versus* 2.0 *M* NaOH were measured. The slopes, B, of plots of ΔT *versus* molarity of substrate, M, were obtained by least-square fitting to the equation $\Delta T = BM$. The relative slope, B_H/B_C, where the subscripts H and C refer to hydrochloric acid and the compound under consideration, respectively, was evaluated. Since the B's were obtained from titrations of dilute solutions run under essentially the same conditions,[6] the relative slope should be identical to the ratio of the molar enthalpies of the reactions. Once B_H/B_C has been determined, a solution of unknown concentration of the compound under consideration can be analyzed by performing a titration of the unknown and following it with a titration of known concentration of hydrochloric acid run under identical conditions. It is easy to show that

$$M_C = M_H\,(\Delta T_C/\Delta T_H)\,(B_H/B_C) \quad \text{(Eq. 1)}$$

where the relative temperature rise, $\Delta T_C/\Delta T_H$, is determined from the enthalpograms, and M_H is the known concentration of hydrochloric acid.

Dosage Forms.—The amounts of active ingredients in the dosage forms were calculated by an absolute method and/or a standard addition method. In the absolute method, the amount of active ingredient was calculated from ΔV and the concentration of the titrant. In the standard addition method, ΔV for the titration of the dosage form was compared, in the obvious manner, to ΔV for a similar titration of the same amount of dosage form plus an accurately weighed portion of active ingredient. In the standard addition method, the purities of the added portions of active ingredients were taken to be exact.

Dosage form analyses were not carried out with ΔT measurements, because the results of the ΔT analyses of the pure compounds indicated that there are uncertainties involved in measuring relative heats without a calibration heater. In addition, there is no assurance in a dosage form titration that all reactions have ceased after the last observed end point. The later condition is necessary for the application of the back-extrapolation method for obtaining ΔT shown in Fig. 2.

RESULTS AND DISCUSSION

Pure Compounds.—Figures 3 and 4 show typical enthalpograms for the 8 titration systems investigated in detail. The 3 systems represented in Fig. 4 involve precipitate formation.

An enthalpogram for the aqueous thermometric titration of chlorpheniramine maleate *versus* HCl, corresponding to the official nonaqueous titration (3), is shown in Fig. 3, curve A. Although the end point is fairly rounded, extrapolation is not difficult. The 1 end point observed results from the protonation of the pyridine nitrogen of the chlorpheniramine; in the official nonaqueous titration, the

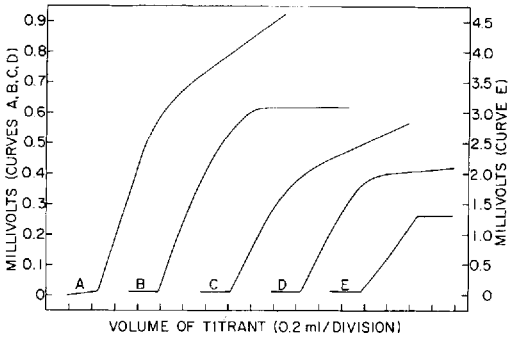

Fig. 3.—Typical enthalpograms. Key: A, 0.01 *M* chlorpheniramine maleate *vs.* 2 *M* HCl; B, 0.008 *M* aminophylline *vs.* 2 *M* NaOH; C, 0.01 *M* niacinamide *vs.* 2 *M* HCl; D, 0.0027 *M* hydrochlorothiazide *vs.* 1 *M* NaOH; E, 0.005 *M* aminophylline *vs.* 2 *M* HCl.

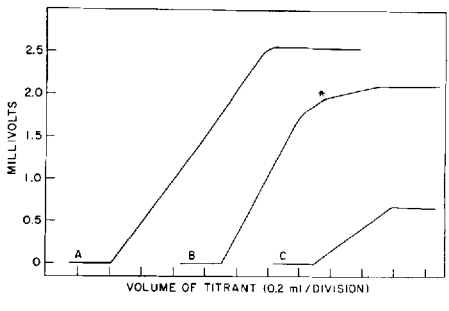

Fig. 4.—Typical enthalpograms. Key: A, 0.005 *M* aminophylline *vs.* 1 *M* $AgNO_3$; B, 0.01 *M* chlorpheniramine maleate *vs.* 2 *M* NaOH; C, 0.01 *M* chlorpromazine hydrochloride *vs.* 2 *M* NaOH.

[6] The only condition which was purposely varied was the bridge sensitivity which was set a factor of 5 more sensitive for the theophylline in aminophylline and hydrochlorothiazide titrations than for the hydrochloric acid titrations. A correction was applied to account for the nonlinearity of the sensitivity scale.

TABLE I.—THERMOMETRIC TITRATIONS OF PURE COMPOUNDS

Compd.	Thermometric Titration Analysis: Concn., 100 ml.	Titrant	Titrations, No.	% Found	U.S.P. Analysis: Titrations, No.	% Found
Chlorpheniramine maleate	0.01 *M*	2.0 *M* HCl	6[a]	99.36 ± 0.30	5	99.64 ± 0.18
	0.01 *M*	2.0 *M* NaOH	5	97.81 ± 0.57[b]		
				99.80 ± 0.23[c]		
Niacinamide	0.01 *M*	2.0 *M* HCl	6	98.62 ± 0.37	5	99.03 ± 0.14
Chlorpromazine hydrochloride	0.01 *M*	2.0 *M* NaOH	6	98.58 ± 0.31	5	99.89 ± 0.20
	0.01 *M*	2.0 *M* NaOH	5[a]	100.15 ± 0.70[d]		
	0.01 *M*	1.0 *M* NaOH	5	99.69 ± 0.33[e]		
Hydrochlorothiazide	0.0027 *M*	1.0 *M* NaOH	5	99.67 ± 0.20[c]	5	100.76 ± 0.29
Aminophylline	0.005 *M*					
Ethylenediamine[f]		2.0 *M* HCl	5	98.90 ± 0.25	5	100.58 ± 0.11
Theophylline[f]		1.0 *M* $AgNO_3$	5	99.93 ± 0.80	5	99.25 ± 0.40

[a] One titration eliminated by Chauvenet's criterion. [b] Based on first end point. [c] Based on second end point. [d] Automatic end point determination apparatus; automatic buret; lag time taken as 0.050 min. (See *Reference 4.*) [e] Automatic end point determination apparatus; manual buret; lag time taken as 0.050 min. (See *Reference 4.*) [f] Percentages based on theoretical content in anhydrous aminophylline (2 moles theophylline:1 mole ethylenediamine).

bimaleate ion is titrated along with the chlorpheniramine.

An enthalpogram for the titration of chlorpheniramine maleate *versus* NaOH is shown in Fig. 4, curve B. Two true end points appear, the first corresponding to the neutralization of the bimaleate ion and the second to the neutralization of the protonated amine moiety of the chlorpheniramine ion. The false "end point" indicated by the asterisk has no stoichiometric relationship and is due to the delayed precipitation of the chlorpheniramine base.

Aqueous titration of niacinamide *versus* HCl, corresponding to the official nonaqueous titration (3), resulted in an enthalpogram (Fig. 3, curve C) with a rounded end point. Extrapolation to the end point is, however, not difficult.

Aqueous thermometric titration of chlorpromazine hydrochloride *versus* HCl, corresponding to the official nonaqueous titration (3), failed to exhibit an end point since the phenothiazine nitrogen is not sufficiently basic to become protonated in aqueous solution. Neutralization of the protonated amine nitrogen with base, however, resulted in an enthalpogram with an extremely sharp end point (Fig. 4, curve C).

The official assay procedure for hydrochlorothiazide requires titration in *n*-butylamine with sodium methoxide in benzene as titrant (3). Aqueous thermometric titration with NaOH involves 2 equivalents of base, since there are 2 sulfonamide groups in hydrochlorothiazide. Midway in the titration branch of the enthalpogram (Fig. 3, curve D), there is a slight change in slope, corresponding to the first end point. The change is not sharp enough to permit precise determination of this end point. The second end point is quite rounded so that extrapolation to either end point is subject to large errors. Thus, for the hydrochlorothiazide determinations, ΔT rather than ΔV analyses were carried out.

Aminophylline is a mixture of 2 moles of theophylline to 1 mole of ethylenediamine. The official assay (3) involves separate procedures for the 2 components. Ethylenediamine is analyzed by aqueous titration with HCl, and theophylline is determined by precipitation with $AgNO_3$ and titration of the excess silver ion with NH_4SCN. Medwick and Schiesswohl (5) have developed a nonaqueous potentiometric analysis for both components which uses acetous perchloric acid as titrant and an acetic acid–acetic anhydride solvent.

Three aqueous thermometric titration systems for aminophylline analysis were investigated. Titration with HCl resulted in an enthalpogram (Fig. 3, curve E) indicating reaction with ethylenediamine only. The change in slope midway in the titration branch is the end point for the protonation of 1 of the amine groups, but this point is difficult to determine precisely. The second end point, however, is extremely sharp.

Aqueous thermometric titration of aminophylline *versus* base is feasible due to the acidic nature of theophylline. The resulting enthalpogram (Fig. 3, curve B) is extremely curved throughout the titration branch so that only ΔT analysis can be performed precisely.

Direct titration of the theophylline in aminophylline *versus* $AgNO_3$ resulted in an enthalpogram (Fig. 4, curve A) with a well-defined end point. There is a very slight change of slope approximately midway in the titration branch which is probably due to initial supersaturation followed by precipitation (similar to the false "end point" in the chlorpheniramine maleate *versus* NaOH system). Keily and Hume have observed similar changes in slope in nonaqueous thermometric titrations involving precipitations (6). The best stoichiometric end point for the aminophylline *versus* $AgNO_3$ titrations resulted from taking an average of the end points obtained by extrapolating segments of the titration branch on both sides of the slight change in slope. However, because the change in slope is so slight, little error (~1%) is introduced if only the final straight portion of the titration branch is used to locate the end point.

The quantitative results of the aqueous thermometric titrations (ΔV analyses) of the 5 compounds under investigation are given in Table I.[7] Included in the table are the results of the official assay procedures. The values obtained from the thermometric analyses are generally slightly lower but still in good agreement with the results of the U.S.P. analyses. The poor results obtained from the first end point in the chlorpheniramine maleate *versus* NaOH thermometric titrations can be ascribed to the difficulty in extrapolating the small segment

[7] All appended error limits in this paper are average deviations unless specified.

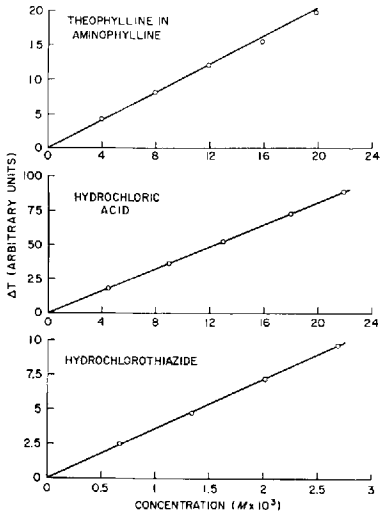

Fig. 5.—Plots of temperature rise *vs.* concentration of substrate for titrations with 2 *M* NaOH. The solid lines represent the least-square fits to $\Delta T = BM$, where the points were weighted as (average deviation)$^{-2}$. The average deviations of the points generally fall within the circles.

between the first end point and the false "end point." The reasonableness of the results of the hydrochlorothiazide *versus* NaOH thermometric titrations must be taken to be fortuitous because of the previously discussed uncertainty in the location of the end point.

Plots of ΔT *versus* concentration for aminophylline *versus* NaOH, HCl *versus* NaOH, and hydrochlorothiazide *versus* NaOH titrations are shown in Fig. 5. The lines drawn through the points were determined by least-square fitting to $\Delta T = BM$ with each point weighted as (average deviation)$^{-2}$. The relative slopes were found to be $B_{HCl}/B_{theophylline} = 4.004 \pm 0.048$ and $B_{HCl}/B_{hydrochlorothiazide} = 1.132 \pm 0.008$, where the appended error limits are estimated standard deviations. These results indicate that the use of Eq. 1 to determine theophylline or hydrochlorothiazide concentrations from thermometric titrations run concurrently with HCl *versus* NaOH titrations should be accurate to ~1%.

Approximately 6 months after the determinations of $B_{HCl}/B_{theophylline}$ and $B_{HCl}/B_{hydrochlorothiazide}$ were made, the ΔT procedure was tested by performing titrations with an entirely new apparatus (bridge, recorder, titration cell, etc.) and calculating the concentrations with Eq. 1. Such analyses yielded for theophylline (0.005 *M* aminophylline; 3 titrations) 94.97 ± 0.36% recovery, as compared to the U.S.P. analysis of 98.60 ± 0.17%. Similar titrations for hydrochlorothiazide (0.0017 *M;* 3 titrations) yielded 95.31 ± 0.44% recovery, as compared to the U.S.P. analysis of 100.76 ± 0.29%. (The U.S.P. analysis result is probably high, due to the broad color change of the indicator.) The poor agreements cast some doubt on the absolute values of the relative slopes.

The proposed method of using an external standard to determine heat changes suffers several disadvantages, especially when it is attempted to translate results from one apparatus to another. Bridge sensitivity nonlinearities are difficult to determine accurately, and they vary, depending on the bridge design, with the age and voltage of the batteries. In addition, it has been found that bridge response changes when switches are opened and closed again. Furthermore, factors such as differences in heat capacities due to differences in composition and volume (it is difficult to dry the titration cell completely between titrations) and distortions of the enthalpograms due to the dilution effect of the titrant can introduce errors. A more reliable method is one in which a calibrated heater immersed in the solution is used as an *internal* heat standard. This method would eliminate most of the possible sources of error discussed above, since the composition and volume of the solution could not change and the bridge need not be turned off or adjusted between calibration and titration. The effects of dilution by the titrant would still be a problem, but these effects should be small if the titrant is always much more concentrated than the solution to be titrated.

Solid Dosage Forms.—Thermometric titration of niacinamide tablets *versus* HCl, chlorpromazine hydrochloride sustained - release capsules *versus* NaOH, hydrochlorothiazide tablets *versus* NaOH, and aminophylline tablets *versus* NaOH resulted in enthalpograms which were almost identical to the enthalpograms obtained from the corresponding titrations of the pure substances. Thus, the inert materials in the dosage forms investigated do not undergo simple acid–base reactions and therefore do not interfere with the titrations of the active ingredients. Thermometric titration of aminophylline tablets *versus* $AgNO_3$ produced an enthalpogram which differed from that of the corresponding titration of pure aminophylline only in that the slight change in slope in the titration branch did not appear. The insoluble materials in the tablets probably act as "seeds" to prevent supersaturation. Enthalpograms for the titration of aminophylline tablets *versus* HCl are shown in Fig. 6. The curves are similar to that of the corresponding titration of pure aminophylline, and the end point is very sharp. The only difference is the fact that in the titration of tablets, the first end point does not appear (curve B) or appears as a slight change in slope (curve A) of opposite direction to that observed in the titration of pure aminophylline. The reason for this difference need not be of concern in strict analytical applications in which the second end point is used.

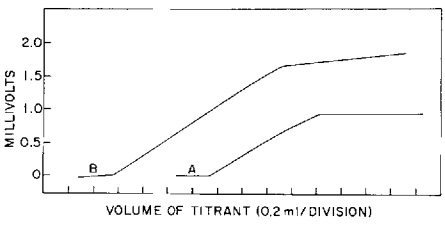

Fig. 6.—Enthalpograms for titrations of aminophylline tablets (100 mg.). Key: A, 2 tablets *vs.* 1 *M* HCl; B, 2 tablets plus 100 mg. aminophylline *vs.* 1 *M* HCl.

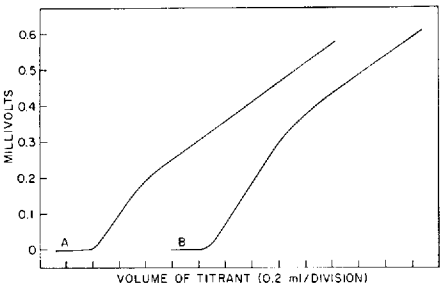

Fig. 7.—Enthalpograms for titrations of chlorpheniramine maleate tablets (4 mg.). Key: A, 30 tablets *vs.* 1 *M* HCl; B, 30 tablets plus 100 mg. chlorpheniramine maleate *vs.* 1 *M* HCl.

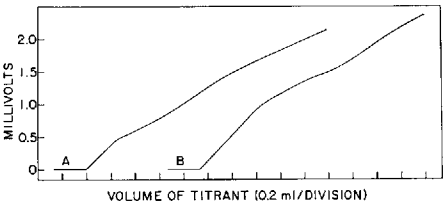

Fig. 8.—Enthalpograms for titrations of chlorpheniramine maleate tablets (4 mg.). Key: A, 30 tablets *vs.* 1 *M* NaOH; B, 30 tablets plus 100 mg. chlorpheniramine maleate *vs.* 1 *M* NaOH.

Enthalpograms for the titration of chlorpheniramine maleate tablets *versus* HCl and of a corresponding standard addition titration are shown in Fig. 7. The roundness at the start of the titration must be due to the reaction of some ingredient in the tablet with the acid. This reaction, however, is not "clean cut," as evidenced by the fact that the region of roundness expanded when additional chlorpheniramine maleate was added prior to the titration. The authors have found no reliable method of determining the initiation of the chlorpheniramine reaction itself. Estimations of ΔV did not yield quantitative results for either the absolute method or the standard addition method.

Titration of chlorpheniramine maleate tablets *versus* NaOH produced an enthalpogram quite different from the curve obtained in the corresponding titration of pure chlorpheniramine maleate. Figure 8 shows typical curves for the titrations of tablets alone and of tablets plus added chlorpheniramine maleate. The high final slopes indicate that some reaction was occurring even after ~2 ml. of 1.0 *M* NaOH had been added (~3 times the amount needed to completely neutralize 120 mg. of chlorpheniramine maleate). None of the breaks is easy to pick out as an end point. Attempts to obtain quantitative results from the first break with both the absolute and standard addition methods were unsuccessful.

The results of the thermometric titrations of the solid dosage forms studied are given in Table II. Although the curvature in the vicinity of the aminophylline *versus* NaOH and hydrochlorothiazide *versus* NaOH end points did not allow accurate extrapolation for absolute analyses, the standard addition method was feasible since errors tend to cancel if the curves are always extrapolated in the same manner. For the hydrochlorothiazide standard additional analyses, "end points" were obtained by extrapolating the initial portions of the titration branches and the final segments of the curves to their intersections; *i.e.*, the fact that there are actually 2 end points was ignored.

Since this phase of the investigation was exploratory in nature, detailed evaluations of precision and accuracy were not attempted. The over-all results are quite favorable and indicate that thermometric titrations of many solid dosage forms without prior separation of the active ingredients are feasible. The rather large error limits encountered with some systems may be due to the fact that individual dosage forms were titrated. The differences between the results of the absolute and standard addition methods may also be due, at least in part, to the use of individual dosage forms as well as to the assumption of 100% purity of the added portion of active ingredient. The latter may be particularly important in the titrations involving aminophylline, which is not always homogeneous.

The most interesting results of these exploratory studies is the fact that, with the exception of the titrations of chlorpheniramine maleate tablets, even the simple acid–base titrations of the solid dosage forms indicated negligible interference due to inert ingredients.

Liquid Dosage Forms.—The general objective of this phase of the investigation was to determine if, without detailed knowledge of the composition of a

TABLE II.—THERMOMETRIC TITRATIONS OF SOLID DOSAGE FORMS

Dosage Form	Dosage Units Titrated, No.	Determinations, No.	Titrant	Amt. Added[a] in S.A. Method, mg.	% Labeled Amt. Absolute	% Labeled Amt. Std. Addition
Niacinamide tablets, 100 mg.	1	3	1.0 *M* HCl	100	107.33 ± 0.31	111.25 ± 0.69
Chlorpromazine hydrochloride sustained-release capsules, 200 mg.	1	2	1.0 *M* NaOH	200	103.51 ± 0.93	105.22 ± 1.45
Hydrochlorothiazide tablets, 50 mg.	1	2	1.0 *M* NaOH	50	[b]	98.76 ± 0.96
Aminophylline tablets, 100 mg.						
Ethylenediamine[c]	1, 2	2	1.0 *M* HCl	100[d]	92.48 ± 0.22	92.29 ± 3.16
Theophylline[c]	1	4	1.0 *M* NaOH	100[d]	[b]	109.92 ± 6.78
Theophylline[c]	1	2	0.5 *M* $AgNO_3$	100[d]	96.56 ± 0.55	101.63 ± 0.48

[a] Purity of added active ingredient taken to be exact. [b] Extreme curvature in vicinity of end point(s) precluded direct analysis. [c] Percentages based on theoretical content in anhydrous aminophylline (2 moles theophylline:1 mole ethylenediamine). [d] Aminophylline.

TABLE III.—THERMOMETRIC TITRATIONS OF LIQUID DOSAGE FORMS

Dosage Form	Titrated, ml.	Determinations, No.	Titrant	Aminophylline Added[a] in S.A. Method, mg.	% Labeled Amt. Absolute	% Labeled Amt. Std. Addition
Aminophylline elixir[b]						
Ethylenediamine[c]	25	2	1.0 *M* HCl	100	94.00 ± 0.07	102.63 ± 0.32
Theophylline[c]	10	1	1.0 *M* NaOH	100	[d]	97.16
Theophylline[c]	10	2	0.5 *M* $AgNO_3$	100	103.88 ± 0.85	102.77 ± 0.40
Aminophylline compound syrup[e]						
Theophylline[c]	10	2	0.5 *M* $AgNO_3$	50	81.33 ± 0.99	82.71 ± 0.36
KI				...	89.87 ± 0.76[f]	...
Compound elixir[g]						
KI	10, 15	2	0.5 *M* $AgNO_3$	...	98.62 ± 0.15	...

[a] Purity of added aminophylline taken to be exact. [b] 12.5 mg. aminophylline and 3.125 mg. diphenhydramine/ml. [c] Percentages based on theoretical content in anhydrous aminophylline (2 moles theophylline:1 mole ethylenediamine). [d] Extreme curvature in vicinity of end point precluded direct analysis. [e] 10 mg. aminophylline, 2.5 mg. diphenhydramine, 7.5 mg. KI, and 2 mg. $CHCl_3$/ml. [f] Average of 4 titrations: 2 of 10 ml. syrup and 2 of 10 ml. syrup plus 50 mg. added aminophylline. [g] 10 mg. KI, 3 mg. theophylline, 0.8 mg. ephedrine sulfate, 0.4 mg. phenobarbital, and 0.167 mg. isoproterenol hydrochloride/ml.

liquid dosage form, one could correlate end points of enthalpograms with the reactions of individual active ingredients. Only a few systems were examined and only sparse quantitative data were obtained.

Titrations of a chlorpheniramine maleate syrup (0.5 mg./ml.) *versus* NaOH and *versus* HCl produced enthalpograms exhibiting single end points which did not correlate with the chlorpheniramine maleate content. When additional chlorpheniramine maleate was added to the syrup prior to the titrations, the titration volumes increased, but no correlation with the original chlorpheniramine maleate content could be made by standard addition analysis. The high final slopes of these enthalpograms indicated that reactions were still occurring even after several times the stoichiometric amounts of titrant had been added.

Results similar to those obtained with the chlorpheniramine maleate syrup titrations were obtained with titrations of a syrup containing 10 mg. aminophylline, 2.5 mg. diphenhydramine, 7.5 mg. KI, and 2 mg. $CHCl_3$ per ml. (this syrup will be referred to subsequently as "aminophylline compound syrup") *versus* NaOH and titrations of a chlorpromazine hydrochloride syrup (2 mg./ml.) *versus* NaOH. In the latter titration system, the volume to the end point did not increase upon the addition of excess chlorpromazine hydrochloride. Titration of aminophylline compound syrup *versus* HCl produced an enthalpogram, shown in Fig. 9, curve A, which does not exhibit any real evidence of reaction.

Thus, in all of the above liquid dosage form titration systems, there is interference from other (inert and/or active) ingredients. In the absence of detailed information as to the compositions of the liquids, any discussion of the natures of the interferences would be highly speculative.

On the other hand, titrations of an elixir containing 12.5 mg. aminophylline and 3.125 mg. diphenhydramine per ml. (subsequently referred to as "aminophylline elixir") *versus* HCl, NaOH, or $AgNO_3$ produced enthalpograms almost identical to those obtained in the corresponding titrations of pure aminophylline. (The end points in the HCl titrations were slightly more rounded than in pure aminophylline *versus* HCl titrations.) Quantitative results obtained from the titrations of aminophylline elixir are given in Table III.

Figure 9, curve B, represents the titration of aminophylline compound syrup (content given previously) *versus* $AgNO_3$. The first end point is fairly sharp while the second is broadly rounded. Titrations with added active ingredients indicated that the first end point corresponds to iodide precipitation and the second end point to theophylline precipitation. Quantitative results from a few titrations are given in Table III. The reason for the low per cent recoveries obtained may be the fact that a sample of syrup from a very old, previously opened bottle whose history was unknown was used for the analyses.

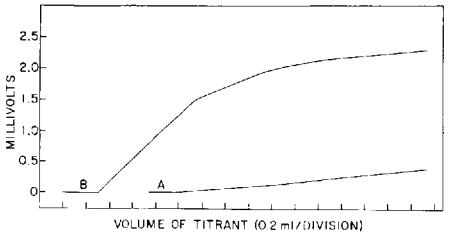

Fig. 9.—Enthalpograms for titrations of aminophylline compound syrup (10 mg. aminophylline, 2.5 mg. diphenhydramine, 7.5 mg. KI, and 2 mg. $CHCl_3$/ml.). Key: A, 25 ml. syrup *vs.* 1 *M* HCl; B, 10 ml. syrup *vs.* 0.5 *M* $AgNO_3$.

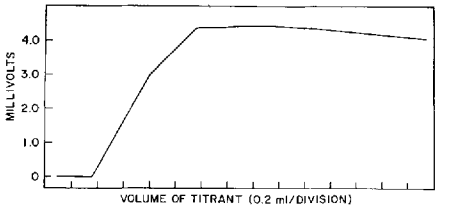

Fig. 10.—Enthalpogram for titration of 15 ml· compound elixir (10 mg. KI, 3 mg. theophylline, 0.8 mg. ephedrine sulfate, 0.4 mg. phenobarbital, and 0.167 mg. isoproterenol hydrochloride/ml.) *vs.* 2 *M* $AgNO_3$.

The last liquid dosage form studied was an elixir containing 10 mg. KI, 3 mg. theophylline, 0.8 mg. ephedrine sulfate, 0.4 mg. phenobarbital, and 0.167 mg. isoproterenol hydrochloride per ml. (This elixir will be referred to subsequently as "compound elixir".) Since several, if not all, of the active ingredients may precipitate silver ion, a complex enthalpogram for titration with $AgNO_3$ would be expected. The actual enthalpogram for such a titration is shown in Fig. 10. Titrations with added KI indicated that the first end point corresponds to iodide precipitation. The volume between the first and second end point is quantitatively in excess of the amount necessary for reaction with all of the other active ingredients combined. When KCl was added to the elixir prior to the titration, this volume increased without introducing any new breaks in the curve. This behavior indicates that the reaction that occurred between the first and second end points was precipitation of excess chloride ion present in the elixir. There is some indication from the enthalpogram that reactions occurred after the second end point, but the breaks are not sharp enough to pick out additional end points. The shape of the curve beyond the second end point was somewhat altered when titrations with added aminophylline were performed, but again no additional end points could be distinguished. Table III includes the results of KI determinations from the first end point in the compound elixir *versus* $AgNO_3$ titrations.

It appears from the rather sparse data presented in this section that simple thermometric titrations of liquid dosage forms are subject to far more interferences than are titrations of solid dosage forms. Some liquid dosage forms may be titrated successfully with nonspecific titrants without prior separation of ingredients. The applicability of such titrations to particular dosage forms must be determined on an individual basis. A more elegant approach, although one requiring knowledge of the composition of the vehicle, would be the use of more selective titrants.

SUMMARY

Aqueous thermometric titrations of chlorpheniramine maleate *versus* HCl or NaOH, niacinamide *versus* HCl, chlorpromazine hydrochloride *versus* NaOH, hydrochlorothiazide *versus* NaOH, ethylenediamine in aminophylline *versus* HCl, and theophylline in aminophylline *versus* NaOH or $AgNO_3$ yielded results with precision and accuracy sufficiently good to propose the methods as alternatives to the official assay procedures (generally nonaqueous titrations). In cases where the end points are extremely rounded (theophylline in aminophylline *versus* NaOH and hydrochlorothiazide *versus* NaOH), the usual volume determinations are not reliable but recourse to temperature-rise determinations is possible. The temperature-rise method used in this investigation employed titrations of HCl *versus* NaOH as heat references. Caution must be exercised if the relative heats determined on one apparatus are used for analyses performed with another apparatus. The use of a calibrated heater is suggested as a more reliable method.

Exploratory studies of aqueous thermometric titrations of solid dosage forms of the compounds listed above were conducted. These titrations employed the same titrants used for the analyses of the corresponding pure compounds. In all except the titrations of chlorpheniramine maleate tablets, negligible interference due to inert ingredients was observed. It appears that successful thermometric titrations of many solid dosage forms can be performed, either by direct volume analysis or by standard addition analysis, without prior separation of active ingredients.

Aqueous thermometric titrations of a few liquid dosage forms were attempted. While in some of the systems studied successful titrations without prior separation of ingredients could not be performed, in other systems no interference from the vehicle was observed. In some titrations of liquid dosage forms with $AgNO_3$, several end points were observed, some of which could be correlated quantitatively with specific ingredients in the mixtures.

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Micromeritics of Granular Pharmaceutical Solids I

Physical Properties of Particles Prepared by Five Different Granulation Methods

By DALE E. FONNER, JR., GILBERT S. BANKER, and JAMES SWARBRICK

Granular pharmaceutical solids, prepared by 5 different granulation methods, were evaluated on the basis of repose angle, hardness, density, number of particles per gram, bulk density, shape volume factor, bulk volume, and geometrical form. Particular attention was given to the development of an accurate technique for evaluating shape volume factor as a means of characterizing the over-all shape of a particle. The findings indicated that repose angle was primarily a function of surface roughness and that geometrical form and shape on a volume basis were inversely proportional to bulk volume. The determination of these easily computed parameters is therefore of value in assessing manufacturing procedures which have as their primary objective the production of smooth, spherical particles suitable for pharmaceutical coating purposes.

THE STANDARDIZATION of processing methods, to achieve uniform product specification, is a constant goal of the pharmaceutical industry. Uniformity of drug distribution and standardization of dosage form characteristics, both within a single dosage unit as well as between dosage units, has become increasingly important in view of the more potent drugs in use today and the continual developments in the area of controlled release dosage forms.

Granulation is a key process in the production of many dosage forms involving the controlled release of a drug from coated or matrix-type particles, regardless of whether conventional granulation methods or the newer techniques of spray drying or fluidized bed processing are employed.

The production of uniform tablet dosage units has been shown to be dependent upon several granular properties. Arambulo *et al.* (1) found that as the granule size was decreased, the variation in tablet weight also decreased, reaching a minimum weight variation at a 400–800 μ granule diameter. Further reduction in granule size led to an increase in tablet weight variation. The particle size distribution also affects tablet weight, as well as exerting a marked influence upon tablet hardness (2). Particle size distribution and other particulate properties, by affecting the internal flow and segregation of a granulation, can vary the composition of compressed tablets (3).

Physical properties of granules, such as specific surface, shape, hardness, surface characteristics, and size, can profoundly influence the rate of dissolution of drugs contained in heterogeneous solid dosage forms. According to Wagner (4), when the granule size exceeds 10 μ the rate of dissolution is directly proportional to the surface area. By using spherical inert granules coated with various thicknesses of tolbutamide, Nelson *et al.* (5) demonstrated the dependence of the excretion rate of the drug upon the available surface area. The effect of the granule size upon the dissolution of tripelennamine hydrochloride (6) and aspirin (7) have been investigated also.

To date, most of the physical studies of the properties of granular pharmaceutical solids have been concerned with the evaluation of repose angle (8–12) and particulate density or porosity (13, 14). The application of such physical parameters as shape, bulk density or packing volume, the number of particles per gram, and particulate hardness to the characterization and utilization of granular pharmaceutical solids has apparently gone unreported. With the development of the many new processing methods designed to produce granular particles (15–21), the need to apply physical measurements of a quantitative nature to pharmaceutical granulations to achieve a rational selection of a particular process or piece of equipment is greater than at any previous time. The purpose of this report is to present simple, reproducible procedures which may be used to estimate certain physical parameters of granular pharmaceutical solids, and to compare, on this basis, granules prepared using 5 different granulation methods.

EXPERIMENTAL

Preparation of the Base Formula and Granulating Solution.—A base formula of 90% lactose and 10% starch was selected as the model system for this study. The powders were passed through a 60-mesh screen in batches of 10 Kg. and mixed in a model c-10 Hobart mixer[1] for 15 min. Portions of this mixed

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[1] Hobart Manufacturing Co., Troy, Ohio.

TABLE I.—GRANULATION METHODS AND CONDITIONS EMPLOYED IN THE MANUFACTURE OF GRANULES

Granulation Method	Batch Size, Gm.	% v/w Granulating Soln. Used	Operating Conditions
Oscillating granulator[a]	2000	11.25	No. 12 screen
Hand screen	2000	12.50	No. 12 screen
Colton upright[b]	2000	12.50	Circular openings equivalent to 12-mesh screen
Liquid-solids V-blender[c]	3100	17.74	.....
Fitzpatrick comminutor[d]	2000	18.75	Knives forward, high speed, no screen

[a] Stokes oscillator, model 43A, F. J. Stokes Machine Co., Philadelphia, Pa. [b] Colton upright granulator, type 3WG, Arthur Colton Co., Division of Cherry-Burrell Corp., Cedar Rapids, Iowa. [c] Liquid-solids V-blender, type LB 2501, Patterson-Kelley Co., East Stroudsburg, Pa. [d] Fitzpatrick comminutor, model M, W. J. Fitzpatrick Co., Chicago, Ill.

powder were massed with the granulating solution and separately processed by each granulation method (Table I). The granulating solution had the following composition:

Gelatin powder	480.0 Gm.
Acacia powder	160.0 Gm.
Distilled water	4000.0 Gm.
Methylparaben	10.0 Gm.
Propylparaben	2.0 Gm.

The granulating solution was prepared by dissolving the solute components in the hot distilled water. The granulating solution was employed at a temperature of 47°. Each granulation was air dried for 72 hr. at about 26° and 15% R.H. and was then sized through a 12-mesh hand screen. The granulations were stored in air-tight jars at room temperature until required.

Sufficient granulating solution was used to make the granules as hard as possible, and so minimize particulate fracture during the evaluative procedures.

In order to minimize variations of those physical parameters dependent upon particle size, a 20/30-mesh sieve fraction of each granulation was used, except where otherwise stated. The sieve fractions were obtained when required by processing 300–400-Gm. samples of the granulation through a nest of sieves shaken for 15 min. on a Cenco-Meinzer sieve shaker[2] operating on speed setting 5.

The moisture content of each granulation was determined using a Cenco moisture balance[2] operating at a temperature of 115°. The relative humidity was recorded using a Serdex relative humidity indicator, model 122-7005.[3]

Physical Measurements.—*Repose Angle, θ.*—Measurements of repose angle were obtained using an apparatus similar to that described by Pilpel (12), except that the cones were formed on a piece of arithmetic grid graph paper rather than on a brass plate.

One hundred milliliters of 20/30-mesh material was introduced into a copper cylinder, 16.5 cm. in length and 3.8 cm. internal diameter, which then was raised vertically through a pulley drive at the rate of approximately 0.2 mm./sec. The height, l, of the deposited granular cone was determined using a microcathetometer[4] and the base diameter, d, of the cone obtained by noting the cone base dimensions on the graph paper and averaging 2 determinations taken 90° apart. The repose angle, θ, was calculated as follows:

$$\theta = \arctan\left(\frac{1}{d/2}\right) \qquad \text{(Eq. 1)}$$

Humidity conditions and the moisture content of the granules were rechecked after repose angle determinations.

Granule Density, ρ_g.—A pycnometer method was chosen for the determination of particulate or granule density due to its ease of operation and reproducibility (22). The capacity of the pycnometer was 30 ml. and approximately 2 Gm. of 20/30-mesh material was used. Benzene, specific gravity 0.879, was employed as the immersion fluid. Due to the rapid evaporation of benzene, the pycnometer stem was marked half way down and the weight recorded as the meniscus reached this mark.

Particulate Hardness.—Many procedures for evaluating hardness were studied. The final selection made on the grounds of simplicity and the sample size used, involved shaking the granules in a closed container for a constant period of time and then measuring the percentage by weight of material retained on a 30-mesh sieve. Twenty grams of 20/30-mesh material was placed in a dry 3-oz. square bottle and shaken on an Eberbach[5] power shaking unit, model 6000, for 5 min. A 3-in. stroke was used with the reciprocating rate set at 216 c.p.m. The contents of the bottle were then placed on a No. 30 sieve and the fines removed by shaking for 15 sec. on the shaker unit. The hardness index, h, was calculated as the weight fraction of material retained as 30-mesh oversize following shaking.

Number of Particles per Gm., N.—One hundred particles of 20/30-mesh material were weighed and the number of particles/Gm., N, computed.

Bulk Density, ρ_b.—The bulk density determination procedure developed by Butler and Ramsey (23) was employed, with the exception that the cylindrical graduate was dropped 20 times instead of the 3 originally proposed. This modification was found to produce significantly more uniform results. A 30-Gm. sample of 20/30-mesh material was placed in a 100-ml. cylindrical graduate and dropped from a height of 0.75 in. onto a hard wooden surface a total of 20 times. Two seconds were allowed to elapse between each drop. If V is the volume in ml. of W Gm. of material in the cylinder, then ρ_b, the bulk density in Gm./ml., is given by:

$$\rho_b = \frac{W}{V} \qquad \text{(Eq. 2)}$$

Shape Volume Factor, α_v.—By definition (24):

$$\alpha_v = \frac{V}{d_e^3} \qquad \text{(Eq. 3)}$$

[2] Central Scientific Co., Chicago, Ill.
[3] Bacharach Industrial Instrument Co., Pittsburgh, Pa.
[4] Gaertner Scientific Corp., Chicago, Ill.
[5] Eberbach Corp., Ann Arbor, Mich.

TABLE II.—VALUES FOR VARIOUS PHYSICAL PARAMETERS OBTAINED FOR 20/30-MESH PARTICLES PREPARED USING 5 DIFFERENT GRANULATION METHODS

Granulation Method	Colton Upright	Oscillating Granulator	Hand Screen	Fitzpatrick Comminutor	Liquid-Solids V-Blender
Repose angle, θ, in degrees	35.78	34.65	35.77	36.23	35.28
Granule density, ρ_g, in Gm./ml.	1.51	1.54	1.50	1.42	1.45
Hardness index, h	.974	.977	.955	.945	.912
No. of particles/Gm., N	5759	6748	7753	6405	5527
Bulk density, ρ_b, in Gm./ml.	.375	.395	.400	.428	.508
Shape vol. factor, α_v	.1520	.1757	.1634	.2163	.2495
Bulk vol./constant vol. of granules, V_b', ml.	80.05	77.97	75.00	66.36	57.09
Geometrical form, k_e	.1809	.2144	.1912	.2639	.3019
Interspace porosity, e_i	.752	.744	.733	.699	.650
Equivalent projected diam., d_e, μ	910	820	810	800	790

where α_v is the shape volume factor, V is the volume of the particle, and d_e is the equivalent projected diameter. d_e is defined as the diameter of a circle having the same area as the particle when the latter is placed in its most stable position on a horizontal plane and is viewed from above.

A series 10 Micro Star[6] microscope, equipped with a projection screen, was used to view the particles, the outlines of which were traced onto paper. The areas of the traced particles were determined using a compensating planimeter.[7]

Extending Eq. 3 to the case of many particles:

$$\alpha_v = \frac{V_n}{\sum_{i=1}^{n} (d_e)_i^3} \qquad \text{(Eq. 4)}$$

where V_n is the volume of n particles. If p is the power of magnification, then d_e can be obtained readily in terms of the projected area, A. Thus,

$$\frac{A}{p^2} = \frac{\pi(d_e)^2}{4} \qquad \text{(Eq. 5)}$$

and

$$(d_e)^3 = \left(\frac{4A}{\pi p^2}\right)^{3/2} \qquad \text{(Eq. 6)}$$

Substituting Eq. 6 into Eq. 4:

$$\alpha_v = \frac{V_n}{\sum_{i=1}^{n} \left(\frac{4A}{\pi p^2}\right)_i^{3/2}} \qquad \text{(Eq. 7)}$$

or

$$\alpha_v = \frac{V_n}{\left(\frac{4}{\pi p^2}\right)^{3/2} \sum_{i=1}^{n} A_i^{3/2}} \qquad \text{(Eq. 8)}$$

Since more than 600 particles were traced, Eq. 8 was programmed in Fortran and the results computed on an IBM 1620 computer.

About 20 granules of 20/30-mesh material were placed on a microscope slide and the projection of each traced. The particles then were transferred to a tared weighing bottle. This procedure was repeated until approximately 150 particles from each granulation had been traced. The weight of the particles was obtained and their total volume calculated as follows:

$$V_n = \frac{M}{\rho_g} \qquad \text{(Eq. 9)}$$

where V_n is the volume, in ml., of n particles, M is the mass, in Gm., of n particles, and ρ_g is the granule density as determined by benzene displacement. The magnification, p, of the microscope used was 30.5×.

Bulk Volume per Constant Volume of Granules, V_b'.—The bulk volume for a constant granule volume was computed, for each granulation, from a knowledge of ρ_g and ρ_b. Thus:

$$V_b' = \frac{V\rho_g}{\rho_b} \qquad \text{(Eq. 10)}$$

where V_b' is the bulk volume in ml. of a fixed particulate volume V (in this instance, 20 ml.), ρ_g is the granule density, and ρ_b the bulk density. The bulk volume per constant volume of granules represents a term comparable to bulk density but which is freed from particulate density values, which varied from granulation to granulation.

Geometrical Form, k_e.—By definition (25):

$$k_e = \alpha_v m n'^{1/2} \qquad \text{(Eq. 11)}$$

where k_e is the geometrical form, m is the ratio of the particle breadth to its thickness, and n' is the length of the particle divided by its width. Since these granules were made by forcing them through square or spherical openings, the breadth can be expected to approximately equal the thickness. In this case, $m = 1$ and:

$$k_e = \alpha_v n'^{1/2} \qquad \text{(Eq. 12)}$$

Values of n' for particles from each granulation were calculated using 20 randomly selected granules. The longest dimension was taken as the length and a line perpendicular to its midpoint was taken as the width.

RESULTS AND DISCUSSION

Physical Methods.—*Repose Angle, θ.*—The repose angle values listed in Table II are the averages obtained from not less than 11 determinations run on particles from each granulation. The average standard deviation was 0.74°. The relative humidity never exceeded 15% during the determinations. In all cases, the moisture content of the granules was less than 2%.

[6] American Optical Co., Buffalo, N. Y.
[7] Keuffel and Esser Co., Germany.

Analysis of variance and contrast techniques (26) were used in the statistical analysis of the data. The repose angle of particles prepared using the oscillating granulator was found to be significantly lower than those values for particles produced using the other 4 methods, when a significance level of 1% was used. Since particles with smooth and even surfaces generally have a low angle of repose (27), the results obtained suggest that particles prepared from the oscillating granulator have the smoothest surface.

The repose angle was unaffected by static charge. Qualitative observations showed that the particles from the liquid-solids V-blender and the Fitzpatrick comminutor possessed a great deal of static charge. However, as shown in Table II, the repose angle of these granules did not vary significantly from those particles produced by the use of the Colton upright and the hand screen granulation methods, which were observed to have a lower static charge. These findings are in agreement with the predictions of Pilpel (12) who deduced, on theoretical grounds, that the magnitude of a static charge is too small to affect the repose angle.

The repose angle for the different granulations was found to be independent of particle shape. This was brought out in the study of shape volume factors. Thus, particles from the liquid-solids V-blender were found to be the roundest (highest α_v), yet their repose angle was quite high in relation to the repose angle of the irregularly shaped particles from the oscillating granulator. It would appear that, for particles of this size, the repose angle is mainly a function of surface roughness, as predicted by Pilpel (12).

Granule Density, ρ_g.—The density values listed in Table II are the average of 2 determinations. The average standard deviation for the reported values was 0.008 Gm./ml.

Since particles containing crevices or entrapped air give low density readings (22), the data in Table II indicate that granules prepared using the liquid-solids V-blender and Fitzpatrick comminutor have pitted surfaces or entrapped air, or both. These particles have very irregular surfaces, since many air bubbles were observed to rise to the surface of the benzene during the preliminary stages of the density determinations carried out using these granules. This was found not to be the case for the particles prepared by the other granulation methods.

Hardness Index, h.—Each of the hardness index values listed in Table II is the average of 3 or 4 determinations. The hardness index increased directly with the granule density, with the exception of those particles prepared from the Fitzpatrick comminutor.

Number of Particles per Gm., N.—Each value of N in Table II is the average of 5 determinations. Analysis of variance and contrast techniques (26) showed that the granules prepared using the Colton upright and the liquid-solids V-blender had N values which were significantly lower than those particles produced from the other 3 methods. Granules prepared using the hand screen method were found to have a value of N significantly higher than the other granules. In all cases, the significance level used was 1%.

Assuming that the samples consisted of spherical particles having a normal size distribution over the 20/30-mesh range, N would have a value of approximately 3500 particles/Gm. Deviation of the size distribution from normality cannot, alone, explain the high values of N obtained, implying that the particles are not spherical. The validity of this conclusion was confirmed by microscopic examination of the particles and a consideration of the calculated shape volume factors given in Table II. For perfect spheres, α_v is equal to $\pi/6$, or 0.524.

Bulk Density, ρ_b.—Bulk densities were determined twice for 20/30-mesh fraction of each granulation. In all cases, the 2 values were identical.

It is apparent from the results in Table II that the packing arrangements for particles prepared by the various granulating methods are quite different. The packing arrangements, in turn, are mainly determined by the shape of the granules in this size range (28), leading to the conclusion that the particles from the different granulations have very dissimilar shapes. This fact will become more apparent in the following discussion on shape volume factors.

Shape Volume Factor, α_v.—The shape volume factors shown in Table II were computed from Eq. 8 using 100–150 particles of each granulation. Since α_v is dependent upon the geometrical form and proportions of a particle (25), the shape volume factor was plotted against bulk density to establish the existence of a correlation between these parameters (Fig. 1). The linear correlation coefficient was 0.952, indicating that the bulk densities of the particles studied are related to their respective shape volume factors.

Since the densities of the particles from the various granulations differ, the bulk density values are not only a function of the void spaces but also a function of the granule densities, ρ_g. Therefore, the bulk volumes per constant volume of granules, V_b', were calculated since, as seen from Eq. 10, they are independent of granule density. Figure 2 is a plot of values for V_b' given in Table II *versus* shape volume factor. The correlation coefficient in this instance was 0.965.

In an effort to obtain even better correlation for terms representing particle shape and packing volume, a factor for the geometrical form, k_e, was calculated. These values, also listed in Table II, have been plotted against the bulk volume per con-

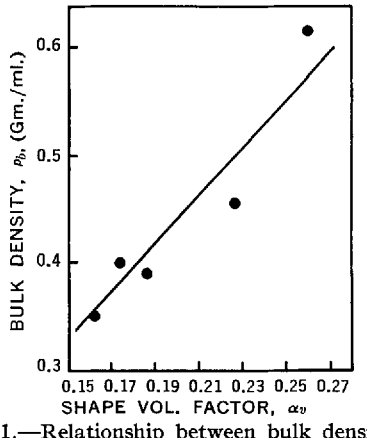

Fig. 1.—Relationship between bulk density and shape volume factor of 20/30-mesh particles prepared by 5 different granulation methods.

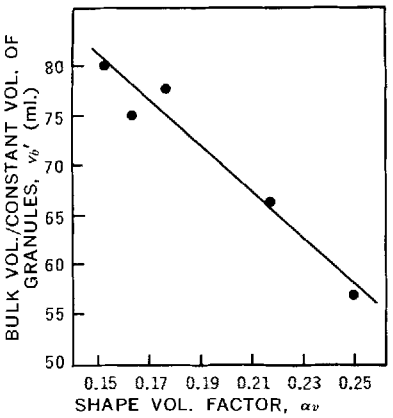

Fig. 2.—Relationship between bulk volume per constant volume of granules and shape volume factor of 20/30-mesh particles prepared by 5 different granulation methods.

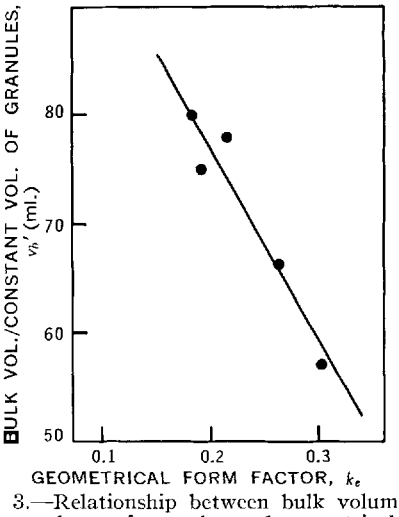

Fig. 3.—Relationship between bulk volume per constant volume of granules and geometrical form factor of 20/30-mesh particles prepared by 5 different granulation methods.

stant volume of granules in Fig. 3. The linear correlation coefficient was 0.961.

The interspace porosity, e_i, is defined as:

$$e_i = 1 - \frac{\rho_b}{\rho_g} \qquad \text{(Eq. 13)}$$

When this parameter was plotted as a function of the shape volume factor, a correlation coefficient of 0.914 was obtained.

Thus, the best correlation was obtained when the bulk volume was plotted against either the shape volume factor or its analog, the geometrical form, indicating that the volume of a packing of particles is a function of their geometrical form and proportions. This finding is important since the easily computed values of V_b' may be used to quickly evaluate procedures in which attempts are being made to obtain spherical particles or granules for coating and/or sustained-release requirements.

To check the accuracy of the computed α_v values, the number of particles per gram, N, was calculated for 20/30-mesh particles from the various granulation methods and compared with those values of N obtained experimentally by counting and weighing techniques. From Eq. 3, and since $N = 1/V\rho_g$ then:

$$N = \frac{1}{\alpha_v \rho_g d_e^3} \qquad \text{(Eq. 14)}$$

where N, α_v, d_e, and ρ_g are as previously defined.

A comparison of the values for N from the 2 techniques is given in Table III. It is readily apparent that the 2 sets of data are in excellent agreement.

Table III.—Comparison of Experimental and Computed Values of N, Number of Particles/Gm., for 20/30-Mesh Fraction

Granulation Method	Exptl. N[a]	Computed N
Liquid-solids V-blender	5527	5606
Colton upright	5759	5782
Fitzpatrick comminutor	6405	6359
Oscillating granulator	6748	6703
Hand screen	7753	7677

[a] These values were obtained from Table II.

Characterization of Granulation Methods.—On the basis of the repose angle studies, those particles prepared from the oscillating granulator were found to have the smoothest surface and can therefore be expected to possess the best flow properties. In addition, these particles were the hardest, even though the percentage of granulating agent used was relatively low (see Table I), indicating that the force of compaction produced by this granulator is high. Due to their relatively high density, the *intraspace* porosity of these particles is low in comparison to granules produced from the other pieces of granulating equipment. These particles are asymmetric, as evidenced by their low shape volume factor.

Since granules possessing smooth surfaces can be uniformly coated more easily than those with uneven surfaces, the oscillating granulator might be expected to produce particles well-suited for film coating purposes. Furthermore, since these were also the hardest granules of those studied, particle breakdown during the coating operation would be at a minimum.

Granules produced from the liquid-solids V-blender and the Fitzpatrick comminutor are relatively porous as evidenced by their granule densities. This was confirmed by the heterogeneous "optical density" of the particles when viewed with the projection microscope. On the basis of shape volume factors, granules from the liquid-solids V-blender and the Fitzpatrick comminutor were the most symmetrical on a volume basis. As a result, these particles assume a very close packing arrangement which is reflected in their high bulk density and low bulk volume. However, because of their surface irregularities, these particles can be expected to have only from average to poor flow properties, since this latter factor is more dependent on surface characteristics than general shape factors. From a consideration of the low density and hardness values for the granules produced by the liquid-solids V-blender and

the Fitzpatrick comminutor, it is apparent that the force of compaction in these 2 pieces of equipment is less than that produced by the oscillating granulator.

Particles produced by use of the hand screen method were intermediate between the 2 groups of granules previously discussed, with respect to repose angle, density, hardness, bulk density, and bulk volume. The number of particles per gram for this granulation was the highest of the 5 granulations; this was due to the low average volume for these particular granules.

Particles produced from the Colton upright were found to be the least spherical, an observation confirmed by the low value for the shape volume factor. These granules, not unexpectedly, also had the lowest bulk density of those studied. The fact that these particles had the highest value for the equivalent projected diameter over the size range of particles studied, accounts for their low degree of symmetry.

SUMMARY

Five standard granulation methods were compared on the basis of the following physical properties of the granules they produced: (*a*) repose angle, (*b*) granule density, (*c*) hardness, (*d*) number of particles per gram, (*e*) bulk density, (*f*) shape volume factor, (*g*) bulk volume per constant volume of granules, and (*h*) geometrical form.

Useful techniques were developed for obtaining some of the more important physical parameters of pharmaceutical granular solids. Particular attention was focused on developing an accurate technique for evaluating the shape volume factor, a parameter important in characterizing the over-all shape of a particle.

The repose angle was found to be primarily a function of surface roughness; in addition, the bulk volume was inversely proportional to particle shape. Thus, the researcher is provided with 2 easy techniques for assessing procedures aimed at producing smooth, spherical pharmaceutical particles.

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Interfacial Properties of Antimicrobial Long-Chain Quaternary Ammonium Salts II

Soluble Films at the Oil/Water Interface

By NORMAN D. WEINER, HELIO C. PARREIRA*, and GEORGE ZOGRAFI†

The adsorption of 3 quaternary ammonium salts, dodecylpyridinium chloride, dodecyltrimethylammonium chloride, and dodecyldimethylethylammonium chloride, has been measured at the hexane-, octane-, decane-, dodecane-, and tetradecane-0.1 *M* KCl interface. Adsorption appears to be influenced by interaction between the oil and both the nonpolar and polar portions of the surface-active agents, even though the surface-active agents are not soluble in the oils. The relative effect of chain length of the oil on surface concentration is a function of the bulk concentration of the quaternary ammonium salts.

In a previous publication (1) a study was reported concerning the adsorption of 3 quaternary ammonium salts, dodecylpyridinium chloride (DPC), dodecyltrimethylammonium chloride (DTAC) and dodecyldimethylethylammonium chloride (DEAC), at the air/water interface. The present study is concerned with the properties of these compounds at a number of different oil/water interfaces.

It is well recognized that the interfacial properties of surface-active agents at the air/water surface are quite different than their properties at the oil/water interface (2–6). Hutchinson (3) has suggested that oil molecules are present at the interface along with adsorbed surface-active molecules, and that a competition exists at the interface between the nonpolar portions of like molecules and the nonpolar portions of the oil and surface-active agents. He came to this conclusion by comparing the π-A characteristics of fatty acids adsorbed at various oil/water interfaces and their relative solubility in the oils. Schulman *et al.* (7) have studied the effect of the oil phase on microemulsion formation, and have concluded that oil molecules associate at the interface with surface-active agents. The presence of oil at any particular surface pressure was said to depend on the oil's ability to interact with film molecules and thus resist ejection with increasing film pressure. Zisman (8) has reported that mineral oil molecules appear to penetrate long-chain alcohol monolayers adsorbed at the oil/water interface, and remain as part of the surface film up to surface pressures of about 30 dynes/cm.

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Davies (9) has suggested that compounds having the same polar group, but differing in chain-length, should have the same π-A relationships at an oil/water interface since interactions between hydrocarbon chains of the compounds do not exist or are very weak. Brooks and Pethica (10, 11) have recently compared π-A curves at heptane–water interfaces for dodecyl, octadecyl, and docosyltrimethylammonium bromides. These π-A curves were obtained by applying the Gibbs adsorption equation in the case of the dodecyl compound, and by spreading insoluble monolayers of the other compounds. The π-A curves for these compounds were in good agreement, thus apparently verifying Davies' conclusions. Since the effect of changing the oil phase has not been considered in these systems, the authors chose to measure adsorption from aqueous solution in the presence of a series of straight-chain hydrocarbons, ranging from hexane to tetradecane. Since the quaternary ammonium salts are not soluble in the oils, it was felt that any effect on adsorption, due to changing oils, must be due to changes occurring in the interfacial region. The use of the 3 compounds under consideration also allowed the authors to observe differences due to the polar group, since chain-length and counter-ion were the same in all cases.

EXPERIMENTAL

Materials.—Hexane,[1] octane,[2] decane,[2] dodecane,[2] and tetradecane[2] were purified as follows: 25 ml. of fuming sulfuric acid was added to 500 ml. of oil, and the mixture was shaken in a separator for 5 min. The fuming sulfuric acid layer, which darkened considerably, was then discarded. This procedure was repeated until 3 successive additions of fuming sulfuric acid produced no visible darkening of the acid layer. The oil phase was then washed

[1] Hexane (spectroanalyzed, certified reagent) was purchased from Fisher Scientific Co.

[2] Each of these hydrocarbons (practical grade) was purchased from Eastman Organic Chemicals.

with water 5 times, with 5% sodium bicarbonate solution 5 times, and with water again 5 times. Charcoal and anhydrous sodium sulfate were then added to the oil, and the mixture was allowed to stand overnight. The oil was then filtered and distilled under vacuum. Only the middle 80% was collected. The surface tension of each oil agreed with reported data (12, 13).

Interfacial Tension Measurements.—The drop-volume method was used for all interfacial tension measurements. The apparatus used is the same as that described previously (1). It is a modification of one described recently by Parreira (14).

Calculations of interfacial tension from the drop-volume method were carried out with the Harkins and Brown equation (15),

$$\gamma = \frac{V(d - d')g}{2\pi r\theta} \qquad \text{(Eq. 1)}$$

where V = volume of the drop, cm.^{3}
g = gravity constant (980.3), cm./sec.^{2}
d = density of the aqueous phase, Gm./cm.^{3}
d' = density of the oil phase, Gm./cm.^{3}
π = 3.142
r = radius of the syringe tip, cm.
θ = correction factor obtained from the data of Harkins and Brown (15) for various values of $(r/V^{1/3})$

A syringe tip having a radius of 0.1895 cm. was used for all interfacial tension measurements. A 0.1895-cm. tip allowed at least 3 drops to form without refilling the syringe, even for the most dilute solutions. As with studies at the air/water interface (1), all values for $r/V^{1/3}$ that resulted from interfacial tension measurements with this syringe tip were within the range that allowed a second-order polynomial expression to be used for the calculation of θ.

The densities of hexane, octane, decane, dodecane, and tetradecane are 0.6595, 0.6986, 0.7258, 0.7459, and 0.7610 Gm./ml., respectively. These values do not change, within experimental error, if the oil is saturated with water. The density of water was also unaffected by saturation with oil.

It was observed that, if the syringe was not thoroughly cleaned between fillings, the volumes of the drops varied considerably, even with the same solution. This phenomenon did not occur in previous surface tension studies. It was possible that, if the syringe was not thoroughly cleaned between runs, a small amount of oil remaining on the tip might have been drawn into the syringe with the sample. If proper technique was used, however, the precision of the measurement was always within 0.2 dynes/cm., and in most cases to within 0.1 dynes/cm. In regions of very low interfacial tension, *i.e.*, below 7 dynes/cm., the results were erratic, apparently due to nonuniform wetting of the tip. A change in the size of the tip or the use of a stainless steel tip did not alleviate this difficulty.

It is interesting to note that the interfacial tensions of DPC, DTAC, and DEAC solutions, at all concentrations, were unaffected by the lack of mutual saturation of the aqueous and oil phases. All interfacial tension measurements, therefore, were made in solutions that were not saturated, with frequent checks being made on saturated systems. All solutions were prepared in duplicate. The volumes of at least 3 drops were measured for each sample; the volume of a drop was independent of time if the drop was allowed at least 5 min. to form. All interfacial tension measurements were, therefore, considered to be equilibrium measurements.

Interfacial tension *versus* concentration data were obtained for DPC and DEAC at the hexane–0.1 *M* KCl and dodecane–0.1 *M* KCl interfaces, and for DTAC at the hexane–0.1 *M* KCl, octane–0.1 *M* KCl, decane–0.1 *M* KCl, dodecane–0.1 *M* KCl, and tetradecane–0.1 *M* KCl interfaces. All measurements were carried out at 25 ± 0.1°.

RESULTS

In order to obtain values for the surface concentration and the surface area of each compound at the various interfaces, the Gibbs adsorption equation was applied. Since all measurements were made in the presence of excess electrolyte, the following form of the equation (16) was used:

$$\Gamma = 1/A = (1/kT)(d\pi/d \ln c) \qquad \text{(Eq. 2)}$$

where

Γ = surface concentration (molecules/cm.^{2})
A = surface area (cm.^{2}/molecule)
k = Boltzmann's constant (ergs/degree-molecule)
T = absolute temperature
π = surface pressure (ergs/cm.^{2})
c = bulk concentration (moles/L.)

The slopes of the surface pressure *versus* log bulk concentration plots were estimated with polynomial equations using the least squares method (1). An IBM 7090 digital computer was utilized to obtain the required data. Fourth-order polynomial equations gave the best fit to experimental data; a typical set of interfacial pressure *versus* logarithm molar concentration curves are shown in Fig. 1. The lines represent the empirical polynomial curves and the points represent experimental data.

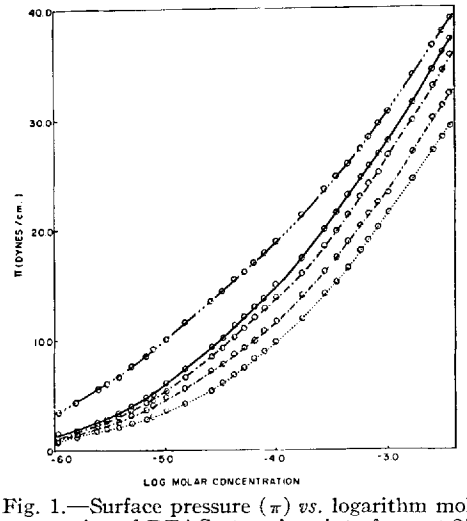

Fig. 1.—Surface pressure (π) *vs.* logarithm molar concentration of DTAC at various interfaces at 25°. (Lines represent empirical polynomial curves.) Key: ·····, hexane–0.1 *M* KCl; ———, octane–0.1 *M* KCl; –·–·–·, decane–0.1 *M* KCl; – – – –, dodecane–0.1 *M* KCl; ....., tetradecane–0.1 *M* KCl.

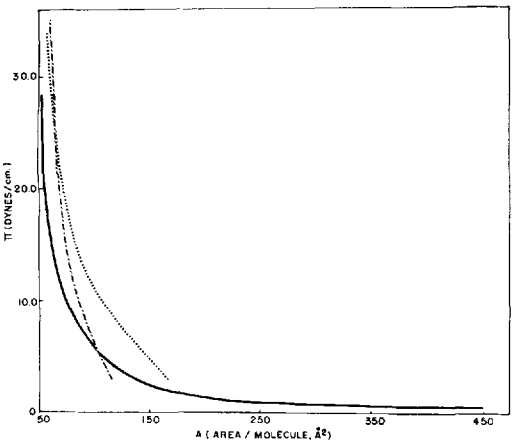

Fig. 2.—Surface pressure (π) *vs.* surface area (A) for DPC at various interfaces at 25°. Key: ———, air–0.1 *M* KCl; . . . . . , dodecane–0.1 *M* KCl; – – – –, hexane–0.1 *M* KCl.

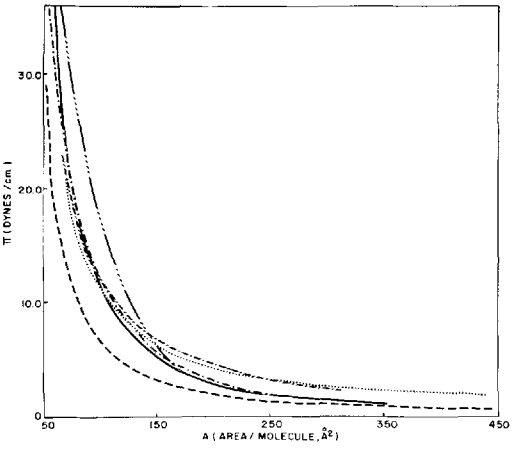

Fig. 3.—Surface pressure (π) *vs.* surface area (A) for DTAC at various interfaces at 25°. Key: – – – –, air–0.1 *M* KCl; —· · ·, hexane–0.1 *M* KCl; ———, octane–0.1 *M* KCl; ·—·—·, decane–0.1 *M* KCl; – — – —, dodecane–0.1 *M* KCl; . . . . . , tetradecane–0.1 *M* KCl.

The π-A curves of DPC, DTAC, and DEAC at the various interfaces are shown in Figs. 2–4. These curves generally indicate that more "condensed" films occur with the lower molecular weight oils (*i.e.*, a marked increased in the surface pressure for a small change in the area per molecule) particularly in regions of high area. The π-A curves for the quaternary ammonium compounds at the air–0.1 *M* KCl, hexane–0.1 *M* KCl, and dodecane–0.1 *M* KCl interfaces are shown in Figs. 5–7, respectively. Whereas the π-A curves of the 3 quaternary ammonium compounds are quite similar at the air/water surface, the π-A curves of DPC appear to be more "condensed" than those of DTAC and DEAC at the oil/water interfaces, particularly at the hexane–0.1 *M* KCl interface.

A region where surface pressure changed linearly with concentration (Traube region) was not observed at any of the oil/water interfaces tested.

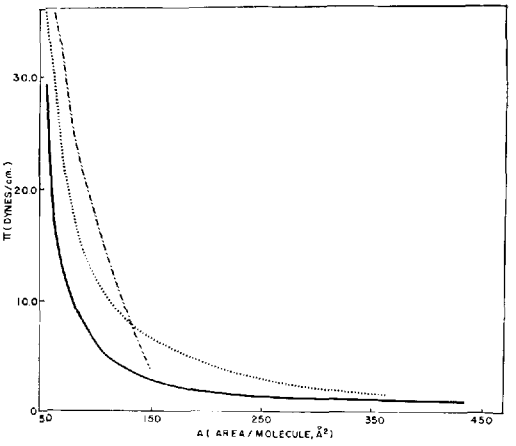

Fig. 4.—Surface pressure (π) *vs.* surface area (A) for DEAC at various interfaces at 25°. Key: ———, air–0.1 *M* KCl; . . . . . , dodecane–0.1 *M* KCl; – – – –, hexane–0.1 *M* KCl.

This observation appears to substantiate Hutchinson's hypothesis (3) that, even for low concentrations of surface-active agents, the hydrocarbon group is immersed in the oil phase, rather than lying parallel to the interface.

DISCUSSION

The greater complexity of the oil/water interface as compared to the air/water surface and differences with different oils is to be expected when one considers the possible interactions between the hydrocarbon portion of the surface-active agent and the oil. Furthermore, it appears that interactions between the polar group of the surface-active agent and the oil are responsible for marked changes in the nature of the surface film. This aspect has not been considered previously.

In order to evaluate the effect of the oil phase, as observed in this study, it will be worthwhile looking at 2 extreme situations. First, the oil does not interact with the hydrocarbon portion of the surface-active agent, and the only interactions of interest are those between the hydrocarbon portion and water at high surface areas, and those between surface-active agent hydrocarbon portions at low areas. In the second case, the oil strongly interacts with the hydrocarbon portion of the surface-active agent and there is minimal hydrocarbon–water interaction at high surface areas, as well as little or no cohesive interaction between surface-active molecules.

If the first case occurs, the hydrocarbon portion of the surface-active agent should be aligned approximately parallel to the interface at high surface areas. This would result in a linear change in surface pressure with concentration (Traube region), as observed at the air/water interface (1), and would mean that the oil phase does not affect the nature of the film. This, of course, is contrary to what is seen in Figs. 3 and 4. At lower areas interaction between the hydrocarbon portions of surface-active agent should result in π-A curves which are the same as those observed at the air/water interface. Again, Figs. 3 and 4 indicate that this is not the case, although at very low areas, expulsion of oil molecules from the surface may be occurring to some extent.

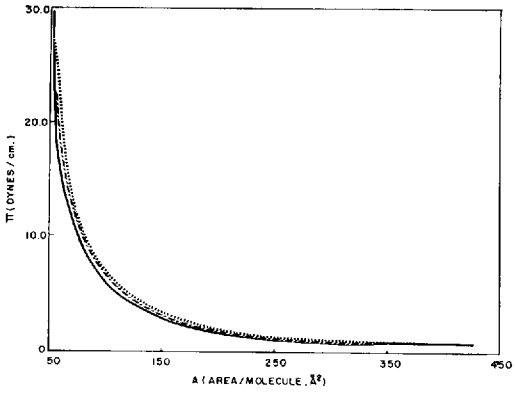

Fig. 5.—Surface pressure (π) *vs.* surface area (A) for the air −0.1 M KCl interface at 25°. Key: ———, DPC; – – – –, DEAC; . . . . . , DTAC.

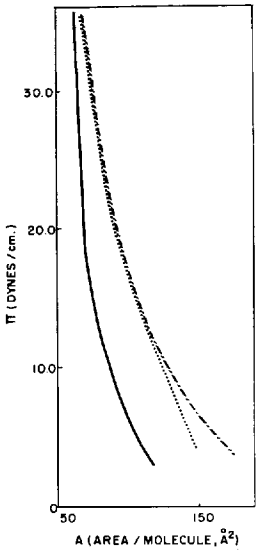

Fig. 6.—Surface pressure (π) *vs.* surface area (A) for the hexane-0.1 M KCl interface at 25°. Key: ———, DPC; – – – –, DEAC; . . . . . , DTAC.

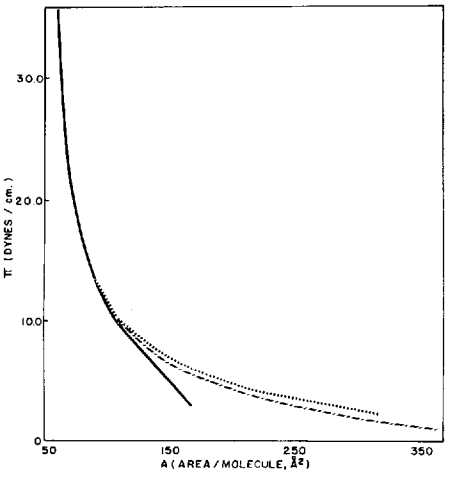

Fig. 7.—Surface pressure (π) *vs.* surface area (A) for the dodecane–0.1 M KCl interface at 25°. Key: ———, DPC; – – – – –, DEAC; . . . . . , DTAC.

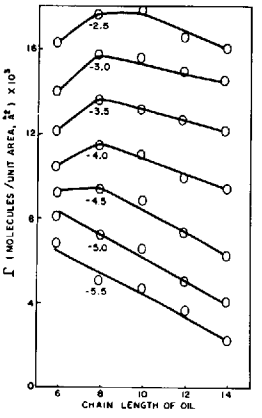

Fig. 8.—Surface concentration (Γ) at the 0.1 M KCl–hydrocarbon interface at 25° *vs.* chain length of hydrocarbon for various bulk concentrations of DTAC (expressed as log of molarity).

If the oil interacts strongly with the surface-active agent, the strong interaction between the hydrocarbon portion of the surface-active agent and the oil should result in an alignment of surface-active molecules approximately perpendicular to the interface, even at high surface areas. It would then be reasonable to expect that the most "condensed" film would result in the dodecane/water interface, since the dodecane–dodecyl quaternary ammonium compound interaction would allow stronger attractive forces than would the other oils tested. Figures 2–4 indicate that this is not the case. In fact, the hexane/water interface results in the most "condensed" films for all 3 quaternary ammonium compounds tested. This extreme would also necessitate the absence of a transition from an "expanded" region (a region where hydrocarbon–water interactions are predominent and small changes in surface pressure produce large changes in area per molecule) to one of saturation adsorption (a region where hydrocarbon–hydrocarbon interactions are predominant and changes in pressure do not produce significant changes in area). Figures 2–4 show that this transition does not always occur, and, in fact, whereas the dodecane/water interface does show such a transition for DTAC and DEAC, the hexane/water interface, at the pressures tested, does not. It therefore appears that the nature of the film is intermediate between the 2 extremes cited. The alignment of the surface-active molecules at the oil/water interface is neither parallel nor perpendicular to the interface, but intermediate between the two.

Plots of the chain length of the oil *versus* the surface concentration of DTAC at various bulk concentrations (Fig. 8) appear to give some indication as to the role of the oil. At low bulk concentrations the surface concentration of DTAC is inversely proportional to the chain length of the oil. At higher bulk concentrations, the octane/water interface contains the highest surface concentration of DTAC. At even higher bulk concentrations, the decane/water interface contains surface concentrations of DTAC which are of equal magnitude to that of the octane/water interface. It seems possible, therefore, that as the bulk concentration of DTAC increases, there is a tendency for the higher chain length hydrocarbons to interact to a greater extent with the DTAC film.

This trend could be accounted for by the fact that, at low bulk concentrations, the DTAC molecules are not aligned perpendicularly to the interface due to

some interaction with water, coiling of the hydrocarbon chains, or both. The size of hexane, as compared to the other hydrocarbons, could make it best suited to interact with the DTAC film at these low concentrations. As the surface concentration increases, it is reasonable to expect that the hydrocarbon portions of the surface-active agents will orient in a more perpendicular direction to the interface. This should improve the chances for the longer-chain hydrocarbons to interact with the exposed groups of the surface-active agents.

The preceding discussion considered only the interactions between the oil and the hydrocarbon group of the surface-active agent. It is important, however, not to neglect the possibility of an interaction between the polar group of the surface-active agent and the oil. Figures 5–7 show that, although the π-A curves of DPC, DTAC, and DEAC are quite similar at the air/water surface, differences between the 3 quaternary ammonium compounds at an oil/water interface do exist. The π-A curves for DPC are "compressed" to a greater extent than those for DTAC and DEAC at the oil/water interfaces, especially at the hexane/water interface. This effect may be due to a dipole-induced dipole interaction between the planer aromatic pyridinium ion and the oil, which is less possible in the case of DTAC or DEAC. Once again, the steric nature of the hexane molecule probably makes it best suited for this interaction.

It appears, therefore, that the nature of the oil phase influences adsorption of water-soluble substances, such as quaternary ammonium salts, and that the oil used in such studies must be considered in any discussion.

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Solubilizing Properties of Bile Salt Solutions I

Effect of Temperature and Bile Salt Concentration on Solubilization of Glutethimide, Griseofulvin, and Hexestrol

By THEODORE R. BATES, MILO GIBALDI and JOSEPH L. KANIG

Data on the micellar solubilization of the poorly water-soluble drugs, griseofulvin, hexestrol, and glutethimide, in 0–0.6 *M* aqueous solutions of the sodium salts of cholic, desoxycholic, taurocholic, and glycocholic acids at 3 temperatures are presented. Employing the pseudo two-phase model for micellar solubilization, the thermodynamic functions, ΔF^0, ΔS^0, and ΔH^0 of partitioning of the drug molecule between the aqueous phase and the micellar phase have been determined for hexestrol and griseofulvin. The physical-chemical ramifications and biological implications in these systems are considered.

AQUEOUS solutions of surfactants exhibit a more or less abrupt change in their physical properties over a narrow concentration range. This distinct change in properties is generally accepted to be due to the formation of oriented aggregates or micelles. The narrow surfactant concentration range at which micelles begin to form is referred to as the critical concentration for micelle formation or CMC. Among the more interesting properties of micellar solutions is their ability to solubilize water-insoluble materials.

Micellar solubilization has been defined by McBain (1) as "the spontaneous passage of solute molecules of a substance, insoluble in water, into an aqueous solution of a surfactant in which a thermodynamically stable solution is formed." This process essentially involves the diffusion of the added solubilizate molecules (*i.e.*, the water-insoluble material being solubilized) from the bulk phase into the surfactant micelle. The solubilized system is in a state of equilibrium.

Micellar solubilization has been broadly classified into 3 types (1–3). (*a*) *Nonpolar (nonspecific) solubilization:* the solubilizate is in-

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AQUEOUS solutions of surfactants exhibit a more or less abrupt change in their physical properties over a narrow concentration range. This distinct change in properties is generally accepted to be due to the formation of oriented aggregates or micelles. The narrow surfactant concentration range at which micelles begin to form is referred to as the critical concentration for micelle formation or CMC. Among the more interesting properties of micellar solutions is their ability to solubilize water-insoluble materials.

Micellar solubilization has been defined by McBain (1) as "the spontaneous passage of solute molecules of a substance, insoluble in water, into an aqueous solution of a surfactant in which a thermodynamically stable solution is formed." This process essentially involves the diffusion of the added solubilizate molecules (*i.e.*, the water-insoluble material being solubilized) from the bulk phase into the surfactant micelle. The solubilized system is in a state of equilibrium.

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corporated into the hydrocarbon center of the micelle, away from the polar head groups. (*b*) *Polar-nonpolar (specific) solubilization:* the solubilizate is incorporated by penetration into the palisade layer of the micelle with the solubilizate molecule oriented in approximately the same manner as is the surfactant molecule in the micelle. (*c*) *Adsorption solubilization:* in this type of solubilization, the solubilizate is adsorbed onto the polar surfaces of the micelle.

In 1936, Hartley (4) predicted that bile salts, like soaps, should form micellar solutions above their CMC. Equivalent conductivity-concentration (5–7), freezing point (5), dye solubilization (8), and small angle X-ray scattering (9) experiments, as well as the solubilization of 20-methylcholanthrene (10), are some of the numerous investigations that conclusively demonstrate that colloidal aggregates form in conjugated and unconjugated bile salt solutions at a certain minimum concentration.

Numerous investigations have demonstrated the solubilizing properties of bile salts for water-insoluble materials. Verzar (11) showed in 1933 that bile salts were capable of solubilizing aniline, calcium carbonate, calcium phosphate, camphor, quinine, strychnine, paraldehyde, quinoline, and benzaldehyde. McBain and co-workers (8) studied the equilibrium solubility of the dye, Yellow AB, in 1% aqueous solutions of the sodium salts of cholic acid, desoxycholic acid, taurocholic acid, and dehydrocholic acid at 25°. Merrill and McBain (12) compared the solubilities of the dyes, Yellow AB and Orange OT, in 1% aqueous solutions of the same 4 bile salts used in the previously cited study (8). Ekwall (13) reviewed some of the solubilization work done by himself and co-workers with a wide variety of insoluble substances in bile salt solutions. This paper includes: (*a*) solubilization of various carcinogenic polycyclic hydrocarbons at 40° (10, 14, 15); (*b*) solubilization of *p*-xylene in solutions of sodium cholate and desoxycholate; (*c*) solubility of cholic and desoxycholic acids in aqueous solutions of their respective sodium salts (16); (*d*) solubilization of cholesterol in sodium desoxycholate, cholate, and taurocholate; and (*e*) solubilization of C_6–C_{18} fatty acids, lecithin and glyceryl monostearate, in 0.09 *M* sodium taurocholate solutions. Ekwall and Sjöblom (17–19) have studied the solubilization of various steroid hormones in bile salt solutions. The solubilization of the nonsteroidal synthetic estrogen, hexestrol, in 5, 10, and 20% solutions of sodium cholate, desoxycholate, dehydrocholate, and glycocholate at 40° was also considered by these investigators.

Bile salts have been shown also to play an important role in the physiological processes of digestion and absorption of dietary lipids. The most modern theory for fat digestion and absorption is the one proposed by Borgström (20). According to his theory, the breakdown products of fat digestion (*i.e.*, fatty acids and monoglycerides) are absorbed across the intestinal mucosa from a mixed micellar solution composed chiefly of fatty acids, monoglycerides, and conjugated bile salts. In connection with this theory of fat absorption, several *in vitro* investigations have appeared in the literature demonstrating the marked solubilizing ability of conjugated bile salts for fatty acids and monoglycerides (21–24).

Although considerable evidence has appeared in the literature concerning the effects of bile salts on endogenous materials, little work has been done to determine the effects of bile salts on drug molecules. Accordingly, little consideration has been given to the possibility that insoluble drugs may be absorbed by a mechanism involving preliminary solubilization of the drug by the bile salt micelles normally present in the intestine. This consideration prompted an extensive physicochemical investigation of the solubilization of 3 water-insoluble pharmaceuticals in dilute bile salt solutions as well as some of the factors which may influence the extent of their solubilization.

This paper reports some of the findings on the effect of temperature and bile salt concentration and structure on the degree of solubilization of the water-insoluble drugs, griseofulvin, hexestrol, and glutethimide, in dilute aqueous solutions of the sodium salts of cholic, desoxycholic, taurocholic, and glycocholic acids.

THEORY

Micellar solubilization of a poorly water-soluble material can be treated as a process in which the poorly water-soluble material is partitioned between an aqueous phase and a micellar phase formed by the surfactant above its CMC (1). The partition coefficient associated with this process is expressed by the equation:

$$K = \frac{[D_M]}{[D_{NM}]} \qquad \text{(Eq. 1)}$$

where $[D_M]$ is the concentration of drug in the micelle and $[D_{NM}]$ is the concentration of drug in the nonmicellar phase.[1] The brackets denote concentrations expressed in terms of the individual phase volumes, rather than the total volume of the system.

Multiplying the numerator and denominator of Eq. 1 by the total volume of the system, V (*i.e.*, $V_{NM} + V_M$), yields the expression,

$$K = \frac{(D_M)}{(D_{NM})} \cdot \frac{V_{NM}}{V_M} \qquad \text{(Eq. 2)}$$

[1] It was assumed that the activity coefficient of the drugs in the nonmicellar phase closely approximated the activity coefficient in the micellar phase.

where the parenthesis denote concentrations expressed in terms of total volume. V_{NM} and V_M represent the nonmicellar and micellar volumes, respectively.

Expressing Eq. 2 in terms of micellar volume yields

$$K = \frac{(D_M)}{(D_{NM})} \cdot \frac{(V - V_M)}{(V_M)} \qquad \text{(Eq. 3)}$$

However, V_M/V may be represented as M, where M is defined as the volume fraction of surfactant (25, 26). Substituting this relationship into Eq. 3 gives the expression,

$$K = \frac{(D_M)}{(D_{NM})} \cdot \frac{(1 - M)}{(M)} \qquad \text{(Eq. 4)}$$

Assuming M is small as compared to the total volume (25, 26) then $(1 - M)$ is approximately equal to unity and Eq. 5 is obtained.

$$K = \frac{(D_M)}{(D_{NM})} \cdot \frac{1}{M} \qquad \text{(Eq. 5)}$$

Above the CMC of a surfactant an equilibrium exists between monomers and micellar aggregates. The solution is saturated with respect to monomers, and further addition of surfactant molecules results in further aggregation. It has been theorized that additional molecules of surfactant produce an increase in the number rather than in the size of the micelles. Accordingly, micellar volume is a direct function of surfactant concentration. Over a limited range, this is considered to be a reasonable approximation (4, 27). This concept may be expressed as Eq. 6.

$$\frac{C_1}{C_2} = \frac{M_1}{M_2} \qquad \text{(Eq. 6)}$$

where C is the molar concentration of the surfactant. The subscripts, 1 and 2, refer to different concentrations above the CMC of the surfactant.

Inserting the relationship between C and M, as expressed in Eq. 6, into Eq. 5 yields the final equation:

$$\Delta D_M = K D_{NM} \Delta C \qquad \text{(Eq. 7)}$$

It can be seen readily from the form of Eq. 7 that a plot of (D_M) *versus* C should yield a straight line above the CMC of the surfactant. The slope of this linear plot divided by (D_{NM}) will give the value of K, from which the thermodynamic constants can be calculated.

The free energy of partitioning (ΔF^0) may be calculated from

$$\Delta F^0 = -2.3RT \log K \qquad \text{(Eq. 8)}$$

The heat of partitioning (ΔH^0) can be determined with the aid of the relationship

$$\frac{\delta \log K}{\delta(1/T)} = \frac{-\Delta H^0}{2.3R} \qquad \text{(Eq. 9)}$$

by plotting log K at various temperatures *versus* $1/T$ (°K.). From the slope of this linear plot, ΔH^0 can be obtained. This method of acquiring ΔH^0 requires that ΔH remain reasonably constant over the temperature range studied.

The change in entropy (ΔS^0) associated with this process of solubilization would follow from the equation

$$\Delta S^0 = \frac{\Delta H^0 - \Delta F^0}{T} \qquad \text{(Eq. 10)}$$

EXPERIMENTAL

Materials.—Hexestrol,[2] griseofulvin,[3] and glutethimide[4] were used as recieved. The pure bile salts, sodium cholate,[5] sodium desoxycholate,[5] sodium glycocholate,[6] and sodium taurocholate[6] were dried *in vacuo* for 36 hr. prior to use.

Equilibration.—The solubility of each drug was measured in a series of aqueous solutions containing various concentrations of the individual bile salts. Hexestrol and griseofulvin were studied at 27, 37, and 45°. The solubility of glutethimide was determined at 27, 32, and 37°.

In each case an excess amount of drug was added to bile salt solution contained in suitably sealed tubes. The tubes were placed in a shaker-incubator[7] and equilibrated for periods usually not less than 1 week's duration. Equilibrium was determined by repetitive sampling.

Assay Procedure.—Each time the tubes were sampled the shaker was turned off to allow most of the excess solid to settle to the bottom of the tubes. The supernatant liquid then was filtered through a filter (Millipore, 0.45 M pore size) to insure that no undissolved solid was present in the sample taken for analysis. To eliminate any temperature differential during the filtration and sampling steps, precautions were taken to maintain the filtration equipment and pipets at the same temperature as that employed for the equilibrium experiments.

Aliquots of the clear drug solutions were diluted with anhydrous reagent methanol, and the drug concentration was determined spectrophotometrically using a Beckman DB recording spectrophotometer. Methanol–water (10:1) served as the blank for hexestrol and griseofulvin, and methanol–water (8:1) was employed for glutethimide. The peak absorbance of griseofulvin and hexestrol (in 1:10 water–methanol solvent mixture) at 292 mμ and 278 mμ, respectively, and glutethimide (in 1:8 water–methanol solvent mixture) at 257.6 mμ was used to prepare Beer's law plots. In the dilutions required for spectrophotometric analysis, no shifts in absorbance maxima were observed as a result of the presence of surfactant. However, the bile salts do absorb slightly at the wavelength of maximum absorbance of these drugs. Therefore, the absorbance of varying concentrations of the bile salts, at the 3 previously mentioned wavelengths, was plotted *versus* bile salt concentration, and a calibration curve was thus constructed.

To determine the amount of drug that had been solubilized, the absorbance value corresponding to the concentration of bile salt in the final dilution

[2] Obtained from Gallard-Schlesinger Chemical Mfg. Co. New York, N. Y.

[3] Generously supplied by Schering Co., Bloomfield, N. J. Marketed as Fulvicin.

[4] Generously supplied by Ciba Pharmaceutical Co., Summit, N. J. Marketed as Doriden.

[5] Obtained from Mann Research Laboratories, Inc., N. Y., special enzyme grade.

[6] Obtained from Southeastern Biochemicals, Morristown, Tenn. Reported to be 98–99% pure by thin-layer chromatography.

[7] Gyratory incubator shaker, model G-25, New Brunswick Scientific Co., N. J.

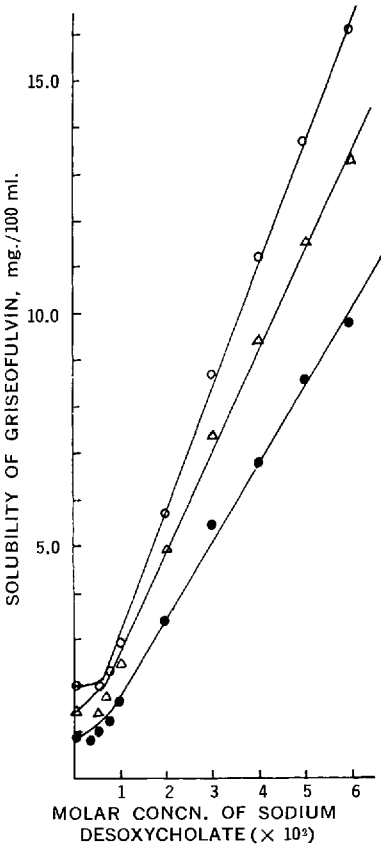

Fig. 1.—Solubility of griseofulvin as a function of sodium desoxycholate concentration and temperature. Key: ●, 27°; △, 37°; ○, 45°.

was subtracted from the observed absorbance values, and the corrected absorbance values were converted to concentrations by the use of the Beer's law plots.

RESULTS AND DISCUSSION

Effect of Bile Salt Type and Concentration on Solubilization.—The solubilization curves for Griseofulvin in varying concentrations of sodium desoxycholate solutions at 27, 37, and 45° are shown in Fig. 1. These data are representative of the type of curves obtained with sodium cholate, sodium glycocholate, and sodium taurocholate solutions. It can be seen from each of these curves that the solubility of griseofulvin increases linearly with bile salt concentration, after a certain minimum concentration of bile salt has been exceeded, *i.e.*, the CMC. The CMC values for the 4 bile salts employed in this study, as determined from the solubilization of griseofulvin and hexestrol, respectively, at 37°, are: sodium cholate (0.014, 0.016), sodium desoxycholate (0.005, 0.010), sodium taurocholate (0.008, 0.014), and sodium glycocholate (0.010, 0.015). These values are in good agreement with those obtained by other investigators employing various solubilizates (9, 13). It should be borne in mind that CMC values determined from solubilization data are only approximate, since the presence of solubilized material may exert an effect on the process of micelle formation (1, 2).

The slope of the linear portion of the solubilization curve, after the CMC, is termed the saturation ratio or capacity, *i.e.*, the ratio of micellar drug to micellar bile salt. The saturation ratios, expressed as moles of solubilized drug per mole of bile salt as well as the inverse of these ratios for griseofulvin in each of the 4 bile salts employed in this study, are presented in Table I. The saturation ratio at 27 and 37° in decreasing order are: cholate ≥ desoxycholate > glycocholate ≥ taurocholate. This sequence also indicates the order of solubility of griseofulvin in a particular concentration of these colloidal electrolytes. At 45° the order of cholate and desoxycholate is reversed, suggesting that at this higher temperature sodium desoxycholate micelles have a higher affinity for griseofulvin than do sodium cholate micelles.

Figures 2 and 3 show the solubility of the synthetic estrogenic hormone, hexestrol, in sodium glycocholate and sodium taurocholate solutions at 27, 37, and 45°. Similar curves were obtained in sodium cholate and sodium desoxycholate solutions. The saturation ratios as well as the inverse of these ratios are listed in Table II. As with griseofulvin, the solubility of hexestrol at all 3 temperatures increases linearly with bile salt concentration above the CMC. The saturation ratios for hexestrol are many times greater than those of griseofulvin, illustrating the well-established fact that the structure of the solubilizate is a critical factor in governing the extent or degree of micellar solubilization (1, 2). The saturation ratios for hexestrol, in decreasing order, at any one temperature, are: glycocholate ≥ taurocholate > cholate > desoxycholate. Thus, in the case of hexestrol, the conjugated bile salt micelles (*i.e.*, sodium glycocholate and sodium taurocholate) show a greater affinity for the solubilizate molecules than do the unconjugated bile salt micelles. The reverse was found to be true for the solubilization of griseofulvin.

Representative solubilization curves for glutethimide in 0–0.06 *M* solutions of sodium cholate at 27, 32, and 37° are shown in Fig. 4. The corresponding saturation ratios (moles of solubilized glutethimide per mole of bile salt) and the inverse of these ratios are reported in Table III. The 32°-

Table I.—Maximum Solubilizing Power of Bile Salts for Griseofulvin

Solubilizer	Saturation Ratio[a] × 10³, moles of Griseofulvin/mole of Solubilizer			Inverse of Saturation Ratio, moles of Bile Salt/mole of Griseofulvin		
	27°	37°	45°	27°	37°	45°
Water	4.59×10^{-4}	7.14×10^{-4}	10.2×10^{-4}	...	...	...
Sod. cholate	5.36	6.18	6.80	187	162	147
Sod. desoxycholate	4.68	6.18	7.54	214	162	133
Sod. taurocholate	3.77	4.90	6.15	265	204	163
Sod. glycocholate	3.85	5.13	6.29	260	195	159

[a] Slope of linear portion of solubilization curve determined by the method of least squares.

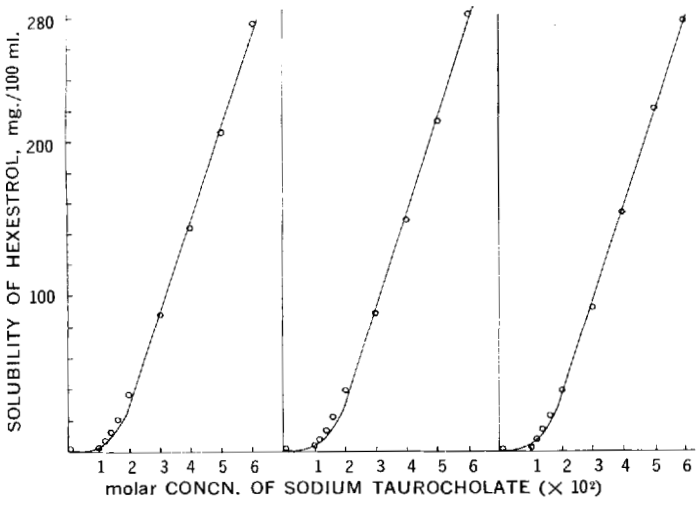

Fig. 2.—Solubility of hexestrol as a function of sodium taurocholate concentration at 27° (left), 37° (middle), and 45° (right).

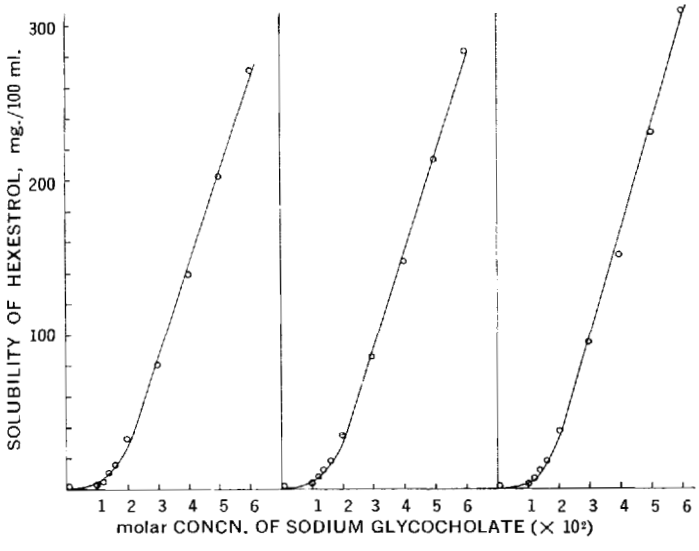

Fig. 3.—Solubility of hexestrol as a function of sodium glycocholate concentration at 27° (left), 37° (middle), and 45° (right).

temperature study was included since at 45° the solubilization curve for glutethimide was not linear, suggesting a possible change in the solubilization mechanism at this higher temperature. All experiments were performed in duplicate, and reasonable replication was obtained. Thus, spurious experimental factors were ruled out as contributing to the nonlinearity. In addition, no change in the U.V. spectra of glutethimide was observed. The saturation ratios in decreasing order are: desoxycholate > cholate ≥ taurocholate > glycocholate.

The differences in the order of the saturation ratios obtained in the various bile salt solutions with each drug used in this study are probably due to differences in the arrangement of the bile salt molecules in the micelle as well as differences in the degree and/or type of interaction between the drug molecule and the bile salt micelle. The penetration of the solubilizate may alter significantly the actual organization, shape, and even the size of the resultant bile salt micelle (13). This would explain not only the differences found in the affinity between these bile salts and the individual drug, but also the differences found in the saturation ratio se-

Table II.—Maximum Solubilizing Power of Bile Salts for Hexestrol

Solubilizer	Saturation Ratio[a] × 10³, moles of Hexestrol/mole of Solubilizer 27°	37°	45°	Inverse of Saturation Ratio, moles of Bile Salt/mole of Hexestrol 27°	37°	45°
Water	4.66×10^{-4}	6.66×10^{-4}	9.32×10^{-4}	...	...	...
Sod. cholate	187	195	197	5.35	5.13	5.08
Sod. desoxycholate	164	167	179	6.10	5.99	5.59
Sod. taurocholate	220	225	223	4.55	4.44	4.48
Sod. glycocholate	221	231	251	4.52	4.33	3.98

[a] Slope of linear portion of solubilization curve determined by the method of least squares.

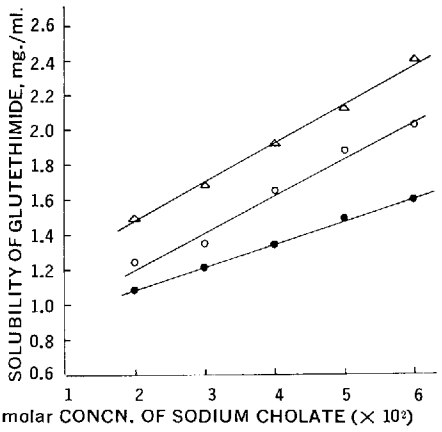

Fig. 4.—Solubility of glutethimide as a function of sodium cholate concentration and temperature. Key: ●, 27°; ○, 32°; △, 37°.

quences of these bile salts and each of the drugs. Unfortunately, at present, very little is known about the structures of the bile salt micelles. However, they are thought to be considerably different from the classical spherical micelles formed by the more common surfactants. A detailed explanation for these observed differences must await a more complete understanding of the molecular arrangement of the bile salt micelles, as well as knowledge of the nature of the interactions that are important in causing micellar aggregation of both the unconjugated and conjugated bile salts.

It can readily be seen from both the saturation ratio and inverse saturation ratio data, presented in Tables I–III, that the solubilities of hexestrol, glutethimide, and griseofulvin in the same bile salt differ greatly. For these solubilizates, the solubilities increase in the following order: griseofulvin < glutethimide < hexestrol. It would appear, based on the higher saturation ratios observed for both hexestrol and glutethimide as compared to griseofulvin, that they are being incorporated predominately into the "palisade layers" of the bile salt micelle as would a relatively polar solubilizate molecule (1–3). On the other hand, griseofulvin is probably more closely associated with the hydrocarbon region of the micelle, similar to a nonpolar solubilizate molecule (1–3).

As previously indicated, knowledge of the exact structure of the bile salt micelles is lacking. In view of this insufficiency, the mechanism by which the molecules of the solubilized substances are incorporated in the micelles must remain speculative until further investigations in this area. It should also be noted that the chemical nature, size, and structure of the solubilized molecules influence in various ways the arrangement of the surfactant molecules in the micelle (9, 13). Experiments presently being conducted in our laboratories, dealing with the effects of amphiphilic and nonpolar additives and inorganic electrolyte on the solubilization characteristics of these drugs should yield an insight to the location of the solubilized drug molecules in the bile salt micelle.

Effect of Temperature on Solubilization.—Temperature is an important factor which has a varying effect on the extent of micellar solubilization. The structure of the surfactant and/or the solubilizate will dictate whether there is an effect and whether it is positive or negative.

Inspection of the saturation ratio values for griseofulvin, presented in Table I, shows that as the temperature of the system increases from 27 to 45° there is a corresponding increase in the degree of micellar solubilization. This positive temperature effect is observed in all of the bile salt solutions studied. Similar temperature effects were observed for both hexestrol (Table II) and glutethimide (Table III). However, temperature appears to have less of an influence on the solubilization of hexestrol in comparison to the other 2 solubilizates.

A positive temperature effect is contrary to the theory proposed for micellar solubilization in solutions of fatty acid soap-type surfactants (3). According to this theory, in a homologous series of fatty acid soaps, below 25°, as the temperature is increased, the CMC of the surfactant decreases, due to an increase in the entropy of the structured water molecules around the hydrocarbon portion of the surfactant molecule. As the temperature is increased above 25°, the kinetic motion of the surfactant molecules in the micelle is enhanced and overshadows this entropy effect. The thermal motion causes a slight increase in the CMC of the surfactant and thus increases the difficulty with which micelles form. Based on these facts, one would expect that at higher temperatures solubilization should decrease.

Although a number of studies have been conducted to examine the effect of temperature on the solubilization process in typical ionic surfactant solutions, relatively few temperature studies have been conducted in bile salt solutions. Merrill and McBain (12) observed a positive temperature coefficient (*i.e.*, the ratio of the saturation ratio at a higher temperature to that at a lower temperature exceeded unity) for the dye Yellow AB in 0.1 *N* sodium desoxycholate solutions. Hofmann observed a similar temperature effect on the solubilization of azobenzene in sodium glycochenodesoxycholate solutions, but observed no effect on the solubilization of the monoglyceride, 1-monoolein (24).

Table III.—Maximum Solubilizing Power of Bile Salts for Glutethimide

Solubilizer	Saturation Ratio[a] × 10³, moles of Glutethimide/mole of Solubilizer			Inverse of Saturation Ratio, moles of Bile Salt/mole of Glutethimide		
	27°	32°	37°	27°	32°	37°
Water	7.13×10^{-2}	8.38×10^{-2}	9.94×10^{-2}	...	...	...
Sod. cholate	59.8	96.2	104	16.7	10.4	9.62
Sod. desoxycholate	103	119	163	9.71	8.40	6.13
Sod. taurocholate	61.2	100	108	16.3	10.0	9.26
Sod. glycocholate	54.3	92.0	71.8	18.4	10.9	13.9

[a] Slope of linear portion of solubilization curve determined by the method of least squares.

TABLE IV.—PARTITION COEFFICIENTS FOR GRISEOFULVIN, HEXESTROL, AND GLUTETHIMIDE BETWEEN THE MICELLAR AND NONMICELLAR PHASE AT 3 TEMPERATURES

Solubilizer	Partition Coefficient, $K \times 10^{-3}$ 27°	32°	37°	45°
		Griseofulvin		
Sod. cholate	11.7	...	8.66	6.67
Sod. desoxycholate	10.2	...	8.66	7.39
Sod. taurocholate	8.21	...	6.86	6.03
Sod. glycocholate	8.39	...	7.18	6.17
		Hexestrol		
Sod. cholate	398	...	294	211
Sod. desoxycholate	352	...	251	192
Sod. taurocholate	472	...	338	240
Sod. glycocholate	475	...	347	269
		Glutethimide		
Sod. cholate	0.840	1.15	1.05	...
Sod. desoxycholate	1.45	1.42	1.64	...
Sod. taurocholate	0.859	1.20	1.09	...
Sod. glycocholate	0.762	1.10	0.722	...

Since micellar solubilization is closely related to micelle formation, one possible explanation for the positive temperature effects observed in the present study is that as the temperature of the system is increased the CMC of the bile salt decreases. However, values determined from the solubilization curves for the individual bile salts show that the CMC values of the bile salts are not significantly altered in the temperature range employed in this study. Thus, the observed temperature effects cannot be explained on the basis of CMC values.

A more plausible explanation has been proposed by McBain and Hutchinson (1). According to these investigators, it may be assumed that the principal effect of temperature is to "change the solubility of the solubilizate in the micelle." Hofmann (24), in his investigations of the effect of temperature on the solubilization of azobenzene and 1-monoolein in sodium glycochenodesoxycholate solutions at 23 and 37°, states that, "the higher saturation ratio observed for the former solubilizate at 37° need not indicate any change in the state of micellar aggregation." This view is in agreement with Hofmann's observations that 1-monoolein had the same saturation ratio at 23 and 37°, thereby indicating that temperature has little effect on the micellar organization.

Thermodynamic Evaluation.—The results of this investigation are consistent with the hypothesis that temperature primarily influences the "solubility" of the solubilizate molecule in the bile salt micelle. Therefore, one would expect that as the temperature of the system is increased so should the degree of interaction between the solubilizate molecule and the bile salt molecules comprising the micelle. In view of this consideration, it was decided to determine the thermodynamics of these bile salt–drug systems so that an appreciation for the magnitude of the energies involved in the process of micellar solubilization in bile salt solutions could be obtained.

In order to determine the thermodynamic constants associated with the solubilization of griseofulvin and hexestrol in bile salt solutions, a pseudo two-phase model was selected. According to this model, the solubilizate molecule is partitioned between an aqueous phase and a micellar phase. This partitioning is similar to that observed for a poorly water-soluble drug between a nonpolar solvent and water. The partition coefficient, K, associated with this process was determined by the use of Eq. 7. The values obtained for griseofulvin, hexestrol, and glutethimide at 3 temperatures are listed in Table IV. The magnitude of these values shows that the poorly water-soluble drugs are preferentially partitioned to the micellar phase.

The data in Table IV also indicate that a decrease in the partition coefficients of both griseofulvin and hexestrol occurs with an increase in temperature. No definite conclusion could be made for the effect of temperature on partition coefficient for glutethimide. A similar decrease in partition coefficient with temperature was observed by Rippie and coworkers (25), in considering the solubilization of a methylprednisolone-21-hemiester in aqueous solutions of polysorbate 80. These investigators partially attribute this negative effect to micellar size changes. In conducting this investigation it was decided to consider the phenomenon on the basis of thermodynamic factors. The heats of solution of

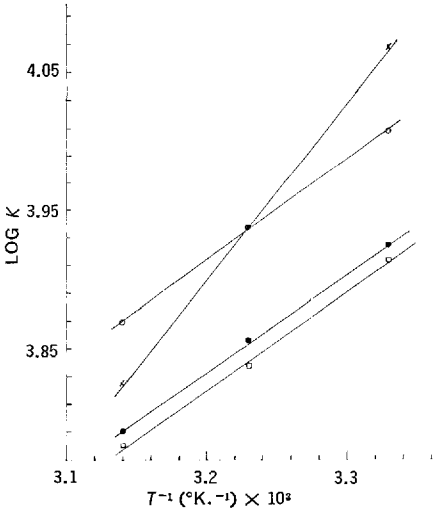

Fig. 5.—Plots of log K *vs.* $1/T$ for griseofulvin. Key: ●, sodium glycocholate; □, sodium taurocholate; ○, sodium desoxycholate; ×, sodium cholate.

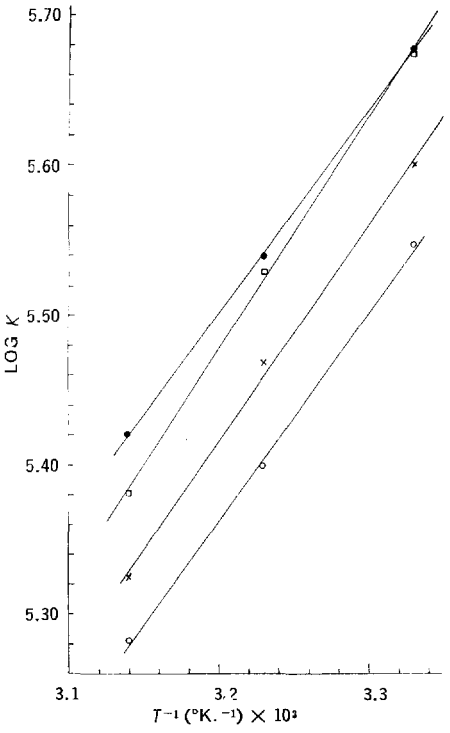

Fig. 6.—Plots of log K *vs.* $1/T$ for hexestrol. Key: ●, sodium glycocholate; □, sodium taurocholate; ○, sodium desoxycholate; ×, sodium cholate.

griseofulvin and hexestrol in water and in each of the bile salt solutions was determined from the slope of a Clausius-Clapeyron-type plot (*i.e.*, a plot of log solubility *versus* $1/T$). The heats of solution ($\Delta H_{soln.}$) in the bile salt micelle ranged from −2.5 to −5.0 Kcal./mole for griseofulvin and from approximately zero to −1.4 Kcal./mole for hexestrol, as compared to −7.3 Kcal./mole for griseofulvin and −8.4 Kcal./mole for hexestrol in water. This indicates that the solubility of the drugs in the aqueous phase is affected by temperature more than it is in the bile salt solutions, resulting in the observed decrease in the partition coefficient.

According to Eq. 9, a plot of log K *versus* $1/T$ should be linear. From the slope of such a plot the standard enthalpy change, ΔH^0, associated with this partitioning process can be obtained. Typical van't Hoff-type plots for griseofulvin (Fig. 5) and hexestrol (Fig. 6) show the excellent linearity observed over the temperature range studied. Treatment of the glutethimide data in a similar manner produced plots which were nonlinear. This suggests the possibility that glutethimide is solubilized by a different mechanism than are hexestrol and griseofulvin.

Values for the standard free energy changes (ΔF^0) and entropy changes (ΔS^0) associated with the solubilization process were determined with the aid of Eqs. 8 and 10, respectively. The values of ΔF^0, ΔH^0, and ΔS^0 for the solubilization of griseofulvin in the 4 individual bile salts are presented in Table V, and those for hexestrol in Table VI.

The negative ΔF^0 values obtained is indicative of the spontaneity of the solubilization process. The standard enthalpies, ΔH^0, for each drug in all of the bile salts are quite similar. However, there is a significant difference between the ΔH^0 values for hexestrol and for griseofulvin. This indicates that the enthalpy function is more dependent on the nature of the drug molecule than on the nature of the bile salt molecule. The negative enthalpy changes obtained are consistent with the hypothesis that micellar solubilization is an exothermic process (1, 25, 26).

It has been proposed that because of the loss of freedom experienced by the drug molecule in going from the aqueous phase to the micellar phase a negative entropy change should accompany the solubilization process (26). The small positive entropy values obtained in this investigation indicate that other factors must be taken into consideration. A possible explanation for the positive entropy values is that loss of water structure in the system counterbalances the restriction placed on the drug molecule when it is solubilized by the bile salt micelle.

Biological Implications.—A comparison of the equilibrium ratio of the amount of drug solubilized by a 0.04 M bile salt solution to that in water for the 3 solubilizates at 37° shows that the ratios in decreasing order are: hexestrol > griseofulvin > glutethimide (Table VII). A bile salt concentration of 0.04 M is considered to be the approximate

Table V.—Standard Thermodynamic Functions for Griseofulvin in Bile Salt Solutions

Bile Salt	ΔH^0,[a] Kcal./mole	ΔF^0, Kcal./mole 300°K.	310°K.	318°K.	ΔS^0, Entropy units 300°K.	310°K.	318°K.
Sod. cholate	−5.9	−5.61	−5.61	−5.59	−1	−1	−1
Sod. desoxycholate	−3.4	−5.53	−5.61	−5.66	+7	+7	+7
Sod. taurocholate	−3.2	−5.40	−5.47	−5.53	+7	+7	+7
Sod. glycocholate	−3.2	−5.42	−5.50	−5.54	+7	+7	+7

[a] Slope of the linear plot of log K *vs.* $1/T$ determined by the method of least squares.

Table VI.—Standard Thermodynamic Functions for Hexestrol in Bile Salt Solutions

Bile Salt	ΔH^0,[a] Kcal./mole	ΔF^0, Kcal./mole 300°K.	310°K.	318°K.	ΔS^0, Entropy units 300°K.	310°K.	318°K.
Sod. cholate	−6.6	−7.73	−7.80	−7.79	+4	+4	+4
Sod. desoxycholate	−6.4	−7.65	−7.70	−7.73	+4	+4	+4
Sod. taurocholate	−7.1	−7.83	−7.88	−7.87	+2	+3	+2
Sod. glycocholate	−6.2	−7.83	−7.90	−7.93	+5	+6	+5

[a] Slope of the linear plot of log K *vs.* $1/T$ determined by the method of least squares.

molarity of the total bile salts present in the small intestine during fat absorption (21, 24, 28). These values show that the bile salts display a significant effect in increasing the solubility of these poorly water-soluble drugs.

TABLE VII.—RATIOS OF THE SOLUBILITIES OF HEXESTROL, GRISEOFULVIN, AND GLUTETHIMIDE IN 0.04 *M* SOLUTIONS OF CONJUGATED AND UNCONJUGATED BILE SALTS TO THAT IN WATER AT 37°

Bile Salt	Drug		
	Hexestrol	Griseofulvin	Glutethimide
Sod. cholate	122	5.7	1.6
Sod. desoxycholate	139	6.7	2.1
Sod. taurocholate	148	5.6	1.6
Sod. glycocholate	147	5.7	1.5

Drug absorption across the gastrointestinal barrier takes place almost exclusively from a solution of the drug (29). Therefore, the drug must be in solution before it can be absorbed. In the case of extremely water-insoluble drugs, dissolution of the drug usually becomes the slow rate-determining step in the absorption process.

Poorly water-soluble drugs will partition between the aqueous phase and a liquid phase, with a relatively large lipid–water partition coefficient. From a physicochemical point of view, one can draw an analogy between these poorly water-soluble drugs and a dietary lipid. Based on this analogy it would be interesting to speculate that the gastrointestinal tract handles these drugs in the same manner as it handles dietary lipids. In view of the *in vitro* evidence it is quite conceivable that relatively water-insoluble drugs may be absorbed by a mechanism involving preliminary solubilization of the drug by bile salt micelles present in the small intestine.

Relative dissolution rate studies indicate that bile salts significantly increase the dissolution rates of griseofulvin and hexestrol over that in water (30). These findings serve to strengthen further the possibility that physiologic surfactants play an important role in the dissolution step of the absorption process.

Lecithin and cholesterol, which are normal components of bile, as well as fatty acids and monoglycerides, which are the normal breakdown products of fat digestion, have been shown to form mixed micelles with the conjugated bile salts in the small intestine. Extensive studies currently are being conducted to determine the effects of these, as well as other additives, on the degree of solubilization of water-insoluble drugs under conditions simulating those existing in the human small intestine during fat digestion and absorption.

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Steroid Tablet Assay Involving Automated Sample Preparation and Blue Tetrazolium Reaction

By WILLIAM F. BEYER

Automated homogenization, chloroform extraction, and blue tetrazolium assay of hydrocortisone, methylprednisolone, and prednisolone tablets are described. The coefficients of variation for the determination of the three steroids were 1.39, 1.51, and 2.33 per cent, respectively. Tablets are analyzed at a rate of 20/hr. with Technicon's solid preparatory unit and proportioning pump in conjunction with a commercially available spectrophotometer and a strip chart recorder.

THE MANUAL assay of steroid tablets by the blue tetrazolium procedure requires somewhat time-consuming techniques. Directions of U.S.P. XVII (1) and N.F. XII (2) require disintegration of tablets in water and extraction with chloroform. Aliquots of chloroform are then evaporated, and steroids are redissolved in alcohol. Color is developed by the reduction of blue tetrazolium to form the formazan in a solution buffered with tetramethylammonium hydroxide. After standing 90 min. in the dark, color is read at about 525 mμ. Automation of certain aspects of this procedure was described by Greely *et al.* at the New York Academy of Science Conference on Automation in January 1965 (3). The need for a system capable of doing many single tablet assays and the requirement of tablet content uniformity by U.S.P. XVII and N.F. XII for specified tablets led to the development of a completely automated system. Details of the procedure and assay data are the subject of this report.

EXPERIMENTAL

Equipment.—Solid preparatory unit (Solidprep) proportioning pump and 37° water bath.[1] Spectrophotometer and recorder.[2]

Reagents.—3A alcohol;[3] blue tetrazolium, 0.1% in 3A alcohol; chloroform, analytical grade; distilled water with 0.5 ml. of polyoxyethylene lauryl ether[4] added to each 5 gal.; tetramethylammonium hydroxide, 10% aqueous solution diluted 1:10 with 3A alcohol, filtered before use.

Standards.—Accurately weighed quantities of hydrocortisone, methylprednisolone, and prednisolone reference standards are dissolved in 75% 3A alcohol so that each 10.0 ml. contains an amount of steroid equivalent to each tablet.

Tablets.—Steroid tablets are placed in sample cups with 10.0 ml. of 75% 3A alcohol. Cups are covered and allowed to stand for 1 hr. or until tablets disintegrate.

Procedure.—Reagents are pumped using the manifold flow system shown in Fig. 1, and instrumental units are standardized to give a zero base line. The Solidprep is adjusted to deliver 75 ml. of distilled water with polyoxyethylene lauryl ether and mixer speed positioned at No. 4 setting. Approximately 4 cups of the appropriate standard are placed in the sampler head, followed by cups of disintegrated tablets. Standards are placed thereafter at regular intervals to minimize reagent changes and instrumental variation. Samples are automatically homogenized in the blender assembly of the Solidprep. Steroids contained in dilute alcohol are then automatically extracted with chloroform using 3 (6 in.) extraction coils. Blue tetrazolium and tetramethylammonium hydroxide are added directly to the chloroform, and the reaction mixture passes through 2 small mixing coils placed in a 37° water bath. Absorbance of the solution is measured at 524 mμ using a 1.0-cm. flow cell and automatically recorded on a strip chart recorder.

Calculations are made using corresponding absorbances of standards and tablet samples.

RESULTS AND DISCUSSION

Preliminary studies were made indicating that chloroform solutions of steroid could be used satisfactorily in the blue tetrazolium reaction. Aspiration of sample directly from the blender of the Solidprep and extraction with chloroform was carried out initially; however, owing to irregular

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[1] Technicon, Chauncey, N. Y.

[2] Hitachi, Perkin-Elmer 139 spectrophotometer with 1.0 cm. Thomas flow cell No. 9120-NO5, Sargent SRL recorder, A. H. Thomas Co., Philadelphia, Pa.

[3] One hundred gallons of ethanol denatured with 5 gal. of methanol (U. S. Industrial Chemicals Co., New York, N. Y.) constitutes 3A alcohol.

[4] Marketed as Brij 35 by Atlas Chemical Industries Wilmington, Del. Technicon reagent, No. AR-110-62.

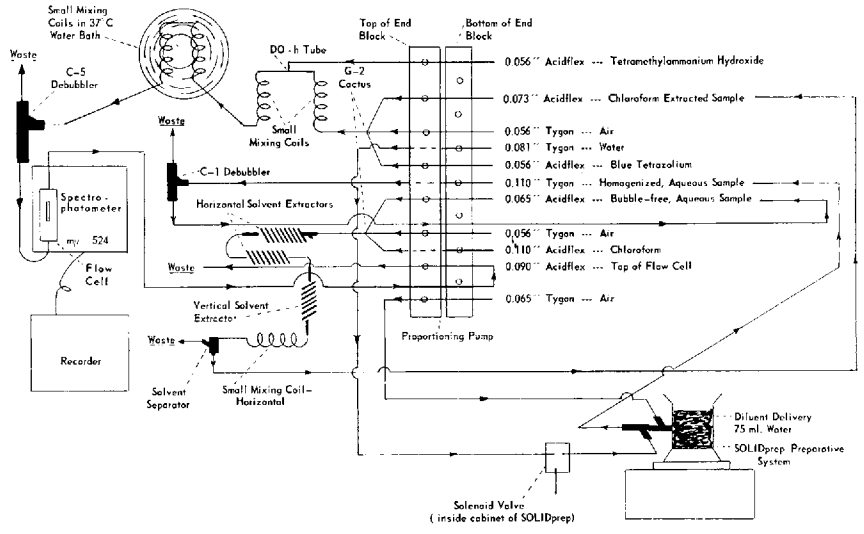

Fig. 1.—Manifold - flow diagram for steroid tablets using AutoAnalyzer Solidprep, solvent extractors and separator, and proportioning pump, in conjunction with a spectrophotometer and strip chart recorder. Transmission lines carrying air or aqueous solutions consist of Tygon tubing, those carrying alcohol or chloroform are acidflex.

sampling, a debubbler was introduced, and a bubble-free sample was withdrawn and extracted. The use of the first two extractors in a horizontal position and the last vertical produced an even pumping action and bubble pattern. Attaching a small horizontal mixing coil to the last extractor permitted satisfactory separation of chloroform and water phases.

On occasion, small acidflex lines would not aspirate reagents until primed with a syringe containing the same solution. Minimal difficulty was encountered when the tubes were placed exactly as shown in Fig. 1. Positioning polyethylene end blocks of the manifold so that the tubes were secured by the rounded ends of the tracts instead of under the projections prevented the acidflex tubes from being sheared. With acidflex tubing larger than 0.090 in., Technicon N-4 nipples had to be used, since pressure from pumping and the action of chloroform on the lines caused a separation of the acidflex tubing from smaller nipples. Wherever possible, acidflex tubing was attached directly to glass fittings.

Following construction of a satisfactory manifold

TABLE I.—PERCENTAGE RECOVERIES OF STEROIDS USING AUTOMATED EXTRACTION AND BLUE TETRAZOLIUM REACTION

Hydrocortisone, 5.0 mg.	Methylprednisolone, 4.0 mg.	Prednisolone, 5.0 mg.
97.6	98.3	100.9
100.4	100.6	102.6
100.6	99.2	98.9
100.0	101.1	95.1
98.4	102.0	96.8
100.4	102.0	101.9
100.0	100.3	100.0
100.0	99.2	101.1
102.6	98.1	100.4
101.0	98.7	101.1
98.4		102.2
Coefficients of Variation		
1.39%	1.51%	2.33%

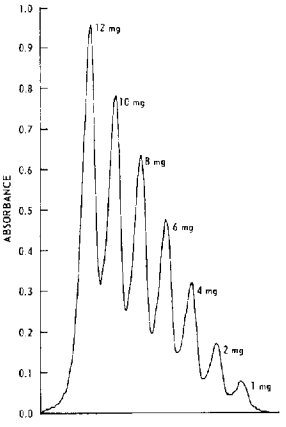

Fig. 2.—Absorbance – concentration recording of hydrocortisone using automatic extraction and blue tetrazolium reaction. Steroid is contained in 10.0 ml. of 75% alcohol, absorbance is measured at 524 mμ, and a 1.0-cm. flow cell is used.

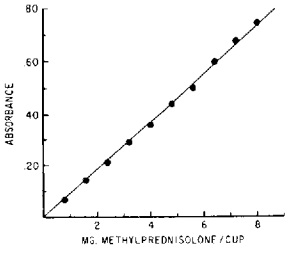

Fig. 3.—Beer's law plot for methylprednisolone.

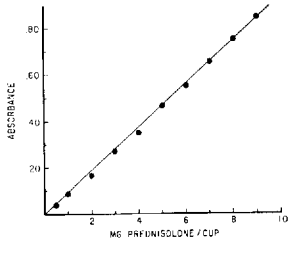

Fig. 4.—Beer's law plot for prednisolone standard.

and optimizing conditions for the blue tetrazolium reaction, the precision of the automated procedure was determined. Replicate standards of hydrocortisone, methylprednisolone, and prednisolone in 75% 3A alcohol were analyzed, giving coefficients of variation of 1.39, 1.51, and 2.33% for the respective steroids. Table I gives results of this study. The precision for the procedure is considered quite satisfactory, considering that this represents the entire assay error from sample preparation, addition of water diluent, homogenization, chloroform extraction, blue tetrazolium reaction, to recording of absorbance peaks. The entire procedure is completed in approximately 8 min. After the first sample, however, additional results are recorded every 3 min.

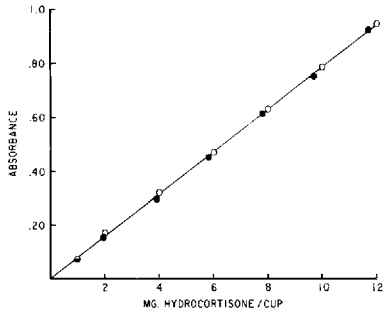

Fig. 5.—Beer's law plot for hydrocortisone originating from standard and tablets. Key: O, hydrocortisone standard; ●, hydrocortisone in pulverized tablet.

Figure 2 shows a recording of various quantities of hydrocortisone standard in 75% 3A alcohol. It can be noted that absorbance is a linear function of concentration. Figures 3 and 4 show that Beer's law is obeyed for methylprednisolone and prednisolone.

To test the recovery of hydrocortisone from varying amounts of 5.0-mg. tablets, 50 tablets were weighed and reduced to a fine powder. Accurately

Table II.—Recovery of Steroids from Various Quantities of Powdered Tablets Using Automated Extraction and Blue Tetrazolium Reaction

Steroid Added from Powdered Tablet, mg.	Amt. of Std. Added, mg.	Total Amt. of Steroid Present, mg.	Amt. of Steroid Recovered, mg.	Recovery, %
		Hydrocortisone Tablets + Std.		
0.98	4.00	4.98	5.00	100.4
1.99	3.00	4.99	5.18	103.8
3.01	2.00	5.01	5.00	99.8
4.00	1.00	5.00	4.73	94.6
		Methylprednisolone Tablets + Std.		
0.78	3.20	3.98	3.87	97.2
1.58	2.40	3.98	3.94	99.0
2.15	1.60	3.75	3.75	100.0
3.19	0.80	3.99	3.87	97.0
		Prednisolone Tablets + Std.		
1.05	4.00	5.05	4.81	95.2
2.17	3.00	5.17	4.98	96.3
3.19	2.00	5.19	5.09	98.1
4.28	1.00	5.28	5.16	97.7

Table III.—Steroid Tablet Assays Using Automated Extraction and Blue Tetrazolium Reaction and Comparisons with a Manual Blue Tetrazolium Procedure

Lot	Automated Procedure: Tablets, No.	Automated Procedure: mg. Steroid/Tablet (± Coefficient of Variation)	Manual Method[a]
	Hydrocortisone Tablets, 5.0 mg./Tablet		
1	10	4.79 ± 3.14%	4.93
2	15	4.93 ± 4.25%	5.04
3	15	5.22 ± 3.91%	5.10
4	15	5.08 ± 4.23%	4.75
5	12	4.52 ± 1.74%	4.90
	Methylprednisolone Tablets, 4.0 mg./Tablet		
6	15	3.91 ± 2.53%	3.96
7	10	3.99 ± 4.27%	4.10
8	15	3.90 ± 3.28%	3.85
9	12	4.00 ± 2.25%	3.90
10	12	3.91 ± 1.79%	4.03
	Prednisolone Tablets, 5.0 mg./Tablet		
11	15	4.89 ± 3.93%	4.83
12	10	5.08 ± 2.95%	5.00
13	10	5.19 ± 2.52%	5.12
14	10	5.20 ± 3.11%	5.17
15	10	5.14 ± 1.95%	4.96

[a] In some instances, the AutoAnalyzer was used for the blue tetrazolium reaction.

weighed quantities of the powder were placed in 10.0 ml. of 75% 3A alcohol and allowed to stand approximately 1 hr. Hydrocortisone standards approximating the steroid content of powder samples were also prepared and analyzed. When absorbances of hydrocortisone standard and powdered tablets are plotted, a single line (Fig. 5) can be drawn through absorbance values for both standard and tablets. Covariance analyses of the data were performed, and no significant differences in results were found.

Studies were performed to determine the efficiency of the automated procedure by adding varying amounts of powdered tablets to a particular standard. Table II shows that recoveries were good.

Assays of steroid tablets were carried out for 5 lots of each particular steroid by the automated procedure and compared to the method essentially as directed by U.S.P. XVII. The data in Table III show good agreement. The automated procedure is suggested for single-tablet assays and should be of value when complying with content-uniformity tests of U.S.P. XVII and N.F. XII.

After completion of this report, several modifications in the automated procedure were made that are considered worthy of reporting. Tetrabutylammonium hydroxide is substituted for tetramethylammonium hydroxide, and filtration of this reagent is no longer a requirement. A small glass wool in-line filter is placed in the flowing stream just prior to the cell debubbler, eliminating the possibility of eroded tubing and other particles entering into the cell.

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Design and Operation of a Laboratory Glass Spray Drier

By JOHN D. TOPHAM

A spray drier made from borosilicate glass is described, which has been used to dry streptomycin without loss of activity and also other pharmaceuticals. Its advantages over other spray driers are: low cost, complete vision of the drying process, and its ease of adaptation to produce sterile powders.

MANY PAPERS have been published on the spray drying of pharmaceuticals (1–5) since the erection of a spray drier at Manchester University in 1939 (6). So far, to the author's knowledge, no work has been published on the spray drying of antibiotics, although one manufacturer has installed a spray drier for this purpose. All the spray driers which are used in industry are made of metal, which is not a satisfactory material when solutions of substances, which are very sensitive to oxidation, are to be dried. Since glass is used to replace metal in the apparatus described, oxidative discoloration does not take place when streptomycin is dried in the apparatus. Also, it is difficult to observe the drying process if metal apparatus is used. These 2 considerations, plus cheapness, persuaded the author to design a spray drier made of borosilicate glass.[1]

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[1] An application has been made to patent the apparatus.

Temperature.—Borosilicate glass softens at 700° and will crack as a result of thermal shock, if sudden temperature fluctuations take place. Consequently, at no time during the operation of the drier should a temperature of 500° be exceeded. In fact, 320° is the maximum temperature to which the apparatus, described in this article, has been subjected. The temperature range within which the spray drier has been used is 140–220°. The normal operating temperature was 160°.

Wetting.—The contact angle between glass and water is zero. Consequently, any drops of solution which come into contact with the walls of the apparatus will adhere. This difficulty has been overcome by silicone coating the apparatus, using a 2% solution of dimethyldichloro-silane in carbon tetrachloride.

Sealing of Joints.—A film of silicone grease was applied to most joints which were then clipped together using simple metal clips.

Spray.—After considerable experimentation it was found that the best spray was produced using an atomizer device constructed from standard laboratory glassware (Fig. 1). This was con-

weighed quantities of the powder were placed in 10.0 ml. of 75% 3A alcohol and allowed to stand approximately 1 hr. Hydrocortisone standards approximating the steroid content of powder samples were also prepared and analyzed. When absorbances of hydrocortisone standard and powdered tablets are plotted, a single line (Fig. 5) can be drawn through absorbance values for both standard and tablets. Covariance analyses of the data were performed, and no significant differences in results were found.

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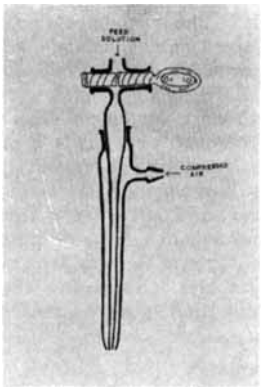

Fig. 1.—Atomizer unit.

nected to an Edwards compression and vacuum pump model RB4, which gave a maximum pressure of 18 lb./sq. in.

Sterilization.—When sterile powders were required, all connecting tubing was sterilized in an autoclave. Sterile filters of nonabsorbent cotton wool 4 cm. thick, supported in calico, were placed at each of the air inlets. The atomizer device and receiver were sterilized by heating in a hot air oven at 160° for 1 hr. The rest of the apparatus was sterilized by passing filtered hot air through the apparatus for 1 hr. at 180–200°.

Method of Operation.—A domestic suction cleaner is used to produce a stream of air at a displacement of approximately 240 L./min. This flows through 4 parallel tubes, each containing a firelighter element of 1.8-kw. capacity. The hot air then enters the drying chamber from above through a fused alumina pipe (Fig. 2). The spray enters the drying chamber alongside the hot air inlet and is dried as it passes through the chamber in the air stream.

The drying temperature can be controlled by switching on and off the heating elements, but greater temperature control is brought about by regulating the rate of flow of the solution through the atomizer.

On leaving the chamber, the dried product and steam pass into a cyclone separator, where the product falls into a previously warmed receiver, and the excess hot air and steam pass out through a dust bag. The receiver must be warmed initially; otherwise water vapor will condense on its inner surfaces, causing the product to become damp.

A positive pressure is required above the surface of the solution to be dried, otherwise air from the atomizer section will blow up the capillary when the feed vessel is almost empty. The rate of drying is 1 L./hr. for concentrated solutions.

Design Considerations.—Ideally, the apparatus should operate from a compressor capable of supplying sterile, oil-free air. This would make possible the continuous production of sterile products.

Originally the hot air inlet tube was made of copper, but it was found that, when streptomycin was spray dried, oxidative discoloration took place, which was due presumably to the catalytic effect of the metal. Hence, the copper tube was replaced by one of fused alumina, although other tubes, constructed of materials which do not contain heavy metals, could be used.

Cleaning.—Each section can be washed in a normal-size sink. The cyclone separator presents some difficulties, but if steel ball bearings are used, the dirt can be "rumbled" away.

EXPERIMENTAL

A 500-ml. quantity of double strength nutrient broth was spray dried in the previously sterilized apparatus. The dry product was collected in 8 sterile containers and sterile water was added to each. These were then incubated at 37° for 3 days, and observed daily for growth. The experiment was duplicated.

Of the 16 containers holding the reconstituted broth 15 were sterile. Growth in the sixteenth container presumably was due to accidental contamination during the transference of the sterile water to the spray-dried product.

After the 3-day incubation period, each sample

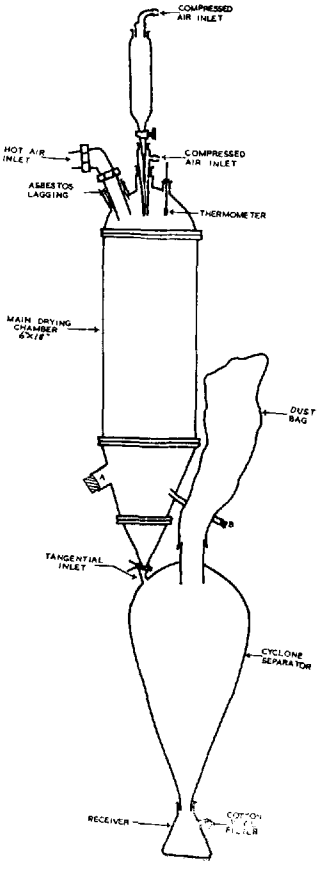

Fig. 2.—Appendages *A* and *B* were part of the original design but are no longer used.

TABLE I.—STREPTOMYCIN ASSAY RESULTS (ZONES OF INHIBITION MEASURED IN mm.)

Plate	Std. S_1	Std. S_2	Spray Dried T_1	Spray Dried T_2
1	16.5	19.5	16.5	20
2	17	20	16.5	20.5
3	17.5	19.5	17.5	19.5
4	17	19	17	19
Total	68.0	78.0	67.5	79.0
Av.	17.0	19.5	16.875	19.75

TABLE II.—PENICILLIN ASSAY RESULTS (ZONES OF INHIBITION MEASURED IN mm.)

Plate	Std. S_1	Std. S_2	Spray Dried T_1	Spray Dried T_2
1	26.5	28.5	26	29
2	26	28	26	28
3	25.5	28.5	25.5	27
4	26	28.5	26	29
Total	104	113.5	103.5	113
Av.	26	28.375	25.875	28.25

was inoculated with a loopful of a culture of *Micrococcus pyogenes*. Every sample became opalescent within 18 hr., indicating that the broth was capable of supporting growth of microorganisms.

Streptomycin sulfate has been spray dried without loss of activity as shown by the assay laid down in the "British Pharmacopoeia," 1958. Four Petri dishes containing seeded nutrient agar were used for each assay. In each dish 4 × 8 mm. holes were bored and the solutions T_1, T_2, S_1, and S_2 added to each plate. The strengths of the standard and test solutions were 5 and 10 units/ml. (Table I). After spray drying, the potency was 100.3% of the original (limits of results 86.7–115.3%).

A similar assay was carried out on sodium benzyl penicillin. The strengths of the test and standard solutions were 3 and 6 units/ml. (Table II). After spray drying, the potency was 96.6% of the original (limits of results 83.9 to 119.1%).

Seaweed extract, coffee, aluminum hydroxide gel, and an aluminum hydroxide complex have also been dried to produce free-flowing powders, which (except for the alumina) readily redissolved in water. Spores of *Bacillus subtilis* have also been dried with a 50% mortality. The powders, when viewed under the microscope, all showed the hollow spheres characteristic of spray-dried powders.

SUMMARY

1. The apparatus permits the drying process to be observed continuously; consequently, any obstruction to flow is readily noticed before damage to the product occurs.

2. It can be used to produce sterile powders more rapidly than freeze drying.

3. It is suitable for drying solutions and suspensions of materials which are prone to oxidation in the presence of metals.

4. The dried products are free flowing and lend themselves to aseptic transfer into sterile containers.

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Notes

Dissolution Rate–Solubility Behavior of 3-(1-Methyl-2-pyrrolidinyl)-indole as a Function of Hydrogen-Ion Concentration

By W. E. HAMLIN and W. I. HIGUCHI*

An investigation of the hydrogen-ion dependence of the dissolution rate of 3-(1-methyl-2-pyrrolidinyl)-indole (U-11028) is reported. Theoretical equations are developed which are in good agreement with the experimental data. This agreement shows clearly that it is the much greater diffusion coefficient of hydrochloric acid (D_H = 3.1 D_B) that is responsible for the deviation from the Noyes-Whitney theory.

CONSIDERABLE evidence has been presented to show that the initial rate of dissolution of a pellet is directly proportional to the solubility of the compound in a test fluid (1). This relationship, derived from the Noyes-Whitney law (2), states that

$$R = kC_s \qquad \text{(Eq. 1)}$$

where R is the initial dissolution rate per unit surface area of the pellet (mg./cm.²/hr.), k is a constant (2.24 for the given test conditions), and C_s is the solubility of the compound (mg./ml.). However, some data do not follow Eq. 1. One such compound which shows a significant positive deviation is 3-(1-methyl-2-pyrrolidinyl)-indole (U-11028). Since this deviation is observed in 0.05 N HCl but

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TABLE I.—STREPTOMYCIN ASSAY RESULTS (ZONES OF INHIBITION MEASURED IN mm.)

Plate	Std. S_1	Std. S_2	Spray Dried T_1	Spray Dried T_2
1	16.5	19.5	16.5	20
2	17	20	16.5	20.5
3	17.5	19.5	17.5	19.5
4	17	19	17	19
Total	68.0	78.0	67.5	79.0
Av.	17.0	19.5	16.875	19.75

TABLE II.—PENICILLIN ASSAY RESULTS (ZONES OF INHIBITION MEASURED IN mm.)

Plate	Std. S_1	Std. S_2	Spray Dried T_1	Spray Dried T_2
1	26.5	28.5	26	29
2	26	28	26	28
3	25.5	28.5	25.5	27
4	26	28.5	26	29
Total	104	113.5	103.5	113
Av.	26	28.375	25.875	28.25

was inoculated with a loopful of a culture of *Micrococcus pyogenes*. Every sample became opalescent within 18 hr., indicating that the broth was capable of supporting growth of microorganisms.

Streptomycin sulfate has been spray dried without loss of activity as shown by the assay laid down in the "British Pharmacopoeia," 1958. Four Petri dishes containing seeded nutrient agar were used for each assay. In each dish 4 × 8 mm. holes were bored and the solutions T_1, T_2, S_1, and S_2 added to each plate. The strengths of the standard and test solutions were 5 and 10 units/ml. (Table I). After spray drying, the potency was 100.3% of the original (limits of results 86.7–115.3%).

A similar assay was carried out on sodium benzyl penicillin. The strengths of the test and standard solutions were 3 and 6 units/ml. (Table II). After spray drying, the potency was 96.6% of the original (limits of results 83.9 to 119.1%).

Seaweed extract, coffee, aluminum hydroxide gel, and an aluminum hydroxide complex have also been dried to produce free-flowing powders, which (except for the alumina) readily redissolved in water. Spores of *Bacillus subtilis* have also been dried with a 50% mortality. The powders, when viewed under the microscope, all showed the hollow spheres characteristic of spray-dried powders.

SUMMARY

1. The apparatus permits the drying process to be observed continuously; consequently, any obstruction to flow is readily noticed before damage to the product occurs.

2. It can be used to produce sterile powders more rapidly than freeze drying.

3. It is suitable for drying solutions and suspensions of materials which are prone to oxidation in the presence of metals.

4. The dried products are free flowing and lend themselves to aseptic transfer into sterile containers.

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Notes

Dissolution Rate–Solubility Behavior of 3-(1-Methyl-2-pyrrolidinyl)-indole as a Function of Hydrogen-Ion Concentration

By W. E. HAMLIN and W. I. HIGUCHI*

An investigation of the hydrogen-ion dependence of the dissolution rate of 3-(1-methyl-2-pyrrolidinyl)-indole (U-11028) is reported. Theoretical equations are developed which are in good agreement with the experimental data. This agreement shows clearly that it is the much greater diffusion coefficient of hydrochloric acid (D_H = 3.1 D_B) that is responsible for the deviation from the Noyes-Whitney theory.

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$$R = kC_s \qquad \text{(Eq. 1)}$$

where R is the initial dissolution rate per unit surface area of the pellet (mg./cm.²/hr.), k is a constant (2.24 for the given test conditions), and C_s is the solubility of the compound (mg./ml.). However, some data do not follow Eq. 1. One such compound which shows a significant positive deviation is 3-(1-methyl-2-pyrrolidinyl)-indole (U-11028). Since this deviation is observed in 0.05 N HCl but

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TABLE I.—SOLUBILITIES AND DISSOLUTION RATES OF 3-(1-METHYL-2-PYRROLIDINYL)-INDOLE AS A FUNCTION OF pH OF THE DISSOLUTION MEDIUM AT 37°

Dissolution Fluid	Initial pH	Solubility at 37°, mg./ml.	Dissolution Rate ±95% C.I. of Rate, mg./cm.²/hr.	Rate-to-Solubility Ratio
0.1 *N* HCl	1.2	21.0	146 ± 17	6.95
0.05 *N* HCl	1.3	10.3	89.5 ± 20.5	8.69
0.01 *N* HCl	2.0	2.35	18.9 ± 1.9	8.04
0.002 *N* HCl (CO_2-free)	2.8	1.02	5.07 ± 0.74	4.97
0.001 *N* HCl (CO_2-free)	3.1	0.837	2.93 ± 0.64	3.50
Distilled H_2O (CO_2-free)	7.0	0.703	1.89 ± 0.21	2.69
Phosphate buffer	7.2	2.36	6.35 ± 0.38	2.69

not in phosphate buffer (pH 7.2), the reason may be the much greater diffusion coefficient for the HCl. The test of this hypothesis is reported.

EXPERIMENTAL

The procedures for determining the initial rate of dissolution and solubility of U-11028 in the test fluids of varying hydrogen-ion concentration were reported previously (1). All tests were run at 37°. The test fluids listed in Table I all have the same ionic strength ($\mu = 0.1$) by adjustment with sodium chloride. Assays were made by ultraviolet spectrophotometry using a Cary model 11 recording spectrophotometer.

The rate of dissolution and solubility data are recorded in Table I as a function of the pH of the test fluid. The rate-to-solubility ratio, equivalent to k in Eq. 1, for each set of data serves as a measure of the deviation from the reference value of 2.69.

An apparent pKa value of 8.9 for this compound was determined by potentiometric titration of a dilute solution in CO_2-free deionized water at room temperature.

RESULTS AND DISCUSSION

Solubility of U-11028 as a Function of Initial H^+.—The important reactions are:

$$B \overset{K_B}{=} BH^+ + OH^- \qquad \text{(Eq. 2)}$$

and

$$OH^- + H^+ \overset{\frac{1}{K_W}}{=} H_2O \qquad \text{(Eq. 3)}$$

Let $(B)_0$ be the unionized base concentration in equilibrium with the solid, and let $(H)_0$ be the initial hydrogen-ion concentration, *i.e.*, the H^+ concentration before addition of any solid base. Let x = moles/L. of BH^+ formed by the reaction given in Eq. 2, and let y = the moles/L. of the reaction in accordance with Eq. 3. Therefore, $x - y$ would be equal to the moles/L. of remaining OH^- at equilibrium and $(H)_0 - y$ the concentration of the remaining H^+.

Therefore,

$$K_B = \frac{x(x - y)}{(B)_0} \qquad \text{(Eq. 4)}$$

and

$$K_W = [(H)_0 - y](x - y) \qquad \text{(Eq. 5)}$$

Neglecting K_W/K_B $(B)_0$ as compared to unity, one obtains from Eqs. 4 and 5

$$x = \frac{(H)_0 + [(H)_0^2 + 4K_B(B)_0]^{1/2}}{2} \qquad \text{(Eq. 6)}$$

Therefore, the total solubility is

$$C_s = (B)_0 + x$$

or

$$C_s = (B)_0 + \frac{(H)_0 + [(H)_0^2 + 4K_B(B)_0]^{1/2}}{2} \qquad \text{(Eq. 7)}$$

Since K_A for U-11028 is about 6×10^{-10}, and since K_W for water is around 2×10^{-14}, $K_B \simeq 3 \times 10^{-5}$. $(B)_0$ may be calculated by taking this value for K_B and the solubility, $C_S = 3.5 \times 10^{-3}M$, for U-11028 in pure water. Noting that $(H)_0 \simeq 1 \times 10^{-7}M$ is negligible compared to $K_B(B)_0$, one obtains from Eq. 7

$$(B)_0 \simeq 3.2 \times 10^{-3}M$$

Therefore, Eq. 7 becomes

$$C_s = 3.2 \times 10^{-3} + \frac{(H)_0 + [(H)_0^2 + 3.8 \times 10^{-7}]^{1/2}}{2} \qquad \text{(Eq. 8)}$$

In Fig. 1, Eq. 8 is plotted (smooth curve) and compared with the experimental data. The agreement of the data with theory is very satisfactory.

Dissolution Rate of U-11028 as a Function of Initial H^+.—Consideration of simultaneous diffusion and chemical reaction leads to the following equation for the initial dissolution rate of a base, B, in HCl solutions (see *Appendix* for derivation). The initial rate, G, is

$$G = \frac{1}{h}\left\{ D_B(B)_0 + \frac{D_H(H)_0 + D_H(H)_0\left[1 + \frac{4D_{OH}K_BD_{BH}(B)_0}{D_H^2(H)_0^2}\right]^{1/2}}{2} \right\} \qquad \text{(Eq. 9)}$$

Here h is the diffusion layer thickness, $(B)_0$ is the free base concentration in equilibrium with the solid, $(H)_0$ is the initial hydrogen-ion concentration in the dissolution medium, and the D's are the respective diffusion coefficients of the species (indicated by subscripts).

Equation 9 may be written

$$\frac{Gh}{D_B} = (B)_0 + \frac{1}{2}\left(\frac{D_H}{D_B}\right)(H)_0 + \frac{1}{2}\left(\frac{D_H}{D_B}\right)(H)_0\left(1 + \frac{4D_{OH}D_{BH}K_B(B)_0}{D_H^2(H)_0^2}\right)^{1/2} \qquad \text{(Eq. 10)}$$

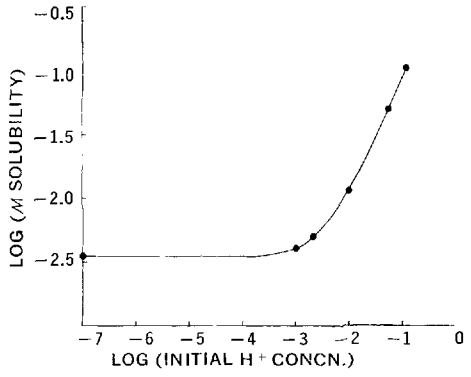

Fig. 1.—Solubility of 3-(1-methyl-2-pyrrolidinyl)-indole as function of initial H^+ concentration. Key: ●, experimental data; ——, theory, Eq. 8.

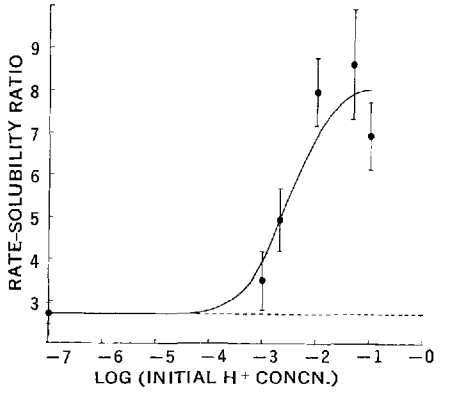

Fig. 2.—The rate-to-solubility ratio for 3-(1-methyl-2-pyrrolidinyl)-indole as a function of initial H^+ concentration in dissolution medium. Key: ●, experimental data; ——, theory (Eq. 10 with D_H/D_B = 3.1); ------, Noyes-Whitney prediction.

It is apparent by comparing Eq. 10 with Eq. 7 that if all of the diffusion coefficients were equal, the dissolution rate would always be directly proportional to the solubility. However, the rate-to-solubility ratio for U-11028 is not constant (Table I) over the range of HCl concentrations. Therefore, consider the possibility that D_H is much greater than D_B.

In Fig. 2, the results of the rate-to-solubility ratios calculated employing Eq. 10 with $D_H = 3.1\ D_B$ are plotted as the smooth curve. In these theoretical calculations, the rate-to-solubility ratio value of 2.69 in distilled water was used to fit the theory to data at this one point.

It is worthwhile to point out that the term involving D_{OH} and D_{BH} in Eq. 10 is small compared to unity, except for the case involving distilled water. Therefore, the choice of values for D_{OH} and D_{BH} is not critical. In the distilled water case, the square root term contributes less than 10% to the rate. So again, the choice of values for D_{OH} and D_{BH} is not critical. Therefore, in these calculations, D_{OH} and D_{BH} were taken to be equal to D_B, the diffusion coefficient for the unprotonated base.

The good agreement of theory with data taken from Table I clearly shows that it is the much greater diffusion coefficient of HCl that is responsible for the deviation from the Noyes-Whitney law. The value of $D_H = 3.1\ D_B$ is reasonable. The diffusion coefficient for HCl in water is about 3.0×10^{-5} cm.^{2} sec.$^{-1}$ at room temperature (3), while that for U-11028 is expected to be as much as 5 times smaller according to Stokes' law. Therefore, although the 3.1 factor appears to be a little low, it is of the right order of magnitude.

As discussed previously (4) where electrolytes are involved, the effective diffusion coefficient of an ion is appreciably influenced by the nature and the concentrations of other ions in the solution. In this regard the primary effect is often the diffusion potential effect which is caused by the greater inherent mobility of 1 ion in the presence of more slowly moving ions of the opposite charge. Thus, the effective diffusion coefficient of the hydrogen ion in an HCl solution is not determined entirely by the inherent mobility of the hydrogen ion, but is influenced greatly by the restraining effect of the chloride ion. Despite the retarding effect of the chloride ion, the diffusion coefficient of HCl is still relatively large. The effective diffusion coefficient of the protonated U-11028 also is influenced by the chloride ion, but, in this instance, the relatively large size of the U-11028 molecule should be the main factor.

It is reasonable then to expect D_H to be relatively large compared to D_B and D_{BH}, with the latter 2 being about the same order of magnitude. While D_{OH} would be expected to be also significantly greater than D_B or D_{BH}, it can be seen from concentration considerations that the OH^- does not play an important role.

APPENDIX

Derivation of Eq. 9.—Consideration of the physical situation allows one to write 2 independent equations for G, the dissolution rate,

$$G = -D_B \frac{d(B)}{dX} - D_{BH} \frac{d(BH)}{dX} \quad \text{(Eq. 1}a\text{)}$$

and

$$G = D_H \frac{d(H)}{dX} - D_B \frac{d(B)}{dX} - D_{OH} \frac{d(OH)}{dX} \quad \text{(Eq. 2}a\text{)}$$

As boundary conditions, at $X = 0$: $(B) = (B)_0$, $(BH) = (BH)_0'$, $(H) = (H)_0'$ and $(OH) = (OH)_0'$, and at $X = h$: $(H) = (H)_0$ and all other species are at 0 concentration. With these boundary conditions and with the approximation that $K_w \ll K_B\ (B)_0$, Eqs. 1*a* and 2*a* may be solved by eliminating $(H)_0'$, $(BH)_0'$, and $(OH)_0'$ to give Eq. 9 in the same manner as was done previously for similar problems (4, 5).

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Alkaloids of *Thalictrum* VI.

Isolation of Obamegine from *Thalictrum rugosum*

By TOSHIAKI TOMIMATSU* and JACK L. BEAL

A description is given of the isolation of a phenolic tertiary base from the roots of *T. rugosum* and the identification of this base as obamegine.

THE PRESENCE of magnoflorine and berberine in the roots of *Thalictrum rugosum* Ait. (*T. glaucum* Desf.) has been reported in the previous paper of this series (1). The present report describes the isolation of a phenolic tertiary base from the roots of *T. rugosum* and the identification of this base as obamegine (I).

The tertiary base, m.p. 172° dec., $[\alpha]_D$ + 241° (chloroform), was obtained in crystalline form from the crude tertiary base fraction referred to in the previous paper (1). The U.V. spectrum suggested a benzenoid nucleus, $\lambda_{max.}$ 285 mμ, while in the I.R. spectrum 2 hydroxyl peaks were detected, 3450 cm.$^{-1}$ and 3260 cm.$^{-1}$. The NMR spectrum indicated the presence of 2 methoxyl and 2 *N*-methyl groups. In addition, the NMR spectrum showed the presence of benzene of crystallization. The molecular formula, $C_{36}H_{38}N_2O_6 \cdot 2C_6H_6$, was assigned on the basis of the elemental analysis which also suggested the presence of a benzene adduct.

On treatment of the phenolic base with diazomethane, a product was obtained which possessed 4 methoxyl and 2 *N*-methyl groups thereby verifying that the base contains 2 phenolic hydroxyl groups. Analytical data indicated the phenolic base to be of the berbamine type. Since obamegine (2, 3) has been described as a phenolic base possessing 2 hydroxyl groups and belonging to the berbamine type alkaloids (4), this base was compared with an authentic sample of obamegine.[1]

The phenolic base was shown to be identical with obamegine by comparision of I.R. spectra and a mixed melting point determination. Finally, this base and its *O,O*-dimethylether and *O,O*-diethylether gave NMR spectra identical with the NMR spectra of obamegine, *O,O*-dimethylobamegine, and *O,O*-diethylobamegine.[1]

This is the first report of the isolation of obamegine from a *Thalictrum* species. Previously, aromoline (thalicrine) was isolated from *Thalictrum thunbergii* D.C. by one of the authors (5–7). Aromoline belongs to the oxyacanthine group of alkaloids. Thus, both the berbamine and oxyacanthine type of alkaloids have been isolated from the genus *Thalictrum*. Both obamegine (I) and aromoline (II) have 2 hydroxyl groups in the 7 and 4′ or 4″ positions.

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[1] The authors thank Dr. M. Tomita and Dr. T. Ibuka, Kyoto University, for an authentic sample of obamegine which was supplied by them, and for the loan of the NMR spectra charts of obamegine, *O,O*-dimethylobamegine, and *O,O*-diethylobamegine.

EXPERIMENTAL[2]

Material.—Roots of *T. rugosum* were obtained from Wayside Gardens, Mentor, Ohio. A minimum of 100 plants are being cultivated in The Ohio State University College of Pharmacy Medicinal Plant Garden. In addition, herbarium specimens have been made and are on file.

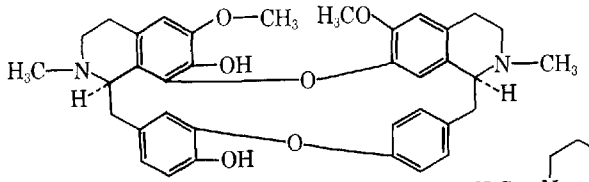
Isolation and Identification of Obamegine from T. Rugosum.—Milled roots, 6.2 Kg., were extracted with methanol in a continuous extractor until a negative test of the extractant for alkaloid was obtained with Valser's reagent. The methanol extract was concentrated almost to dryness, *in vacuo*, and poured while stirring into warm 5% acetic acid. The insoluble material was extracted repeatedly with 5% acetic acid until a negative test was obtained with Valser's reagent. The acidic solution was freed from acidic and neutral substances by extraction with ether, and then made alkaline with ammonium hydroxide solution and extracted exhaustively with ether to remove ether-soluble bases. The ether solution was extracted with 5% potassium hydroxide to remove the phenolic base. The solution containing the phenolic base was made weakly acidic by adding hydrochloric acid and then basic with ammonium hydroxide solution. The alkaline solution was extracted with benzene which was then dried over anhydrous sodium sulfate, filtered, and evaporated to dryness to yield 4.8 Gm. of colorless crystalline residue. Repeated recrystallization from benzene gave colorless needles, m.p. 172° dec. $[\alpha]_D^{30}$ + 241° (chloroform), ultraviolet $\lambda_{max.}$, (ethanol) 285 mμ. The infrared spectrum (chloroform) showed 3450 cm.$^{-1}$, 3260 cm.$^{-1}$ (OH). The NMR spectrum ($CDCl_3$) showed τ 7.60, 7.73 (*N*-methyl), 6.27, 6.36 (*O*-methyl), 2.72 (benzene).

Anal.—Calcd for $C_{36}H_{38}N_2O_6 \cdot 2C_6H_6$: C, 76.80; H, 6.66; N, 3.73. Found: C, 76.52; H, 6.59; N, 3.76.

The melting point was not depressed on admixture with an authentic sample of obamegine, m.p. 172° dec. In addition, the infrared spectrum in potassium bromide of the sample was identical with that of an authentic sample of obamegine.

O,O-Dimethyl Ether.—A 0.1-Gm. quantity of the above phenolic base was dissolved in 15 ml. of methanol and added to an ethereal solution of diazomethane which was made from 4.3 Gm. of *p*-tolylsulfonylmethylnitrosamide and 0.8 Gm. of potassium hydroxide in 20 ml. of 96% ethanol. After standing 3 days, the ether and the excess diazomethane were evaporated on a steam bath. The residue was dissolved in 20 ml. of 5% acetic acid solution which was then made basic with aqueous sodium hydroxide and extracted exhaustively with ether. The ether solution was dried

[2] Melting points were determined with a Thomas-Hoover melting point apparatus. Infrared spectra were obtained using a Perkin-Elmer Infracord spectrophotometer, model 237. The NMR spectra were obtained using a Varian Associates 60 megacycle spectrophotometer. The authors thank Dr. David Dalton, Department of Chemistry, for the NMR interpretations and valuable suggestions.



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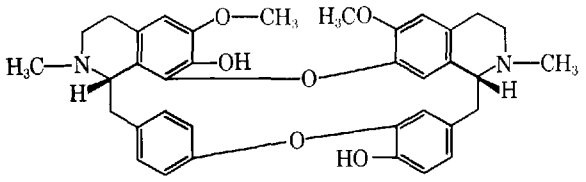

II

over anhydrous potassium carbonate and the ether then evaporated on a steam bath. The residue was dissolved in benzene and chromatographed on a column of Woelm, grade 1, neutral alumina (10 Gm., column 1 cm. × 6 cm.) using benzene as the solvent. A single compound was obtained in the eluate. The NMR data ($CDCl_3$) indicated τ 7.46, 7.76 (*N*-methyl), 6.12, 6.29, 6.41, and 6.87 (*O*-methyl), and was identified with that of *O,O*-dimethylobamegine.

O,O-Diethyl Ether.—A 0.1-Gm. sample of the phenolic base was dissolved in 10 ml. of methanol and added to an ethereal solution of diazoethane which was made from 7 Gm. of nitrosoethylurea and 40 Gm. of 50% potassium hydroxide solution. After standing 4 days at room temperature, the solution was treated by the usual method and the residue crystallized from acetone as colorless rosette crystals (0.098 Gm.), m.p. 203–204.° The NMR spectrum ($CDCl_3$) showed τ values at 7.39, 7.71 (*N*-methyl), 6.22, and 6.40 (*O*-methyl). The NMR spectrum was superimposable with that of known *O,O*-diethylobamegine.

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Tosylation of *N*-Phenyl-*N'*-(3-hydroxypropyl)urea

By ARTHUR F. FERRIS*, O. LEROY SALERNI, and BEVERLY A. SCHUTZ

Two unexpected products, *N*-phenyl-*N'*-(3-chloropropyl)urea (II) and 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine (III), are formed from the reaction of *p*-toluenesulfonyl chloride and *N*-phenyl-*N'*-(3-hydroxypropyl)-urea (I).

As part of a program dealing with the synthesis of potential antiradiation drugs, it became necessary to carry out the reaction of *p*-toluenesulfonyl chloride and *N*-phenyl-*N'*-(3-hydroxypropyl)urea (I). The authors have found that this reaction in pyridine solvent gives 2 unexpected products: *N*-phenyl-*N'*-(3-chloropropyl)urea (II) and 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine (III) in total yield of 86%.

Addition at 0–10° of an equivalent of *p*-toluenesulfonyl chloride to the hydroxypropyl urea (I) (prepared from 2 equivalents of phenyl isocyanate with 3-aminopropanol and subsequent basic hydrolysis of the product),[1] followed by acidification gave a white solid. The infrared spectrum of the solid revealed a tosylate ester band at 1340 cm.$^{-1}$ (2), and was totally void of tosylate anion absorption. When dried in a vacuum oven at 55° or allowed to stand at room temperature for 3 days, the solid decomposed. The resulting syrup did not crystallize on cooling, and its infrared spectrum showed intense tosylate anion bands at 1005 and 1020 cm.$^{-1}$ (3). When dissolved in a minimum of ethanol and poured into water, a white solid precipitated; its infrared spectrum did not show tosylate anion bands. A sodium fusion of this material revealed the presence of chlorine, and elemental analysis for C, H, N, and Cl agreed with that calculated for *N*-phenyl-*N'*-(3-chloropropyl)-urea. The yield was 48%.

The conversion of —OH to —Cl during a tosylation reaction is not unprecedented. Under certain conditions and with phenols bearing nitro groups in the nucleus, an abnormal reaction (4, 5) takes place in which the OH group is replaced by chlorine.

The filtrate from the chloro-urea was made strongly basic with 10% sodium hydroxide, and additional white solid precipitated. Again, the infrared spectrum was void of tosylate anion bands. A sodium fusion for sulfur and chlorine was negative and elemental analysis agreed with that calculated for 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine. The yield was 38%.

These observations can be rationalized in the following way. The tosylation reaction gives a mixture of the chloro-urea (II) and *N*-phenyl-

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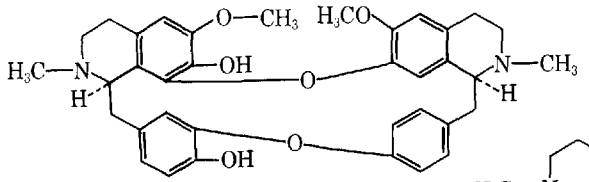
* Deceased.
[1] The procedure used for the tosylation reaction was essentially that of Marvel and Sekera (1).



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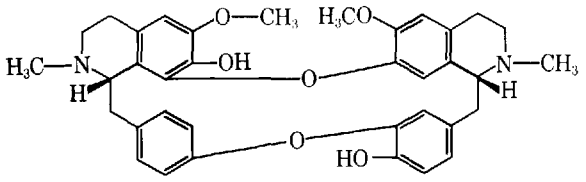

II

over anhydrous potassium carbonate and the ether then evaporated on a steam bath. The residue was dissolved in benzene and chromatographed on a column of Woelm, grade 1, neutral alumina (10 Gm., column 1 cm. × 6 cm.) using benzene as the solvent. A single compound was obtained in the eluate. The NMR data ($CDCl_3$) indicated τ 7.46, 7.76 (*N*-methyl), 6.12, 6.29, 6.41, and 6.87 (*O*-methyl), and was identified with that of *O,O*-dimethylobamegine.

O,O-Diethyl Ether.—A 0.1-Gm. sample of the phenolic base was dissolved in 10 ml. of methanol and added to an ethereal solution of diazoethane which was made from 7 Gm. of nitrosoethylurea and 40 Gm. of 50% potassium hydroxide solution. After standing 4 days at room temperature, the solution was treated by the usual method and the residue crystallized from acetone as colorless rosette crystals (0.098 Gm.), m.p. 203–204.° The NMR spectrum ($CDCl_3$) showed τ values at 7.39, 7.71 (*N*-methyl), 6.22, and 6.40 (*O*-methyl). The NMR spectrum was superimposable with that of known *O,O*-diethylobamegine.

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Tosylation of *N*-Phenyl-*N'*-(3-hydroxypropyl)urea

By ARTHUR F. FERRIS*, O. LEROY SALERNI, and BEVERLY A. SCHUTZ

Two unexpected products, *N*-phenyl-*N'*-(3-chloropropyl)urea (II) and 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine (III), are formed from the reaction of *p*-toluenesulfonyl chloride and *N*-phenyl-*N'*-(3-hydroxypropyl)-urea (I).

As part of a program dealing with the synthesis of potential antiradiation drugs, it became necessary to carry out the reaction of *p*-toluenesulfonyl chloride and *N*-phenyl-*N'*-(3-hydroxypropyl)urea (I). The authors have found that this reaction in pyridine solvent gives 2 unexpected products: *N*-phenyl-*N'*-(3-chloropropyl)urea (II) and 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine (III) in total yield of 86%.

Addition at 0–10° of an equivalent of *p*-toluenesulfonyl chloride to the hydroxypropyl urea (I) (prepared from 2 equivalents of phenyl isocyanate with 3-aminopropanol and subsequent basic hydrolysis of the product),[1] followed by acidification gave a white solid. The infrared spectrum of the solid revealed a tosylate ester band at 1340 cm.$^{-1}$ (2), and was totally void of tosylate anion absorption. When dried in a vacuum oven at 55° or allowed to stand at room temperature for 3 days, the solid decomposed. The resulting syrup did not crystallize on cooling, and its infrared spectrum showed intense tosylate anion bands at 1005 and 1020 cm.$^{-1}$ (3). When dissolved in a minimum of ethanol and poured into water, a white solid precipitated; its infrared spectrum did not show tosylate anion bands. A sodium fusion of this material revealed the presence of chlorine, and elemental analysis for C, H, N, and Cl agreed with that calculated for *N*-phenyl-*N'*-(3-chloropropyl)-urea. The yield was 48%.

The conversion of —OH to —Cl during a tosylation reaction is not unprecedented. Under certain conditions and with phenols bearing nitro groups in the nucleus, an abnormal reaction (4, 5) takes place in which the OH group is replaced by chlorine.

The filtrate from the chloro-urea was made strongly basic with 10% sodium hydroxide, and additional white solid precipitated. Again, the infrared spectrum was void of tosylate anion bands. A sodium fusion for sulfur and chlorine was negative and elemental analysis agreed with that calculated for 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine. The yield was 38%.

These observations can be rationalized in the following way. The tosylation reaction gives a mixture of the chloro-urea (II) and *N*-phenyl-

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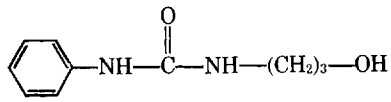
* Deceased.
[1] The procedure used for the tosylation reaction was essentially that of Marvel and Sekera (1).



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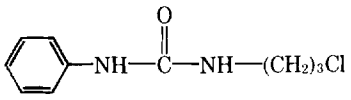
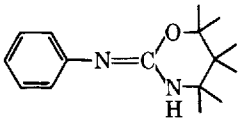
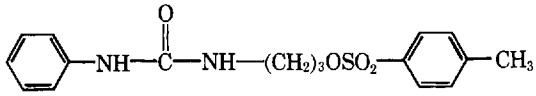


II

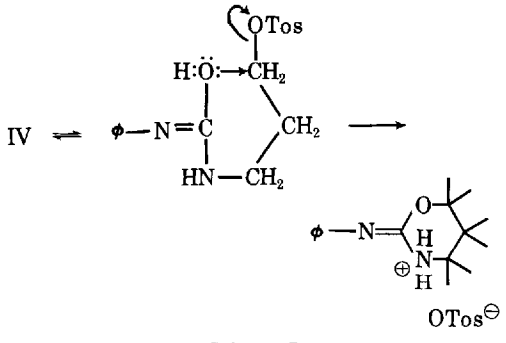


III



IV

N'-(3-*p*-toluenesulfonyloxypropyl)urea (IV). Such a mixture would explain the lack of tosylate anion absorption in the infrared spectrum of the crude reaction product. On standing or warming in a vacuum oven, the tosylate ester (IV) undergoes cyclization to the oxazine salt as illustrated, thus accounting for the intense tosylate anion bands in the infrared spectrum of the decomposed material. (Scheme I.)



Scheme I

When dissolved in ethanol and poured into water, the insoluble chloro-urea precipitates and the soluble oxazine salt remains in solution. On basification of the filtrate, the free base is liberated from its salt and precipitates.

EXPERIMENTAL[2]

The *N* - phenyl - *N'* - (3 - *N* - phenylcarbamoylpropyl)urea was prepared by the method of Dyer and Read (6).

N - Phenyl - N' - (3 - hydroxypropyl)urea (I).—To a solution of 117.4 Gm. (0.375 mole) of *N*-phenyl - *N'* - (3 - *N* - phenylcarbamoylpropyl) urea in 700 ml. of 95% ethanol was added a solution of 60.0 Gm. (1.5 moles) of sodium hydroxide in 200 ml. of water. The resulting solution was refluxed for 14 hr. with mechanical stirring. A solid (sodium carbonate) separated during the reflux period. The ethanol was removed under reduced pressure, and 500 ml. of water was added to the residue. An oil (aniline) and some solid separated. The mixture was made acid (pH 3–4) by the cautious addition of hydrochloric acid with cooling and stirring. The oil disappeared (aniline hydrochloride was formed) and carbon dioxide evolved. A tan solid separated and was recovered and dried. The yield was 56.0 Gm. (77%), m.p. 106–112°. After 2 recrystallizations from acetonitrile, the compound gave a m.p. 111–113°. This sample was identical to the *N*-phenyl-*N'*-(3-hydroxypropyl)urea prepared by the method of Dyer and Read (6) from phenyl isocyanate and 3-aminopropanol.

N-(3-Chloropropyl)-N'-phenylurea (II) and 2-Phenylimino - 2,4,5,6 - tetrahydro - 1,3 - oxazine (III).—To a solution of 46.0 Gm. (0.237 mole) of *N*-phenyl-*N'*-(3-hydroxypropyl)urea in 127.5 Gm. (1.61 moles) of pyridine was added 50.5 Gm. (0.266 mole) of *p*-toluenesulfonyl chloride. The acid chloride was added in portions, with stirring and cooling, keeping the temperature below 20°. After the addition was complete, the reaction mixture was stirred at 20° or less for 4 hr. A solution of 240 ml. of concentrated hydrochloric acid dissolved in 560 ml. of water was then added with stirring. The temperature was kept below 30°. White solid separated and, after recovery and drying *in vacuo* at room temperature, it amounted to 87.6 Gm., m.p. 84–86°. The solid was then heated *in vacuo* at 50–60° for about 1 hr. It melted[3] and did not solidify on cooling. The syrup was taken up in 125 ml. of warm 95% ethanol and poured into about 1 L. of ice and water. White solid separated which, after drying, amounted to 24.3 Gm. (48%) of 1-(3-chloropropyl)-3-phenylurea (II), m.p. 115.5–121°. Three recrystallizations from acetonitrile afforded an analytical sample, m.p. 128–129.5°.

The filtrate from the chloro-urea was made strongly basic with 10% sodium hydroxide. The solid which separated was recovered and dried *in vacuo*. It amounted to 15.7 Gm. (38%) of 2-phenylimino-2,4,5,6-tetrahydro-1,3-oxazine (III), m.p. 131–134°. Two recrystallizations from acetone gave an analytical sample, m.p. 132–133.5°.

Anal.—(II) Calcd. for $C_{10}H_{13}ClN_2O$: C, 56.47; H, 6.16; Cl, 16.67; N, 13.17. Found: C, 56.60; H, 6.27; Cl, 16.41; N, 13.01.

Anal.—(III) Calcd. for $C_{10}H_{12}N_2O$: C, 68.15; H, 6.87; N, 15.90. Found: C, 68.16; H, 6.90; N, 15.80.

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[2] All melting points were taken on a Thomas-Hoover capillary melting point apparatus and are uncorrected. All elemental analyses were carried out by Galbraith Laboratories, Inc., Knoxville, Tenn.

[3] Similar results were obtained when the solid was allowed to stand at room temperature for 3 days.

NMR 1,3-Diaxial Deshielding Effect of the Hydroxyl Group on Ring Hydrogens Studied from Partially Deuterated Six-Membered Ring Compounds

By ALAIN C. HUITRIC, JOHN B. CARR, and WILLIAM F. TRAGER*

The deshielding effect of the hydroxyl group on axial ring protons through 1,3-diaxial spatial interaction has been studied in *trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol-3,3,6,6-d_4 (II) and *trans*-2-*o*-tolyl-*cis*-4-hydroxycyclohexanol-3,3,6,6-d_4 (III) in deuterated chloroform, acetone-d_6, methanol-d_4, acetic acid, and pyridine; in 4-*tert*-butyl-*trans*-1,4-cyclohexanediol-3,3,5,5-d_4 (VI) in methanol-d_4 and pyridine; and in 4-*tert*-butyl-*cis*-1,4-cyclohexanediol-3,3,5,5-d_4 (VII) in pyridine. A consistent downfield shift of about 0.50 p.p.m. was found for the signal of H-1 of II in the first 4 solvents, but a considerably larger shift of 0.90 p.p.m. was obtained in pyridine. A downfield shift of about 0.58 ± 0.05 p.p.m. was found for the signal of H-2 in III in the first 4 solvents while a shift of 0.97 p.p.m. was obtained in pyridine. The deshielding effect of the tertiary hydroxyl groups in VI and VII was found to be smaller than that of the secondary hydroxyl in II and III in a given solvent, but the magnitude of the pyridine solvent effect was about the same in the 2 series.

THE DESHIELDING effect of the hydroxyl group through 1, 3-diaxial spatial interaction is well established in NMR spectroscopy of 6-membered ring compounds. The effect was observed on the NMR signals of angular methyl groups in steroid molecules by Shoolery and Rogers in 1958 (1) and has since been the object of systematic investigations by several authors (2–6). Much of the work has centered around the effect on angular methyl groups in steroids, where the average downfield shift for a large number of steroids measured in chloroform was found to be in the neighborhood of 0.25 p.p.m. (2) but examples of deshielding of axial protons are also known (6). Bhacca and Williams give tabulated data covering both cases (7).

A number of partially deuterated 6-membered ring compounds have been synthesized in this laboratory in recent years (8, 9). Some of these compounds provide excellent models for the investigation of the deshielding effect of an axial hydroxyl group on ring protons in 1,3-diaxial orientation to the hydroxyl group. Remote deuteration greatly simplifies the spectra and often allows more reliable chemical shifts and coupling constants. This is demonstrated in Fig. 1. The deshielding was investigated by comparing the chemical shifts of H-1 in compounds I and II, and H-2 in I and III in 5 different solvents, and by comparing the chemical shifts of the axial protons at positions 2 and 6 in IV and VI in pyridine and deuterated methanol, and V and VII in pyridine. The results are given in Tables I and II.

The origin of the magnetic anisotropy responsible for the 1,3-diaxial long-range deshielding by a hydroxyl group is not clearly understood. Early in the course of this investigation a large difference was observed in the deshielding effect in compounds II and III in pyridine compared to deuterated chloroform. Additional solvents were therefore selected in order to determine if a pattern of solvent effects could be found on the deshielding effects of the axial hydroxyl group in solvents that can associate with the hydroxyl group through hydrogen bonding only by acting as proton acceptors *versus* solvents which can hydrogen bond by acting as proton donors as well as proton acceptors. No such pattern was found in comparing the shift in acetone with that in methanol, acetic acid, and chloroform. In compound II the shift is identical in these 4 solvents; in III there is a slightly larger shift in acetone than in methanol and acetic acid, but the variation is not very large. A much larger difference in the shift was found between pyridine and all other solvents used. It is interesting to note that the downfield shift is not so large in VI and VII as in II or III in the corresponding solvents, but that the difference in the shift between pyridine and methanol is of similar magnitude for VI as for II and III. The smaller shift caused by a tertiary hydroxyl group in the *cis* and *trans* isomers of 4-*tert*-butylcyclohexanediol compared to the shift caused by a secondary hydroxyl

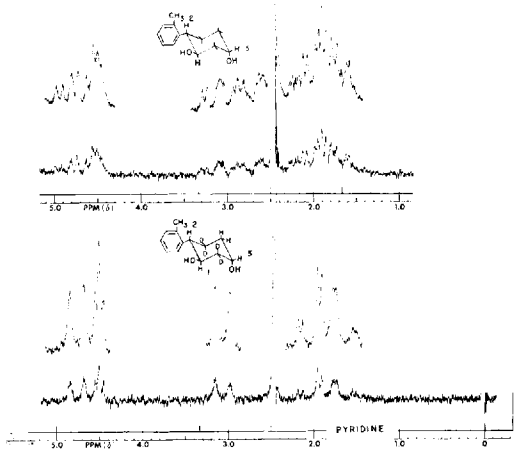

Fig. 1.—NMR spectra of *trans*-2-*o*-tolyl-*trans*-5-hydroxycyclohexanol and the corresponding 3,3,6,6-tetradeutero analog; 60 mc., about 1 *M* in pyridine with TMS as internal reference.

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* Public Health Service Predoctoral Fellow, GPM-18,507, 1962–1965.
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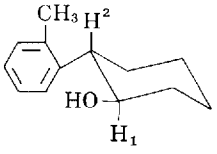
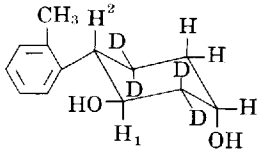
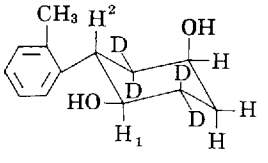
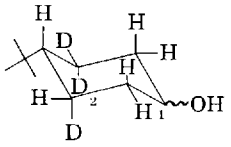
TABLE I.—CHEMICAL SHIFTS (ν) IN C.P.S. AT 60 MC. AND DESHIELDING EFFECT OF AXIAL HYDROXYL GROUP ON AXIAL HYDROGENS

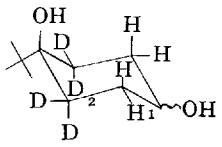
	Solvents	ν, c.p.s. at 60 mc. H-1	H-2	CH_3	Deshielding by Axial OH
 I	$CDCl_3$	220	161	138	
	Acetone-d_6	225	165	138	
	Methanol-d_4	222	163	138	
	AcOH	232	168	139	
	Pyridine	232	173	140	
					($\nu_{II} - \nu_I$) for H-1
 II	$CDCl_3$	249	162	140	29 c.p.s.
	Acetone-d_6	255	167	140	30
	Methanol-d_4	252	165	140	30
	AcOH	261	171	140	29
	Pyridine	285	184	148	53
					($\nu_{III} - \nu_I$) for H-2
 III	$CDCl_3$	228	198	143	37
	Acetone-d_6	232	203	140	38
	Methanol-d_4	226	196	141	33
	AcOH	236	200	140	32
	Pyridine	246	231	145	58

group in II and III in a given solvent could possibly be the result of a greater restriction to rotation about the C—O bond in the 4-*tert*-butyl compounds, causing the OH group to have a different preferred conformation on a time average than in II and III. Any deshielding effect resulting from the anisotropy of the O—H bond or of the unshared electrons of the oxygen atoms will be affected by the orientation of the hydroxyl group.



IV, *cis*, OH axial

V, *trans*, OH equatorial



VI, *trans*-diol, OH-1 axial

VII, *cis*-diol, OH-1 equatorial

TABLE II.—CHEMICAL SHIFTS (ν) IN C.P.S. AT 60 MC. AND DESHIELDING EFFECT OF AXIAL HYDROXYL GROUP ON AXIAL HYDROGENS

Solvent	ν, c.p.s. at 60 mc. Axial H-2 and H-6	Equatorial H-2 and H-6	Deshielding by Axial OH
IV Pyridine	86	119	
Methanol-d_4	85	109	
V Pyridine	83	129	
			($\nu_{VI} - \nu_{IV}$), axial H-2 and H-6
VI Pyridine	132	112	46
Methanol-d_4	110	95	25
			($\nu_{VII} - \nu_V$), axial H-2 and H-6
VII Pyridine	127	121	44

Table I indicates that the introduction of an axial hydroxyl group at C-5 in II has a negligible effect on the chemical shift of H-2, and the introduction of an axial hydroxyl group on C-4 of III has a negligible effect on the chemical shift of H-1 in III in all but the pyridine solvent. The chemical shift of the aromatic methyl group shows very little variation in all solvents for all 3 compounds. Although not given in Table I, the chemical shift of H-5 in II was found to be 251, 254, 252, 260, and 270 c.p.s. in deuterated chloroform, acetone-d_6, methanol-d_4, acetic acid, and pyridine, respectively, and that of H-4 in III was 246, 244, 238, 248, and 257 c.p.s. in the respective solvents.

The spectra of the deuterated compounds II and III allow the determination of accurate coupling constants between H-1 and H-2 from simple AX systems. Compound II gave J_{12} values of 10.5, 10.3, 10.3, 10.4, and 10.5 c.p.s. in chloroform-*d*, acetone-d_6, methanol-d_4, acetic acid, and pyridine, respectively; compound III gave J_{12} values of 10.0, 10.2, 10.3, 10.5, and 10.5 c.p.s. in the same respective solvents. These constant values of J_{12} indicate that there is practically no difference in conformation of these 2 compounds in the various solvents used. The coupling constants of about 10 c.p.s. indicate that H-1 and H-2 have a diaxial orientation and that II and III exist almost exclusively in a chair conformation with the aromatic group in an equatorial orientation in the 5 solvents used (8).

EXPERIMENTAL

trans-2-*o*-Tolylcyclohexanol (I) is a known compound (10).

trans-2-*o*-Tolyl-*trans*-5-hydroxycyclohexanol-3,3,-6,6-d_4 (II) and *trans*-2-*o*-tolyl-*cis*-4-hydroxycyclohexanol-3,3,6,6-d_4 (III) were prepared by the method previously reported for the corresponding nondeuterated compounds (11) except that butadiene-1,1,4,4-d_4 (12) was used in the Diels-Alder condensation step.

cis-4-*tert*-Butylcyclohexanol-3(axial)4,4-d_3 (IV) and the corresponding *trans* isomer (V) have been reported in a previous communication (13). Detailed synthesis of these 2 compounds will be reported in a subsequent publication.

4-*tert*-Butyl-*trans*-1,4-cyclohexanediol-3,3,4,5-d_4 (VI) and the corresponding *cis*-diol (VII) have been reported previously (9).

The NMR spectra were determined with a Varian A-60 spectrometer using tetramethylsilane as internal reference.

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Elucidation of the Configuration of an Intermediate in the Synthesis of *cis*-β-Acetoxystyrene II

By BIPIN B. CHAUDHARI*, DONALD T. WITIAK†, and ROGER M. CHRISTIANSEN‡

The configuration of *erythro*-α-hydroxy-β-toluene-*p*-sulfonyloxypropionic acid (III) was proved through conversion to the known methyl *erythro*-β-phenyl-α,β-ditoluene-*p*-sulfonyloxypropionate (VIII). Compound III represents the key intermediate in the conversion of *trans*-β-phenylglycidic acid (II) to *cis*-β-acetoxystyrene (I) and elucidation of the *erythro* configuration for III substantiates the original proposal that decarboxylative elimination of the probable acetate derivative (IV) occurred *trans*.

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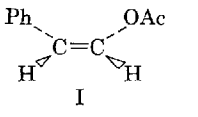

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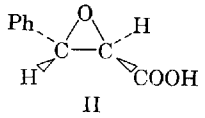

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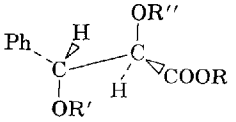

III, R = R'' = H, R' = Ts
IV, R = H, R' = Ts, R'' = Ac
V, R = R' = R'' = H
VII, R = CH_3, R' = Ts, R'' = H
VIII, R = CH_3, R' = R'' = Ts
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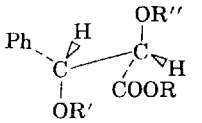

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* Present address: Research Center, Chicago Division, The Kendall Co., Barrington, Ill.
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[1] *cis*-β-Acetoxystyrene was formed directly from compound III. Formation of the enol acetate was rationalized on the basis of the instability of the α-carboxy-β-tosyloxy system.

EXPERIMENTAL

trans-2-*o*-Tolylcyclohexanol (I) is a known compound (10).

trans-2-*o*-Tolyl-*trans*-5-hydroxycyclohexanol-3,3,-6,6-d_4 (II) and *trans*-2-*o*-tolyl-*cis*-4-hydroxycyclohexanol-3,3,6,6-d_4 (III) were prepared by the method previously reported for the corresponding nondeuterated compounds (11) except that butadiene-1,1,4,4-d_4 (12) was used in the Diels-Alder condensation step.

cis-4-*tert*-Butylcyclohexanol-3(axial)4,4-d_3 (IV) and the corresponding *trans* isomer (V) have been reported in a previous communication (13). Detailed synthesis of these 2 compounds will be reported in a subsequent publication.

4-*tert*-Butyl-*trans*-1,4-cyclohexanediol-3,3,4,5-d_4 (VI) and the corresponding *cis*-diol (VII) have been reported previously (9).

The NMR spectra were determined with a Varian A-60 spectrometer using tetramethylsilane as internal reference.

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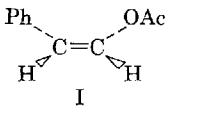
Elucidation of the Configuration of an Intermediate in the Synthesis of *cis*-β-Acetoxystyrene II

By BIPIN B. CHAUDHARI*, DONALD T. WITIAK†, and ROGER M. CHRISTIANSEN‡

The configuration of *erythro*-α-hydroxy-β-toluene-*p*-sulfonyloxypropionic acid (III) was proved through conversion to the known methyl *erythro*-β-phenyl-α,β-ditoluene-*p*-sulfonyloxypropionate (VIII). Compound III represents the key intermediate in the conversion of *trans*-β-phenylglycidic acid (II) to *cis*-β-acetoxystyrene (I) and elucidation of the *erythro* configuration for III substantiates the original proposal that decarboxylative elimination of the probable acetate derivative (IV) occurred *trans*.

RECENTLY, the authors reported (1) a stereoselective synthesis for *cis*-β-acetoxystyrene (I) from *trans*-β-phenylglycidic acid (II). The key intermediate in the synthesis involved formation of the α-hydroxy-β-tosyloxy compound (III) through reaction of *trans*-β-phenylglycidic acid with *p*-toluenesulfonic acid in dry ether.

Assignment of the *erythro* configuration to compound III was originally based on the stereoselective formation of *cis*-β-acetoxystyrene (I) which was proposed to have formed by *trans* decarboxylative elimination of the corresponding α-acetoxy derivative (IV). Since the α-acetoxy derivative (IV) was not isolated when compound III was treated with acetic anhydride in pyridine (1)[1] and since some openings of benzylic epoxides with various Bronsted acids in nonpolar medium have been shown to occur with retention of configuration (2) independent evidence for the configuration of the β-tosyloxy-α-



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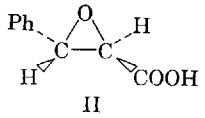

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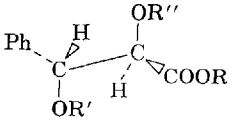

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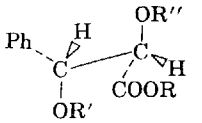

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[1] *cis*-β-Acetoxystyrene was formed directly from compound III. Formation of the enol acetate was rationalized on the basis of the instability of the α-carboxy-β-tosyloxy system.

hydroxy acid (III) was desirable. Such evidence would confirm our original proposal that the decarboxylative elimination did indeed occur *trans*.

Our earlier attempts (1) to prepare stable derivatives of compound III which could subsequently be compared to derivatives of the known *erythro* (V) and *threo* (VI) diols (3) were unsuccessful. This was probably due to the instability of the α-carboxy-β-tosyloxy system which readily decomposed under relatively mild conditions. In order to obtain stable derivatives, intermediate III was converted to its methyl ester (VII) through treatment with ethereal diazomethane. Subsequent reaction of compound VII with *p*-toluenesulfonyl chloride in pyridine afforded the ditosyl derivative (VIII) which was identical in all properties to the ditosylated methyl ester which we prepared according to the method of Linstead and co-workers (4) from the known *erythro* diol methyl ester (IX).

Admixture of VIII and the ditosylated methyl ester (X) prepared from the known *threo* diol methyl ester (XI) gave a depression in melting point while admixture with the known *erythro* diol methyl ester gave no such depression. In addition, the infrared spectra of the *erythro* and *threo* ditosylated methyl esters (VIII and X) differed in the region between 8 and 16 μ. The *erythro* ditosylates prepared by either method had additional peaks at 9.70, 10.92, and 11.89 μ, while the *threo* ditosylate exhibited peaks at 10.96 and 12.01 μ. These data prove that the α-hydroxy-β-tosyloxy intermediate has the *erythro* configuration and that decarboxylative elimination of the probable acetate intermediate occurred *trans*.

EXPERIMENTAL[2]

Erythro - α,β - dihydroxy - β - phenylpropionic Acid (V).—A suspension of 28 Gm. (0.15 mole) sodium *trans*-β-phenylglycidate in 420 ml. of 10% sodium hydroxide was heated at reflux for 1.5 hr. After cooling to room temperature, the mixture was acidified with hydrochloric acid and extracted with ether. The ether solution was dried over anhydrous sodium sulfate, filtered, and the solvent was removed under reduced pressure affording a yellow oil. The oil crystallized affording 7.2 Gm. (32.8%) of white crystalline solid. Recrystallization from acetone–ether–skellysolve B yielded 6 Gm. (28%) of product, m.p. 121–121.5°. [Reported m.p. 122° (3).]

Threo - α,β - dihydroxy - β - phenylpropionic Acid (VI).—Utilization of the above procedure with sodium *cis*-β-phenylglycidate (5) afforded the *threo* dihydroxy acid in 67% yield. Recrystallization from ether yielded a white crystalline compound, m.p. 140–141°. [Reported m.p. 141–142° (3).]

Methyl Erythro - α - hydroxy - β - phenyl - β-toluene - p - sulfonyloxypropionate (VII) from Erythro - α - hydroxy - β - phenyl - β - toluene - p-sulfonyloxypropionic Acid (III).—A suspension of 5 Gm. (0.016 mole) of *erythro*-α-hydroxy-β-phenyl-β-toluene-*p*-sulfonyloxypropionic acid in 200 ml. of anhydrous ether was cooled to 0°. To this suspension was added an ethereal solution of diazomethane (6) until evolution of nitrogen ceased and the solution acquired a pale yellow color. Excess diazomethane was destroyed by dropwise addition of glacial acetic acid. Removal of the solvent under reduced pressure afforded 3.5 Gm. (67%) of oil which could not be crystallized in our hands but which was converted directly to the *erythro* ditosylate (VIII).

Methyl Erythro - β - phenyl - α,β - ditoluene - p-sulfonyloxypropionate (VIII) from Methyl Erythro-α-hydroxy-β-phenyl-β-toluene - p - sulfonyloxypropionate (VII).—Methyl *erythro*-α-hydroxy-β-toluene-*p*-sulfonyloxypropionate (1.75 Gm., 0.005 mole) was dissolved in 5 ml. of pyridine and the solution was cooled to 0°. To this solution was added 1.5 Gm. (0.005 mole) of *p*-toluenesulfonyl chloride with vigorous stirring. The mixture was shaken and allowed to stand for 30 min. at 25°, poured onto 50 Gm. of ice, and extracted with two 100-ml. portions of ether. The ether layer was washed with two 100-ml. portions of 5% hydrochloric acid and with 100 ml. of water. The ether solution was dried over anhydrous sodium sulfate, filtered, and the solvent was removed under reduced pressure. A white, solid residue remained weighing 1.5 Gm. (59%). Recrystallization from methanol yielded white needles, m.p. 129–131°. Mixed melting point with an authentic sample of *erythro* ditosylate prepared from the known *erythro* diol was 129–131°. Mixed melting point with the known *threo* ditosylate 110–112°.

Anal.—Calcd. for $C_{24}H_{24}O_8S_2$: C, 57.1; H, 4.8; S, 12.4. Found: C, 56.9; H, 4.5; S, 12.5

[2] All melting points are corrected and were taken with a Thomas-Hoover melting point apparatus. Infrared spectra were taken with a Beckman IR-5A spectrophotometer. Elemental analyses were run by Clark Microanalytical Laboratory, Urbana, Ill., and Crobaugh Laboratories, Charleston, W. Va.

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Inhibition of Isolation-Induced Attack Behavior of Mice by Drugs

By EDWARD T. UYENO

Three dose-response experiments indicated that psilocybin, mescaline, and 2-brom-lysergic acid diethylamide (BOL-148) inhibited isolation-induced attack behavior of 11-week-old Swiss-Webster mice, tested at the time of peak effect. The 3 dose-response curves show that the per cent inhibitory effects of these compounds are increasing monotonic functions of dose. The ED_{50} and confidence limits for psilocybin, mescaline, and BOL-148 are 2.096 mg./Kg. (1.334–3.291), 5.413 mg./Kg. (2.904–10.090), and 1.183 mg./Kg. (0.676–2.069), respectively.

In a previous study it was found that the per cent inhibitory effect of 0.1–1.6 mg./Kg. of *d*-lysergic acid diethylamide (LSD-25) on the attack behavior of mice, tested 5 min. after intraperitoneal injection (i.p.) was an increasing monotonic function of dose (1).

The present experiment was conducted in order to compare the results of the LSD-25 study with those obtained with 2 other hallucinogens: psilocybin (an indole derivative like LSD-25) and mescaline (belonging to the phenylethylamine group). Moreover, it was deemed important to compare the effects of 2-brom-lysergic acid diethylamide (BOL-148), a congener of LSD-25, reported to produce LSD-25-like psychic effects (2). More specifically, the effects of psilocybin, mescaline, and BOL-148 on isolation-induced attack behavior of Swiss-Webster male mice were investigated.

METHOD

Six-week-old Swiss Webster male mice were socially isolated for 5 weeks in 18 × 10 × 12 cm. metal cages which had wire mesh fronts and floors. Another group was housed together (30/cage) in 44 × 26 × 16 cm. metal cages, constructed in the similar manner as the smaller cages. All animals were fed *ad libitum* some standardized dietetically optimum pellets.[1]

After 5 weeks, a 5-min. pretest was initiated by placing a "group-housed" mouse into the cage of an isolated mouse until attack behavior was observed. An attack was defined as an aggressive contact involving biting. When an attack occurred, the attack latency was recorded and the "group-housed" mouse was returned to its cage. (The group-housed animals did not attack, and hardly any of them retaliated when they were attacked.) On the basis of their attack latencies, the attackers were matched and assigned to control and 3 experimental groups (3 drugs). Each experimental group was divided into 4 subgroups (4 subgroups/drug).

The Time of Peak Effect.—Experimental animals (n = 20 or 30/subgroup) were tested at 5, 15, 30, or 45 min. after i.p. injection of 4 mg./Kg. of psilocybin, 20 mg./Kg. of mescaline base, administered as the sulfate, or 2 mg./Kg. of BOL-148 in saline solution. The control animals were injected with 0.9% saline solution and were tested for attack behavior 30 min. later. A standard volume of 10 ml./Kg. of drug–saline and saline solution was administered to the experimental and control animals, respectively. Each attacker was paired with the same group-housed animal with which it had been paired in the pretest. The test was conducted in a manner similar to the pretest.

Dose-Response.—Dose-response experiments were conducted with 6-week-old Swiss-Webster male mice that were essentially similar to those used in the peak time study. At the end of a 5-week isolation period, the pretest was given. On the basis of attack latencies, they were assigned into a control and 3 experimental groups (3 drugs) with 4 graded dose groups per drug. (N = 20 or 30/subgroups.) As before, the control animals were injected with 0.9% saline solution. After injection, the control and experimental animals were tested for attack behavior at the time of peak effect as determined from the earlier studies.

RESULTS AND DISCUSSION

In the time of peak effect and dose-response experiments, all control animals attacked the group-housed animals, but many experimental animals did not attack. In the peak time study, the sum of nonattackers in each time group was expressed as a percentage of all the animals in that group (per cent effect). Per cent effect was plotted against post-injection time (Fig. 1). The time of peak inhibitory effects of 4 mg./Kg. of psilocybin and 2 mg./Kg. of BOL-148 was 30 min. after the i.p. injection. For 20 mg./Kg. of mescaline, the peak time was between 15 and 30 min. after the injection.

In the dose-response study the sum and percentage of nonattackers in each dose group were calculated for each compound (Table 1). Per cent effect was plotted against dose on logarithmic probability paper (Figs. 2 and 3). The data show that the per cent inhibitory effect of each compound is an increasing monotonic function of dose. Probit

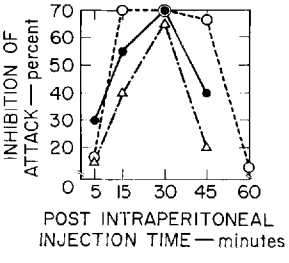

Fig. 1.—Time of peak inhibitory effect of 20 mg./Kg. of mescaline (N = 30/plot), 4 mg./Kg. of psilocybin (N = 20/plot), and 2 mg./Kg. of BOL-148 (N = 20/plot) on attack behavior. Key: △, BOL-148; ●, psilocybin; ○, mescaline.

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The author is indebted to Mr. H. Althouse, Sandoz Pharmaceuticals, Inc., for supplies of psilocybin and BOL-148.

[1] White Diet supplied by Simonsen Laboratories, Inc., Gilroy, Calif.

TABLE I.—PERCENTAGE OF NONATTACKERS IN EACH DOSE GROUP

Compd.	Dose, mg./Kg.	N	Non-attackers	% Effect
Psilocybin	8	20	16	80
	4	20	14	70
	2	20	11	55
	1	20	5	25
Mescaline	30	30	24	80
	20	30	21	70
	10	30	16	53
	1	30	8	27
BOL-148	2.0	20	13	65
	1.5	20	10	50
	1.0	20	9	45
	0.5	20	7	35
LSD-25[a]	0.8	20	20	100
	0.5	20	17	85
	0.4	20	15	75
	0.3	20	10	50
	0.2	20	6	30
	0.1	20	4	20

[a] Data obtained from *Reference 1*.

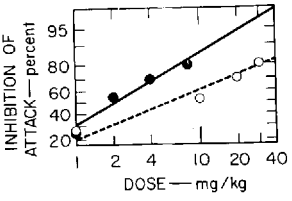

Fig. 2.—Dose response curves. Key: ●, 1.0–8.0 mg./Kg. of psilocybin (N = 20/dose); ○, 1.0–30 mg./Kg. of mescaline (N = 30/dose).

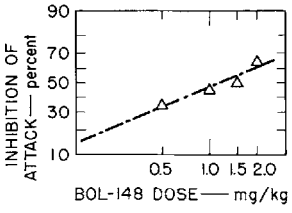

Fig. 3.—Dose response curve. Effect of 0.5–2.0 mg./Kg. of BOL-148 on attack behavior (N = 20/dose).

analysis of the data was conducted by an electronic computer (3). The ED_{50} and the confidence limits for psilocybin, mescaline, and BOL-148 are 2.096 mg./Kg. (1.334–3.291), 5.413 mg./Kg. (2.904–10.090), and 1.183 mg./Kg. (0.676–2.069), respectively. The equations of the 3 curves are: psilocybin, $Y_1 = 4.469 + 1.652\ (X_1)$; mescaline, $Y_2 = 4.314 + 0.935\ (X_2)$; BOL-148, $Y_3 = 4.916 + 1.160\ (X_3)$.

In general, experimental mice withdrew to the rear of the cage, remained inactive [as LSD-25 mice (1)], and did not attack. The latencies of a few that attacked were longer than their pretest latencies. In contrast, the latencies of the control animals were shorter than during the pretest. The inactive reaction of the psilocybin mice appeared to be consistent with that observed by Cerletti (4), who reported that this compound produced a distinct reduction of motor activity in mice, rabbits, and monkeys. Moreover, in humans, Hollister (5) and Delay *et al.* (6) found that an oral dose of 150 mcg./Kg. and 130 mcg./Kg., respectively, induced physical responses indicative of depressant behavior, such as drowsiness, fatigue, and weakness. The results of the mescaline experiment corroborate those of Saxena *et al.* (7), who found that in fish (*colisa lalia*) the fighting response to another male was completely blocked by intramuscular injection of 0.1–0.2 mcg. of mescaline sulfate. The lowered activity and withdrawal reaction of the mescaline mice in the present study are consistent with results reported for cats given 0.3–1.0 mg./Kg. of this compound (8). The depressant effect of mescaline was also observed in a pole climbing performance of rats that increased their latencies when they were injected 20 mg./Kg. i.p. of this compound 15 min. before test (9).

The results indicate that the times of peak effect of 4 mg./Kg. of psilocybin, of 20 mg./Kg. of mescaline, and of 2 mg./Kg. of BOL-148 were later than that (5 min. after the i.p. injection) of 0.4 mg./Kg. of LSD-25 (1), suggesting that the 3 former compounds may have slower rates of passage into the brain. Like LSD-25, the 3 compounds made most of the animals inactive and inhibited their isolation-induced attack behavior. Mescaline with the highest ED_{50} (5.413 mg./Kg.) and LSD-25 (1) with the lowest ED_{50} (0.265 mg./Kg.) seem to suggest the former is the least and the latter the most potent inhibitors of isolation-induced attack behavior.

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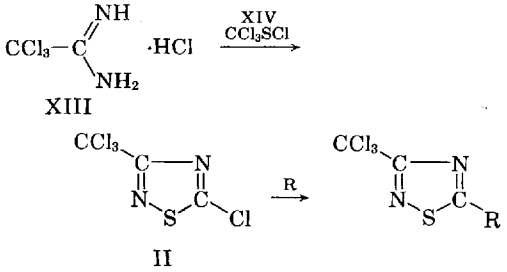
Antifungal Agents I.
5-Substituted-3-(trichloromethyl)-1,2,4-thiadiazoles

By V. L. NARAYANAN, JACK BERNSTEIN, and J. WILLIAMS

Some 5-substituted-3-(trichloromethyl)-1,2,4-thiadiazoles were synthesized by the nucleophilic displacement of the Cl^- ion of 5-chloro-3-(trichloromethyl)-1,2,4-thiadiazole for evaluation as soil fungicides. An interesting O → N migration of the β-hydroxyethyl substituent was observed during the synthesis of 5-(2-hydroxyethoxy)-3-(trichloromethyl)-1,2,4-thiadiazole. All these compounds retained 60–80 per cent of the over-all fungicidal activity of the analogous 5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole.

THE DISCOVERY of 5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole as an effective soil fungicide (1, 2) led the authors to synthesize the compounds listed in Table I (compounds III–VIII). The objectives were to decrease the volatility of the parent compound and to determine the structure-activity relationships in this series.



Trichloroacetamidine hydrochloride (XIII) was reacted with trichloromethanesulfenyl chloride (XIV) to give a 56% yield of II (3). The displacement of the Cl^- ion of II by the appropriate nucleophiles gave compounds III through VIII. The reactions were achieved by the slow addition of the appropriate nucleophiles, dissolved in suitable solvents, to a solution of II, and stirring for several hours at room temperature. The above general procedure is necessitated because of the instability of the allylic chlorines toward strong bases, especially at elevated temperatures. Furthermore, it takes advantage of the high reactivity of the chlorine at the 5-position toward nucleophilic displacements.

Compound VIII was obtained in almost quantitative yields following the general procedure outlined. A v.p.c. analysis[1] indicated the material to be of 90–95% purity. However, distillation of VIII *in vacuo* led to extensive desulfurization, and a 30% yield of an isomeric mixture (X) was obtained. On the basis of the spectral data,[2] the mixture was estimated to contain approximately 75% of VIII, the major component of the remainder being the *N*-alkylated product (XI). The thermal isomerization could proceed through the mechanism shown in Scheme I.

The fact that the propoxy derivative (VII) distilled unchanged and that it could not be isomerized by heating at 155–165° suggests that the transition state (XII) for the formation of XI may have some ionic character, being stabilized by the polar —OH group.

Attempts to obtain IX by reacting II with the anion $-P(OC_2H_5)_2$ (4), following the general procedure, failed. Although its formation was indicated [I.R., λ $CHCl_3$ 9.7 μ (aryl phosphate)] (5), isolation procedures led to extensive decomposition, and a mixture of unidentified products was obtained. The nature of this reaction was not studied further.

EXPERIMENTAL

All melting points were taken on a Thomas-Hoover melting point apparatus and are uncorrected. The v.p.c. separation was performed on K-22A column at 142° and 30 ml./min. gas flow. The infrared spectra were determined with samples in the form of Nujol mulls, and NMR spectra in deuteriochloroform in the presence of deuterium oxide with tetramethylsilane as the internal standard.

Substituted Thiadiazoles (Table I; III–VIII). General Procedure.—The appropriate nucleophile (0.1 mole) dissolved in a suitable solvent (chloroform or the alcohols from which the nucleophiles were generated) was added dropwise with stirring to a solution of 0.1 mole of 5-chloro-3-(trichloromethyl)-1,2,4-thiadiazole (II) dissolved in the same solvent. The mixture was then stirred for 4–6 hr., and allowed to stand overnight at room temperature. After removal of the solvent *in vacuo*, the product was isolated either by distillation under reduced pressure or by crystallization.

Pertinent data are listed in Table I.

5-(2-Hydroxyethoxy)-3-(trichloromethyl)-1,2,4-thiadiazole (VIII).—Sodium methylate, 2.83 Gm., was dissolved in 30 ml. of absolute methanol, and to this solution 31 Gm. of ethylene glycol, dissolved in 20 ml. of absolute methanol, was added. The mixture was concentrated to remove the methanol, and the remaining solution was then added dropwise at room temperature to a solution of 11.9 Gm. of II in 100 ml. of ether. The reaction mixture was stirred overnight, washed with water, and then dried over $MgSO_4$. Removal of ether gave 12.1 Gm. (92.4%) of the product as a thick pale yellow viscous oil. λ 2.95 μ, broad (bonded OH), and 6.55 μ (C=N), τ 5.25 (t, O—CH_2) and τ 5.95 (t, CH_2).

Anal.—Calcd. for $C_5H_5Cl_3N_2O_2S$: C, 22.51; H, 1.88; N, 10.84. Found: C, 21.87; H, 2.09; N, 10.99.

A v.p.c. analysis indicated the material to be 90–95% pure.

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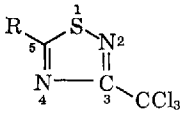
[1] The v.p.c. analysis was determined by Mr. A. Niedermayer.

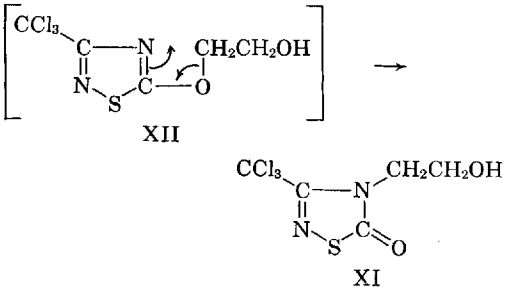
[2] The spectral data were furnished by Miss B. Keeler and Dr. A. Cohen.

TABLE I.—COMPOUNDS SYNTHESIZED



Compd.	R	M.p. or b.p., °C. (mm.)	Formula	Anal., %[a] Calcd.	Found
I	$-OCH_2CH_3$				
II	$-Cl$	53–55/0.05			
III	$-OCH(CH_3)_2$	70/0.02	$C_6H_7Cl_3N_2OS$	Cl, 40.66	40.68
				N, 10.71	10.96
				S, 12.26	12.61
IV	$-NH-NH_2$	182–183	$C_3H_3Cl_3N_4S$	Cl, 45.57	45.50
				N, 24.00	23.76
V	$-NH-OH$	170–171	$C_3H_2Cl_3N_3OS$	Cl, 45.37	45.36
				N, 17.92	17.60
VI	$-NH-O-CH_2-\phi$	153–155	$C_{10}H_8Cl_3N_3OS$	Cl, 32.78	32.94
				N, 12.95	12.97
VII	$-O-CH_2-CH_2-CH_3$	103.5–104/1.4	$C_6H_7Cl_3N_2OS$	Cl, 40.66	40.99
				N, 10.71	10.88
				S, 12.26	12.49
VIII	$-O-CH_2-CH_2-OH$				
IX	$-P(=O)(OC_2H_5)_2$				

[a] The microanalyses were conducted by Mr. J. Alicino.



XII

XI

Scheme I

Isomerization of 5-(2-Hydroxyethoxy)-3-(trichloromethyl)-1,2,4-thiadiazole (VIII → XI).—Ten grams of VIII was distilled *in vacuo* under nitrogen, and the fraction that distilled at 153–158°/0.6–0.7 mm. was collected as a thick yellow liquid, weighing 2.8 Gm. λ 2.95 μ broad (bonded OH), 5.85 μ (C=O) and 6.55 μ (C=N); τ 5.25 (t, O—CH_2), τ 5.51 (t, N—CH_2), and τ 5.95 (t, CH_2).

Anal.—Calcd. for $C_5H_5Cl_3N_2O_2S$: C, 22.51; H, 1.88; Cl, 40.48; N, 10.84. Found: C, 22.79; H, 1.92; Cl, 40.37; N, 10.63.

An attempted v.p.c. separation on K-122A column was not successful.

On the basis of the relative intensities of the C=N band at 6.55 μ, XI was estimated to be present to the extent of about 25%.

BIOLOGICAL TESTING

Compounds III–VIII were screened as soil fungicides[3] against 3 different pathogenic fungi, *Fusarium*, *Rhizoctonium*, and *Pythium*. Although no definite pattern of structure-activity relationships was discernible, all these compounds retained about 60–80% of the over-all activity of the analogous 5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole. However, in general, they were more phytotoxic than I.

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[3] The authors thank Dr. S. Cohen for the biological results.

Quantitative Analysis of Anhydrotetracycline on Microcrystalline Cellulose

By D. L. SIMMONS, C. K. KOORENGEVEL, R. KUBELKA, and P. SEERS

A quantitative TLC method for the determination of anhydrotetracycline alone and in the presence of tetracycline has been developed on microcrystalline cellulose. It is a rapid and precise method for determining small amounts of this compound in tetracycline samples, provided one works within the range of 10–40 mcg. of anhydrotetracycline per spot.

IN GENERAL, the separation of tetracycline antibiotics has been performed by chromatographic methods based on pH control of the adsorbent. Early investigators employed complex solvent systems with buffered paper (1, 2) or paper impregnated with sequestering agents (3). The quantitative separation of these antibiotics using a column of powdered cellulose impregnated with tartrate buffer was reported in 1961 by Hrdy and Vesely (4). In 1962 Addison and Clark (5) described a method for the separation of tetracycline from epitetracycline and anhydrotetracyline using a modified Whatman cellulose phosphate cation exchange paper. Kelly (6) employed a column of silicate containing a buffered EDTA solution to effect the resolution of anhydrotetracycline and epianhydrotetracycline in the presence of large quantities of tetracycline.

The application of microcrystalline cellulose to thin-layer chromatography has only recently been investigated (7, 8). Wolfrom and co-workers (9) reported that TLC on a microcrystalline form of cellulose[1] has been more efficient than paper chromatography in resolving certain water-soluble sugars, sugar derivatives, amino acids, and related compounds. This material has almost completely displaced the papergram in their laboratory, and in most cases was found to be superior to silica gel where both systems were applicable. These results combined with the published data on the resolution of tetracycline on paper led the authors to investigate the behavior of anhydrotetracycline on microcrystalline cellulose.

EXPERIMENTAL

Preparation of Anhydrotetracycline.—A solution of tetracycline hydrochloride (15 Gm.) in 0.1 *N* hydrochloric acid (500 ml.) was warmed on a steam bath at 70° for 1 hr. After cooling, the precipitate was collected by filtration and suspended in water (500 ml.). The suspension was adjusted to pH 4.5 with 5 *N* sodium hydroxide solution and extracted with ethyl acetate (2 × 400 ml.). The dried organic extracts were concentrated to approximately 50 ml. and cooled. The product (5.0 Gm.) was collected by filtration and recrystallized from benzene as yellow needles, m.p. 217.5°. [Lit. m.p. 215–220° (10).]

Anal.—Calcd. for $C_{22}H_{22}NO_7$: C, 61.96; H, 5.19. Found: C, 62.18; H, 5.08.

Preparation of Plates.—Microcrystalline cellulose (50 Gm.) was passed through a 100 mesh sieve and mixed in a mortar and pestle for 2 min. with 0.05% ammonium chloride solution (180 ml.). Since microcrystalline cellulose is commonly used in the pharmaceutical industry as a gelling agent, it was decided to employ a dilute salt solution such as ammonium chloride instead of water alone so that the gelling process was minimized. This modification permitted easier manipulation of the cellulose slurry. A 0.25-mm. layer of this homogeneous slurry was then applied to 5 clean glass plates (20 × 20 cm.) with a Desaga variable applicator. Prior to spotting, the plates were allowed to dry at room temperature for 10 min. and then heated in an oven at 90° for 30 min. Unlike silica gel plates, no special storage conditions were necessary.

Plate Development and Recovery of Anhydrotetracycline.—The plates were spotted by applying aliquots (10, 15, 20. . . 60 μl.) of a freshly prepared standard solution of anhydrotetracycline in methanol (1 mcg./μl.) approximately 3 cm. from the edge of each chromatoplate. These quantities are equivalent to 10–60 mcg. of anhydrotetracycline per spot. The plates were then placed in a developing chamber containing 0.1% aqueous ammonium chloride solution (160 ml., pH 5.6); filter paper saturated with the eluant lined the interior chamber walls. The plates were allowed to develop for 20 min., removed from the chamber, and dried for 10 min. under ambient conditions. An area (4 × 6 cm.) was marked off around the visible yellow spot (R_f 0.35), and the surrounding adsorbent was removed by a straight edge spatula and discarded. Individual spots were scraped off and collected in 3-ml. sintered-glass filter funnels, and the anhydrotetracycline was recovered from the adsorbent by washing the contents of the funnel with hot methanol directly into a 10-ml. volumetric flask under vacuum in a vacuum bell jar. The absorbances of the resulting filtrates were determined at 428 mμ against a methanol blank on a Beckman DU spectrophotometer. It was not necessary to employ a methanolic extract of cellulose for blank determinations at this wavelength. The procedure was repeated 5 times for each concentration.

Similar aliquots of the standard solution were transferred directly to 10-ml. volumetric flasks, adjusted to volume with methanol, and the absorbances read at 428 mμ.

Results are summarized in Table I.

Estimation of Anhydrotetracycline in Tetracycline Standard Mixture.—Tetracycline hydrochloride (80 mg.) was dissolved in 8.0 ml. of anhydrotetracycline standard solution in a 10-ml. volumetric flask, and the contents were adjusted to volume with methanol. An aliquot (0.04 ml., equivalent to 32 mcg. of anhydrotetracycline and 320 mcg. tetracycline hydrochloride) was removed by micrometer

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[1] Marketed as Avicel by the American Viscose Co.

TABLE I.—ANALYSIS OF ABSORBANCES[a] FOR DILUTIONS OF ANHYDROTETRACYCLINE STANDARD SOLUTION (1 mcg./μl.)

Concn., mcg./10 ml.	Absorbances	
	Direct Dilution	Dilution After TLC
10.0	0.020 ± 4.4%	0.021 ± 4.0%
15.0	0.029 ± 4.9	0.031 ± 3.2
20.0	0.041 ± 1.5	0.041 ± 1.9
25.0	0.052 ± 1.2	0.052 ± 1.4
30.0	0.062 ± 1.2	0.063 ± 2.1
35.0	0.072 ± 2.4	0.071 ± 2.4
40.0	0.082 ± 0.54	0.081 ± 1.8
45.0	0.093 ± 0	0.088 ± 0.71
50.0	0.102 ± 0.98	0.096 ± 0.78
60.0	0.122 ± 0.82	0.116 ± 2.0

[a] ± = per cent standard deviation.

syringe and spotted on a cellulose coated plate. The development and recovery of anhydrotetracycline from the mixture was performed in triplicate according to the procedure described previously. Anhydrotetracycline was visible as a yellow spot at R_f 0.35, whereas tetracycline required ultraviolet light for detection at the solvent front. The quantity of anhydro-derivative recovered from the 0.04-ml. application was obtained by multiplying the observed absorbance (A_0) by the concentration of standard (C_{st}) over the corresponding absorbance of standard (A_{st}).

$$\frac{A_0 \times C_{st}}{A_{st}}$$

The total anhydrotetracycline in the sample was then determined by accounting for dilutions and weight conversion (Table II).

RESULTS AND DISCUSSION

Results indicate that quantitative isolation of anhydrotetracycline by microcrystalline cellulose TLC is possible over the 10–40 mcg. range, but deviations from the standard occur at quantities above 40 mcg. (Table I). Fairly extensive tailing of the chromatogram was observed in the 45–60 mcg. range; this is probably due to overloading of the film with anhydrotetracycline and/or its solvent. The anhydrotetracycline can only be recovered quantitatively in this range by extracting the entire cellulose area between origin and spot.

TABLE II.—RECOVERY OF ANHYDROTETRACYCLINE FROM TEST SAMPLE (32 mcg./SPOT)

Absorbance	mcg. Anhydro/Spot
0.064	31.2
0.065	31.7
0.065	31.7

An interesting feature of the procedure described is that significant changes to the pH (1.5–7.8) of the solvent system did not alter the behavior of the chromatogram. This is in contrast to the exacting pH requirements of previous methods.

Initially this chromatographic procedure helped to demonstrate the purity of the anhydrotetracycline. After each recrystallization from benzene, a standard solution (1 mcg./μl.) was prepared and subjected to TLC on cellulose. When the absorbance readings of the direct dilutions of the standard were identical to the dilutions obtained following TLC, the compound was judged to be of analytical purity. Subsequent elemental analysis substantiated this judgment.

Additional evidence for the purity of the anhydrotetracycline standard was obtained by demonstrating the absence of epianhydrotetracycline, an anticipated contaminant, by subjecting the compound to a chromatographic method established for this purpose (3). This method also showed that epimerization of the anhydrotetracycline did not take place under the TLC conditions described in this paper and is in agreement with the findings of Addison and Clark (5). These investigators found that tetracycline in solution in aqueous ammonium chloride remained stable over several hours.

A mixture of anhydrotetracycline and tetracycline did not have an adverse effect on the chromatogram. The authors' demonstrate that the anhydro-derivative can be quantitatively isolated and determined from a test mixture containing anhydrotetracycline and a large quantity of tetracycline.

Since the foregoing method for the quantitative determination of anhydrotetracycline in tetracycline mixtures was established, the authors have developed procedures for the separation and determination of anhydro- and epianhydrotetracyclines in standard tetracycline mixtures and degraded tetracycline products. These procedures will be reported in a future paper.

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Structure and Activity in the Schistosomicidal Thiaxanthone and Xanthone Derivatives

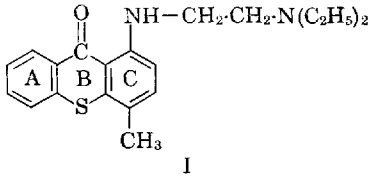
Sir:

1-(2-Diethylaminoethylamino)-4-methyl thiaxanthone (I) possesses schistosomicidal and antitumor activity. Also its analog, 6-chloro-1-(2-diethylaminoethylamino)-4-methyl xanthone (II) shows similar but less potent activity (1, 2).

In both areas of application, the activity depends mainly upon the presence of the methyl group at the 4-position *para* to an amino side-chain at the 1-position of ring C. Changes in these groupings caused loss of activity (3).

In previous communications, it was reported that both of the groupings attached to ring C could undergo dehydrogenation through the enzymatic action of peroxidase in the presence of hydrogen peroxide (4). Also, the 4-methyl group could undergo chlorination (5) and Mannich (6) reactions under the appropriate experimental conditions.

This particular reactivity may be explained by the presence of the polarizable carbonyl group adjacent to the 1-position and the heteroatom (oxygen or sulfur) neighboring the 4-position. As both heteroatoms bear 2 pairs of free electrons



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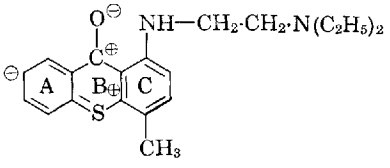

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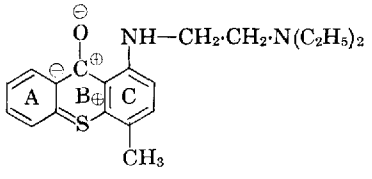

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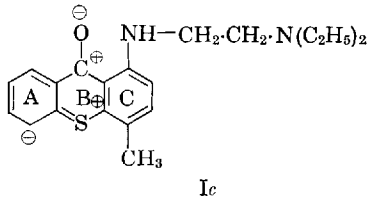

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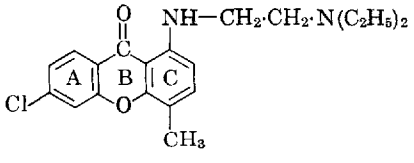

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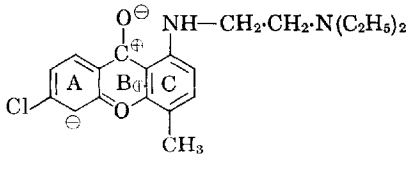

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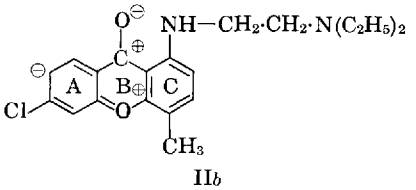
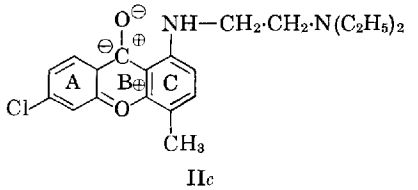
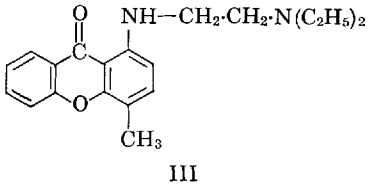


IIb



IIc



III

and the structures of both molecules (I and II) permit, to different extents, extensive delocalization of these free electrons to be involved in the resonance hybrids I, I*a*, I*b*, I*c*, and II, II*a*, II*b*, II*c* with ring A, the delocalization of the free electrons on the heteroatom preferentially extends toward ring A as ring C bears electron-donating substituents, and this would not favor the resonance hybrids to involve ring C.

This tendency is more pronounced with the sulfur present in I than with the oxygen present in II since oxygen is more electronegative than sulfur. Also, oxygen has an atomic radius of 0.66 Å., which would allow more orbital overlap with the attached carbon atom (atomic radius 0.77 Å.), and this would inhibit to an extent the electron delocalization tendency. This might explain the difference in the biological activity between I, II, and the structural analog, 1-(2-diethylaminoethylamino)-4-methyl xanthone (III) which does not show any noticeable biological activity. In II, the chlorine atom, through its inductive effect, helps to overcome the inhibiting factors of the delocalization of the electrons on oxygen. This effect is missing in III, and thus it is deprived of activity.

In these resonance hybrids, the heteroatom acquires positive charge and ring B becomes loaded with positive charges at both poles, which is electrostatically unfavorable. It tends to compensate for these positive charges by withdrawing electrons in this direction, thus increasing the acidity of the hydrogens attached to the 4-methyl group and the dissociability of that attached to nitrogen at the 1-position of the amino side-chain.

In confirmation of this hypothesis, the NMR measurements of I and II showed that the hydrogens of the 4-methyl group appeared at 7.5 τ which is at the lower part of the field. Aromatic methyl usually appears at 8 τ; thus, slight acidity is implied. In III, it appeared at 7.88 τ, which is at a higher part of the field. Also, the dipole moment of thiaxanthone itself is 5.4 *D* while that of xanthone is 3.11 *D*.

From the biological standpoint, when the carbonyl group in any of the drugs was reduced to the corresponding hydrol, then the activity was lost. In addition, when the sulfur in I was oxidized to sulfone, the drug was no longer active (3).

(1) Kikuth, W., Gonnert, R., and Mauss, H., *Naturwissenschaften*, **33**, 253(1946).
(2) Blanz, E., and French, F., *J. Med. Chem.*, **6**, 185(1963).
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(4) Nabih, I., and ElSheikh, M., Abstracts of papers presented to the American Chemical Society, Atlantic City meeting, September 1965.
(5) Nabih, I., and ElSheikh, M., *J. Pharm. Sci.*, **54**, 1672 (1965).
(6) *Ibid.*, **54**, 1821(1965).

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Role of Sulfate Formation in Biotransformation of Salicylamide in Man

Sir:

Salicylamide is eliminated in man mainly by biotransformation to the ether glucuronide and the ester sulfate (1, 2). Studies of the kinetics of salicylamide sulfate formation as a function of dose, which will be described in detail in a subsequent report (3), have shown that this process reaches a maximum rate and exhibits characteristics of apparent zero-order kinetics in the usual dose range. This preliminary report is presented in view of the theoretical and practical importance of such unusual kinetic characteristics in the elimination of a commonly used drug.

Salicylamide was administered to healthy adult males as an aqueous solution on empty stomach after an overnight fast. It was given in single doses of 150 and 1000 mg. In addition, a single dose of 1000 mg. salicylamide was given with L-cysteine which was administered every hour for 7 doses starting 3 hr. before salicylamide administration. Total urine collections were made every 0.5 hr. for 4 hr., then every hour for 4 hr., finally at convenient intervals up to 24 hr. after drug administration. Total salicylamide, salicylamide glucuronide, and salicylamide sulfate in the urine were determined by a combination of chemical and enzymatic methods (3). The results of these experiments in 2 representative subjects are shown in Table I. Essentially all of the administered drug was recovered in the urine in the form of salicylamide metabolites. About 50% was excreted as salicylamide sulfate after administration of 150 mg. of drug; this fraction decreased to about 30% when the dose was increased to 1000 mg. The maximum excretion rate of salicylamide sulfate increased

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This tendency is more pronounced with the sulfur present in I than with the oxygen present in II since oxygen is more electronegative than sulfur. Also, oxygen has an atomic radius of 0.66 Å., which would allow more orbital overlap with the attached carbon atom (atomic radius 0.77 Å.), and this would inhibit to an extent the electron delocalization tendency. This might explain the difference in the biological activity between I, II, and the structural analog, 1-(2-diethylaminoethylamino)-4-methyl xanthone (III) which does not show any noticeable biological activity. In II, the chlorine atom, through its inductive effect, helps to overcome the inhibiting factors of the delocalization of the electrons on oxygen. This effect is missing in III, and thus it is deprived of activity.

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TABLE I.—EFFECT OF DOSE AND CYSTEINE ADMINISTRATION ON SULFATE CONJUGATION OF SALICYLAMIDE

	Subject 1			Subject 2		
Dose, mg.	150	1000	1000 + Cysteine	150	1000	1000 + Cysteine
Urinary recovery, % of dose	101	97	99	99	92	98
Per cent excreted as sulfate[a]	53	28	52	49	32	49
Max. sulfate excretion rate, mg./hr.[b]	43	109	268	45	121	241

[a] Per cent of amount recovered in urine. [b] Expressed in terms of salicylamide; based on 0.5 hourly urine collection.

less than threefold despite the 6.7-fold increase in the administered dose. The peak excretion rate of salicylamide sulfate after administration of 1000 mg. salicylamide represents a maximum which is not increased by use of larger doses and apparently reflects the maximum capacity of the body for this conjugation process. Coadministration of L-cysteine with 1000 mg. of salicylamide had a pronounced effect on the formation of the sulfate conjugate. The fraction excreted in the form of this metabolite increased to the value found with the lower dose, and the maximum excretion rate became essentially proportional to the administered dose when compared to the lower dose. Apparently, L-cysteine serves as a precursor for sulfate and thereby increases the capacity of the process responsible for salicylamide sulfate synthesis in the body. The results of these experiments show that man has a limited capacity for salicylamide sulfate formation and suggest that the limiting factor is the availability of sulfate.

Some important implications of these findings are: (*a*) the relative rate of salicylamide elimination decreases with dose, (*b*) pharmaceutical dosage form characteristics which affect the absorption rate of salicylamide will also affect the metabolic fate of this drug, (*c*) studies of salicylamide metabolism for which unusually large doses are employed for assay convenience yield quantitative results which do not apply to lower doses, (*d*) the elimination rate of salicylamide in high doses may be increased by administration of L-cysteine (and probably by absorbable sulfate or other sulfate precursors).

Until recently, ethanol was the only drug known to be metabolized in man by apparent zero-order kinetics in the therapeutic dose range. Salicylate (4) and salicylamide (specifically, glycine conjugation of the former and sulfate formation of the latter) can now be added, and there is preliminary evidence that at least 1 other type of biotransformation process exhibits saturation kinetics in man (5). The general implications of these findings, particularly as reflected in items (*a*) to (*c*) in the preceding paragraph, require therefore serious consideration.

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REVIEWS

Interpretation of NMR Spectra: An Empirical Approach. By ROY H. BIBLE, JR. Plenum Press, 227 West 17th St., New York, N. Y. 10011, 1965. x + 150 pp. 15.5 × 23.5 cm. Price $12.50.

The aim of the author is (*a*) to teach organic chemists to interpret their proton NMR spectra and (*b*) to provide handbook data for the job. In the latter aim he has done excellently. As to the first aim, his success is relative to the depth of the interpretation envisioned. He has given little more than recipes for the assignment of chemical shift values and spin-spin coupling constants. There is almost no discussion of physical interpretation.

Though it consists almost exclusively of empirical generalizations, the book is easy to read, owing to the author's lucidity and his use of a generous number of examples. Most of the points discussed in the text are ultimately summarized in tabular form. There are adequate references to the literature.

The earlier chapters describe the variation in chemical shift and coupling constant with structure, with numerous tables and graphs summarizing the experimental data. A wall chart of characteristic chemical shift ranges is furnished. The intensity patterns of first-order splitting comprises one chapter, which is followed by a discussion of the complications arising from higher order splitting. This chapter includes the method of numerical analysis of the ABX spectrum. Methods for simplifying complex spectra for interpretation are presented, and a final chapter outlines an over-all analysis of the spectrum. The appendix includes a glossary to the jargon of the field, which has largely grown out of electronic engineering and quantum physics.

This book will have its best application by those whose primary interest is the routine use of NMR in structural problems. It will also serve a beginner in the field by making easier the reading of Pople, Schneider, and Bernstein. As a virtual summary of the 500 pages of the latter, it is curious that it comes at almost the same price.

Reviewed by L. D. Tuck
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University of California
San Francisco

Handbook of Ultraviolet Methods. By ROBERT G. WHITE. Plenum Press, 227 W. 17th St., New York, N. Y. 10011, 1965. 356 pp. 17 × 26 cm. Price $17.50.

This book contains more than 1600 references to ultraviolet methods of analysis which have appeared in technical journals during the past 25 years. The references cited deal primarily with substances of biological significance. Emphasis is placed on the material or compound being analyzed rather than on instrumentation or techniques. All entries describe the subject treated and some contain considerable detail. The items are arranged alphabetically by senior author; specific substances are indexed by chemical name with cross-indexing to generic and trivial names. Chemists may find some of the abbreviations used, particularly ASTM CODEN for reference citations, unfamiliar initially. However, this volume should prove a useful and rapid reference to available literature on ultraviolet spectrophotometric methods of analysis.

Reviewed by Carolyn Damon
Drug Standards Laboratory
A.Ph.A. Foundation

NOTICES

Caries-Resistant Teeth. Ciba Foundation Symposium. Edited by G. E. W. WOLSTENHOLME and M. O'CONNOR. Little, Brown and Co., Boston, Mass. 02106, 1965. xii + 338 pp. 14 × 21 cm. Price $12.50.

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Drug Presentation and Prescribing. By W. R. L. BROWN and J. W. HADGRAFT. Pergamon Press Inc., 44-01 21st St., Long Island City, N. Y. 11101, 1965. viii + 118 pp. 13 × 19.5 cm. Price $3.00. Paperbound.

Pharmaceutical Abstracts. Vol. V, Issue No. 5. Edited by H. M. BURLAGE. College of Pharmacy, University of Texas, Austin, Texas 78712, 1964. 21 × 28 cm. Price $4. Paperbound. Issues 1 and 2 combined and issues 3 and 4 combined are available at $2.50 postpaid. A listing of previous issues with prices is available upon request. Vol. VI, Issue No. 1, April, 1965. Price $2.75 postpaid.

Index Nominum, Supplement 1965. Edited by the Laboratory of the Swiss Pharmaceutical Society, Zürich. Publishers: Swiss Pharmaceutical Society, Sihlstr. 37, 8001 Zürich, Switzerland. (To be inserted in place of the 1964 supplement.) 135 pp. 17 × 24 cm. Price: Swiss francs 22.—. Price of the entire Index Nominum 1963/65, Swiss francs 95.—, binders I and II included.

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Review Article

Biological and Phytochemical Screening of Plants

By NORMAN R. FARNSWORTH

CONTENTS

Received from the School of Pharmacy, University of Pittsburgh, Pittsburgh, Pa.

The author expresses his appreciation to the many people who aided in certain phases of this review. In particular, the staff of the Maurice and Laura Falk Library of the Health Professions and graduate students and staff of the Department of Pharmacognosy, University of Pittsburgh, Pittsburgh, Pa., were most helpful in the acquisition and compilation of references. Special thanks are extended to Dr. H. H. S. Fong and Dr. R. N. Blomster, who aided through their continual interest, criticisms, and helpful suggestions.

Certain of the material presented in this review was determined in our laboratories through support from research grant CA-08228 from the National Cancer Institute, U. S. Public Health Service, Bethesda, Md., from Eli Lilly and Co., Indianapolis, Ind., and from Riker Laboratories, Northridge, Calif.

INTRODUCTION

THE IMPORTANCE of plant-derived medicinals in modern medicine is often underestimated. Such useful compounds as digitoxin, rutin, papain, morphine, codeine, papaverine, atropine, scopolamine, quinine, quinidine, reserpine, ergotamine, ergonovine, cocaine, vincaleukoblastine, leurocristine, *d*-tubocurarine, protoveratrines A and B, ephedrine, sparteine, physostigmine, pilocarpine, colchicine, and caffeine—to mention a few—present a broad and representative range of pharmacologic activities. In addition, crude drugs such as *Digitalis purpurea* leaf and *Rauwolfia serpentina* root are still preferred by many physicians in their practice; whereas extracts from *Podophyllum peltatum* (podophyllin), *Rhamnus purshiana* (anthraquinones), *Cassia* species (anthraquinones), and *Plantago* species (mucilage) are widely utilized for their medicinal activity. In fact, a recent survey has pointed

out that 47% of some 300 million new prescriptions written by physicians in 1961 contained, as one or more active ingredients, a drug of natural origin. Further, between 1950 and 1960, prescriptions containing drugs of natural origin increased by 7.7% (1).

A knowledge of the biological activities and/or chemical constituents of plants is desirable, not only for the discovery of new therapeutic agents, but because such information may be of value in disclosing new sources of such economic materials as tannins (2), industrial oils (3–9), gums (10), precursors for the synthesis of complex chemical substances (11), etc. Also, a novel chemical structure, isolated from plant sources, often prompts the chemist to a successful series of modified semisynthetic compounds, *e.g.*, atropine →homatropine, reserpine→syrosingopine, morphine→*N*-allylnormorphine, which may have some medicinal or otherwise useful economic value. On the other hand, attempts at modification of model natural compounds to enhance their activity have, of course, been unsuccessful (12). A knowledge of the chemical constituents of so-called "toxic" plants could place the treatment of plant poisonings of both humans and animals on a more rational and specific basis. The possibility of discovering new research tools for use in pharmacology, *e.g.*, as has been served by the hallucinogens, should not be discounted. A knowledge of the chemical constituents of plants would further be valuable to those interested in the expanding area of chemotaxonomy (biochemical systematics), to those interested in biosynthesis, and to those interested in deciphering the actual value of folkloric remedies.

This review is intended to present the various approaches used by investigators primarily interested in the discovery of new biologically active plant principles. *Phytopharmacologic Approaches* will consider those methods which involve, as a first step, the observation or detection of biological activity induced by plant products. *Phytochemical Screening Approaches* will describe and evaluate methods used for the detection of phytochemical classes of compounds, examples of which are known to elicit some desirable biological response.

The problems of the natural product investigator interested in biologically active compounds are complex and differ distinctly from those of the organic chemist who synthesizes or manipulates molecules using structure-activity relationships as his theoretical motivation to design. Natural product investigators must initially select their plants to investigate from a total number of available species that has been estimated to be as high as 750,000, excluding the bacteria and fungi (Table I). When this selection has been made, whether it be on theoretical grounds or on the basis of preliminary experimentation, the problems of acquisition and the variability of investigational plant material become complicating factors. These problems have been discussed recently in relationship to drug plants (14). Next, the natural product investigator must enlist the aid of a cooperative pharmacologist, or make other arrangements to insure a suitable biological evaluation for his extracts and isolated compounds. The problems inherent in the biological evaluation of crude plant extracts are in themselves unique, and several will be delineated in this review. Many of these problems remain to be solved, and the personal experiences of this author cause him to believe that those who are concerned with biological evaluations have little interest in crude plant extracts. In effect, priority is usually given to the biological evaluation of crystalline, water-soluble compounds. However, it should be remembered that in natural product studies, these pure compounds are realized only after initial biological tests on crude extracts provide justification for a phytochemical investigation. This lack of interest in the biological evaluation of crude plant preparations will probably continue as the major block to progress in the study of natural products.

The selection of research plant material by the investigator interested in the discovery of new biologically active phytoconstituents is a real problem. Approaches outlined in this review are representative of those which have either been used in the past, or are currently in progress. Each has obvious advantages as well as disadvantages, and the final method or combination of methods to be used by an individual will necessarily reflect his own background, training, interests, and available research facilities.

PHYTOPHARMACOLOGIC APPROACHES

Medicinal Folklore Evaluation

From about the 11th to the 18th centuries a dogma known as the Doctrine of Signatures was almost the sole means by which man attributed medicinal value to certain plants. This dogma held that the color, shape, habitat, or other physical characteristics of a plant were indicative of its medicinal value. Thus, the worm-shaped embryo of chenopodium (wormseed) suggested it to be of value as an anthelmintic, the yellow color of saffron served to point out its value in liver disorders, the serpentine shape of rauwolfia roots (snakeroot) indicated that they should be useful in treating snakebite, etc. Obviously,

TABLE I.—ESTIMATED NUMBER OF PLANT SPECIES[a]

Thallophyta	
Bacteria	1,500
Fungi	100,000
Algae	19,000
Lichens	20,000
Bryophyta	14,000
Pteridophyta	10,000
Spermatophyta	
Gymnosperms	700
Angiosperms	
Monocots	200,000
Dicots	500,000

[a] As put forth by Schultes in 1963 (13).

no rational justification for the use of drugs selected in this manner can be made. However, as man experimented, he found by trial and error, certain plants useful for the treatment of some of his myriad illnesses. Many of the uses of these plants were, in the course of time, documented in various herbals, books on medical botany, in ethnobotanical manuscripts, and even as notes on herbarium specimens. Uses of other alleged medicinal plants remain undocumented in the literature and exist only in the lore of primitive people. Let us consider some of these sources of medicinal folklore.

Herbals.—Space will permit only a token mention of the hundreds of herbals available for scrutiny by those interested in pursuing this approach to uncover new leads to plants with potential medicinal attributes. "The Herbal," by Arber (15), tabulates many of those which are known. A further list of herbals and related works, many of which are rare, has been prepared for the holdings of the Hunt Botanical Library (16, 17). The use of such materials as sources of ideas for new drug plants has been discussed (13, 18, 19).

Medical Botany.—One could spend a lifetime surveying the available published books and periodicals describing the native flora of various regions and the medical uses ascribed for each plant. De Laszlo (20) has compiled a list of some 1500 references on books, journals, and periodicals concerning phytotherapy and Dragendorff's "Die Heilpflanzen der Verschienden Volker und Zeiten" (21) should not be overlooked as a source of new leads. Steinmetz's "Codex Vegetabilis" (22) also lists hundreds of plants together with their synonyms, constituents, and reported uses; however, references to source material are not included in this work. Perhaps because of the Indian system of Ayurvedic medicine, the plants of that country have been reported medicinally useful more often than perhaps those of any other country. At least one gains this impression from the host of available references (23–28). Other countries represented by books or review publications on medical botany are Mexico (29, 30), Poland (31), New Guinea (32), the Philippines (33), Nigeria (34), the U.S.S.R. (35, 36), China (37–39), Burma (40), Puerto Rico (41), Malaya (42), Africa (43), Greece (44), Australia (45), New Zealand (46–49), Taiwan (50, 51), and Haiti (52), as well as others (53–57). These references are to be taken only as selected examples illustrating the type of source material available to the interested investigator. Schultes has recently pointed out, in an excellent article, the opportunities for investigation through an appreciation of medical botany (13).

Of course, simply reading through these works will not automatically assure one of success in his selection of plants for investigation. A great deal of common sense, a broad background in the medical sciences, and some knowledge of plant constituents and of chemotaxonomic relationships are all necessary for one to select the most promising plants for study.

Ethnobotany.—An evaluation of those publications by persons who have studied plants in their relationship to human affairs could uncover many species with potential biological activity. The papers by Train *et al.* (58–61), which describe plants used by the Nevada Indians, are certainly worth examining for new leads. Any person interested in the discovery of new biologically active compounds in plants would do well to become acquainted initially with the ethnobotanical writings on narcotic drug plants by Schultes and others (62–76), since they contain a wealth of information found in few other references.

An interesting ethnobotanical report has described 3 plants, all members of the *Araceae*, that are used by natives in Colombia as oral contraceptives (77). Several years ago, we had occasion to investigate *Dieffenbachia picta* (*Araceae*) because reports forwarded to our laboratory indicated that natives in South America were known to utilize this plant as an oral contraceptive. Our animal experiments failed to confirm this use for *D. picta*, but subsequently an excellent review article on the toxicity of *Dieffenbachia* species was located which described in detail many cases wherein these species had been utilized for their antifertility effects (78). Extracts of *D. seguine* were shown to produce sterility in both male and female rats after 30 to 90 days of either oral or subcutaneous administration. On histological examination it was revealed that complete or partial atrophy of the entire male or female genital apparatus had been induced in the treated animals, thus rendering them sterile (79). This obviously is an undesir-

able effect for an oral contraceptive drug and interest in *Dieffenbachia* species for this purpose appears to have been curtailed. In retrospect, however, it is unusual that natives in South America and in certain of the Caribbean islands chew the leaves of *D. seguine* to impart a 24- to 48-hr. period of sterility, whereas in laboratory animals, extracts of this plant must be administered for several weeks in order to obtain the same effect (78).

Perhaps a specific example, pointing out the value of ethnobotanical studies in the search for new biologically active compounds, would be in order. During early 1956 this author had occasion to review an ethnological manuscript, representing the doctoral dissertation of Philips (80), for possible publication in the form of a book by the University of Pittsburgh Press. The manuscript was concerned, in part, with Lebanese folk cures, the idea for the dissertation arising from contacts with people in Lebanon as well as Lebanese immigrants in Brooklyn. The author found, as her investigation progressed, that the customs of the Lebanese people were greatly influenced by the use of plant drugs as a facet of their culture. Information was derived from personal interviews with 843 people and resulted in a rather extensive list of plants used as drugs. Many of the native uses for these plants could be accounted for on rational grounds through a knowledge of chemical compounds existing in the plants which had known biological activity. Others could not be explained on these bases and seemed worthy of future investigation. As I read the manuscript, a list was prepared of those plants which appeared to be used by the greatest number of people for specific, well defined, disease conditions. The completed list included 15 plant names, the alleged uses for which I could not explain at the time. Several months later an article was noted concerning the isolation and pharmacology of an alkaloid named petaline chloride, in addition to others, from *Leontice leontopetalum* (*Berberidaceae*) (81). Ironically, this plant headed my list taken from the ethnobotanical manuscript as being most likely to yield interesting and biologically active entities. *Leontice leontopetalum* had been used as a folk medicine in Lebanon for years as an aid to the treatment of epilepsy. Petaline chloride was shown to be a central nervous system depressant in mice and rabbits, and the investigators claimed antiacetylcholine activity on isolated frog skeletal muscle. Additional studies to indicate more clearly antiepileptic activity, *i.e.*, effect on chemically or electrically induced convulsions in animals, were not performed, presumably due to an inadequate supply of petaline. To my knowledge, additional pharmacologic evaluations of this alkaloid have not been reported.

Other ethnobotanical manuscripts that offer similar possibilities, or give interesting background material on this approach to the discovery of new drug plants, have been written by Tantaquidgeon (82), Gunther (83), Grover (84), and Schultes (85, 86).

Herbaria.—A rather unusual approach to the search for new leads to biological activity in plants is being pursued at Harvard University. This involves a search of individual herbarium specimens for field notes entered by the botanist making each particular collection. In many instances, as pointed out by von Reis (87), information appears on some specimens that indicates a particular medicinal use for a plant as observed by the collector. The project consists of a sheet by sheet study of the entire collection of flowering plants in the Harvard University Herbarium, which includes some 2,200,000 specimens from the collections of the Arnold Arboretum and the Gray Herbarium. Advantages to this type of study have been summarized by von Reis (87). In the first 6 months of the study, 6 families had been searched, *i.e.*, *Apocynaceae*, *Asclepiadaceae*, *Convolvulaceae*, *Lythraceae*, *Myristicaceae*, and *Rubiaceae*. About 400 species of interest were recorded and separated into several categories, *i.e.*, medicinal uses (40%), suggested medicinal applications or possible physiological activity (33%), substances eaten, but not as normal staples of the diet (13%), poisons (8%), plants collected expressly for the purpose of chemical analysis and possible drug use (3%), and plants which animals were said to be attracted to or that they especially avoided as food or contact substances (1%). It has been conservatively estimated that the study, when completed, should yield some 360 notes concerning new potential medicinal applications for plants.

The only other study of this type which has been publicized is that being conducted by Perry involving a compilation of medicinal flora of southeastern Asia. Included as a part of this effort is a study of field notes on herbarium specimens (88).

Field Exploration.—Throughout history, botanical collecting expeditions have been organized for the specific task of discovering new drugs. In 1716, Peter the Great requested the botanist Breynius, in Danzig, to organize an expedition to explore the botanical resources of Russia for new drugs. The botanist Messer-

schmidt from 1720–1727 made vast collections in Siberia of plants reputed to have medicinal value (89). In 1602 Bartholomew Gosnold, in a ship chartered by Sir Walter Raleigh, sailed the coast of New England specifically to obtain the bark of *Sassafras albidum* for export to England (89). Parke Davis and Co. sponsored expeditions into South America in 1885 which led to the discovery of *Rhamnus purshiana* (cascara) and *Guarea rusbyi* (cocillana), 2 drugs still in use today (89). In 1942, when the quinine source of the world was cut off by the invasion of the Dutch East Indies by the Japanese, American botanists Steere, Fosberg, Camp, and others relocated and identified several quinine-yielding species in South America (89). More recently, the *Strophanthus* hunt in Africa, as well as the search for new steroidal sapogenin and alkaloid-bearing plants of the world, testify to the value of field exploration for new plant products of medicinal and/or economic value. These, along with accounts of the most striking plant explorations of the past, have recently been documented by Kreig (90).

There is controversy regarding the optimal approach to plant exploration if one is to uncover information on drug plants of potential value. Some believe that a semirandom collection of plants which contain specific types of chemicals, *e.g.*, alkaloids, will be most rewarding (91–94). Others consider that a random selection of plants in combination with subsequent broad pharmacological screening will lead to new therapeutic agents (95–98). Still others feel that recording the names of plants used in native medicine by witch doctors, medicine men, etc., directly from the individuals who use the products, will give a greater insight to the selection of plants for subsequent biological evaluation (99, 100).

Schultes, who spent some 12 years in the Amazon, feels that natives are more apt to reveal their secrets (drug plants) only after acquaintance with an invading investigator for a long period of time (13). He has cited several examples from personal experiences that bear out this point (13). Furthermore, Schultes is of the opinion that the most successful explorations for new plant drugs will not be those set up with the express purpose of looking for medicinals and nothing else, but rather those primarily designed to acquire new botanic and ethnobotanic knowledge (13). In the course of such investigations, information regarding new drug plants and related materials should be uncovered during the normal course of events.

Recognizing that the latter approach has merit and could well be the most effective method presented, the need for an inception of such a program is immediate. That is because, as civilization spreads into primitive areas, the first aspect of primitive culture to be lost is knowledge on the use of plants as medicine (13). Schultes, with respect to this matter, has stated, "The rapidity of this disintegration (knowledge of medicinal plants) is frightening" (13). Unfortunately, trained manpower to carry out this type of field exploration program is not available for an extensive undertaking at the present time (13).

Poisonous or Toxic Plants

A documentation of toxicity for any plant material is usually evidence for the presence of biologically active material in the plant cited. The terms "poison" and "toxic" are, of course, often relative where biological activity is concerned. If one discounts reports concerning contact poisons and considers mainly plants reported to cause systemic toxicity, it is possible that proper dose administration of extracts from the particular plant in question will uncover knowledge of pharmacologic activity worthy of further study. A number of books and other periodicals are available which point out plants that have been reported to cause toxicity. Interesting historical background information on this subject has been published by Kingsbury (101) and an excellent review of the toxic plants of the United States and Canada, which is thoroughly referenced, is found in Kingsbury's recent book (102). Other references to toxic plants and fungi of the U. S. are those of Muenscher (103), Duncan *et al.*, (104, 105), Morton (106, 107), and O'Leary (108). African (43), Australian (45), Indian (109, 110), British (111), Venezuelan (112), and the Pacific region (113) toxic plants, have been similarly documented. Again, the references cited above are not meant to constitute a complete listing of all available references to toxic plants. Rather, they are offered as an introduction to the person interested in studying toxic plant constituents and the relationship of these constituents to possible therapeutic applications.

Phytopharmacological Surveys

A number of interesting surveys have been conducted in which plant extracts have been evaluated for various biological activities. Although the data presented in certain of these surveys must be considered as negative, *i.e.*, those regarding antineoplastic evaluations published by the Cancer Chemotherapy National Service Center (114–118) as well as by others (119), these reports are quite helpful to investigators

engaged in similar studies who might not want to duplicate negative efforts. Biological evaluation of plant material presents certain inherent and apparently uncontrollable problems. If one considers the well known fact that phytoconstituents can vary based on climate, habitat, soil nutrients, and time of harvest, in addition to other factors (120–122), and that distinct chemical races of plants are known to exist (123–127), variation in results from one lot of plant material to the next must be expected. Furthermore, the selection of an appropriate extraction solvent, when the chemical nature of the potentially active phytoconstituents is unknown, presents a problem. Also, the conditions to be used in preparing the extracts of plant material could be the difference between demonstration of biological activity and obtaining essentially negative results. The presence of antagonistic substances, as regards to biological activity, could result in a failure to detect either of the 2 or more individually active materials. Since many plants are known to accumulate rather large quantities of toxic inorganic constituents, *i.e.*, selenium, nitrates, copper, etc. (102), the predominant action of any one of these in a plant extract containing organic compounds with potentially interesting biological activity could lead to the dismissal of further interest in the plant. It is interesting to note that we recently isolated leurosine, an alkaloid with a high order of activity against the P-1534 leukemia in DBA/2 mice, from a crude fraction of *Catharanthus lanceus* alkaloids that by itself was shown to be devoid of activity (128). Svoboda (129) also has pointed out that leurocristine and leurosidine, both highly active against the P-1534 leukemia, have also been isolated from crude alkaloid fractions that were devoid of activity against the same neoplasm.

There appears to be a great race to determine the biological activity of plant extracts, but little is being done to investigate the problems mentioned above. When, and if these problems are solved, all negative data obtained with present methods may have to be re-examined, and perhaps duplication of similar studies will be warranted.

Antineoplastic Activity.—The plant kingdom should be a prospective and fruitful hunting ground for new tumor inhibitors. This has been illustrated by the isolation, characterization, and structure elucidation (130) of vincaleukoblastine (vinblastine) (131, 132) and leurocristine (vincristine) (129) from the apocynaceous shrub *Catharanthus roseus* (*Vinca rosea, Lochnera rosea*). Vincaleukoblastine is used clinically for the treatment of Hodgkin's disease (133) and for choriocarcinoma (133), whereas leurocristine is effective in the treatment of acute leukemia in children (133).[1] Noble *et al.* (131) were the first to publish on the antineoplastic and leukopenic activity of *C. roseus* and the isolation of crude vincaleukoblastine. Their studies were initiated by reports that *C. roseus* was used extensively as an oral hypoglycemic agent in folk medicine. Furthermore, the discovery of anticancer activity was made during their blood sugar work, only on the basis of followup studies on a toxicity observed in *C. roseus*-treated animals. Antitumor activity of *C. roseus* extracts also was noted by Svoboda in his laboratory at about the same time that Noble and co-workers made their observations of this activity. Svoboda's observations came about through a routine screen for antitumor activity, as his initial interest in this plant was a potential source of hypoglycemic compounds (134). Subsequent studies by Svoboda (129) led to the isolation of leurocristine, as well as leurosine (132) and leurosidine (129), 3 additional *C. roseus* dimeric antineoplastic alkaloids (133). However, in another laboratory, an alcoholic extract of *C. roseus* (*Lochnera rosea*) whole plant provided negative results when screened against the sarcoma 180 and the L-1210 leukemia (114). Further negative results have been reported for an aqueous extract of *C. roseus* (*Vinca rosea*) seeds on testing against the sarcoma 180, the adenocarcinoma 755, and the L-1210 leukemia (114).

Hartwell (135) has discussed the role of the Cancer Chemotherapy National Service Center (CCNSC) in screening plant extracts, as well as other compounds, for antineoplastic activity. A comprehensive plant collection program by the U. S. Department of Agriculture (USDA) as well as by others, serves to supply plant material to the CCNSC for evaluation. Most plants are semirandomly selected for testing, but home remedies alleged to be useful for the treatment of cancer are not discounted (135). Since its inception, the CCNSC has screened about 26,000 plant extracts. No figures are available to indicate the number of species represented by this number, but it has been estimated that an average of 2 plant parts for each species are tested, and for most plant materials, 2 extracts (*i.e.*, water, ethanol) are prepared. Therefore, one could estimate that about 6500 species have been evaluated to date by the CCNSC. About

[1] Dr. Gordon Zubrod, Director of Intramural Research, National Cancer Institute, Bethesda, Md., told a Fountain Subcommittee hearing on April 23, 1964, that leurocristine is the . . . "Most effective antileukemia agent that has been discovered in the last 5 or 6 or 10 years." [*F-D-C Reports—The Pink Sheet*, **26** (17) (April 27, 1964).]

10% of these species have been found to be "active" against one or more tumors and are considered candidates for phytochemical isolation work. The CCNSC expects that this program will be continued at about its present rate (136).

Although the CCNSC program is still in its infancy, on the basis of the number of compounds and plants that are available for evaluation, reports in the current literature are beginning to identify some of the antitumor and/or cytotoxic constituents of plant extracts that have been determined active in this screening sequence. Kupchan *et al.* (137) have isolated aristolochic acid, active against the adenocarcinoma 755, from *Aristolochia indica* (*Aristolochiaceae*). Aristolochic acid has been found to exert a high order of renal toxicity which will preclude its use in clinical studies (138). Undoubtedly, structural relatives of this compound will be prepared in an attempt to reduce toxicity and yet maintain a high order of antineoplastic activity. Monocrotaline, from *Crotalaria spectabilis* (*Leguminosae*) has also been found to be an active tumor inhibitor (AC-755) (139). Phytochemicals which are cytotoxic for the 9-KB cell culture have been isolated and found to be apocannoside and cymarin from *Apocynum cannabinum* (*Apocynaceae*) (140), podophyllotoxin from *Juniperus virginiana* (*Pinaceae*) and other *Juniperus* species (141), calotropin from *Asclepias curassavica* (*Asclepiadaceae*) (142), cissampareine from *Cissampelos pareira* (*Menispermaceae*) (143), eupatorin from *Eupatorium semiserratum* (*Compositae*) (144), and gaillardin from *Gaillardia pulchella* (*Compositae*) (145). Semipurified fractions from *Rumex hymenosepalus* (*Polygonaceae*) exert significant activity against the Walker 256 tumor and the sarcoma 180 in mice (146). The active material is thought to be a polyphenolic flavonoid tannin. A proteinaceous fraction from *Gutierrezia sarothrae* (*Compositae*) is inhibitory for sarcoma 180 in mice (147). Although many other examples concerning the isolation of active antitumor or cytotoxic plant principles could be cited, the above should serve to point out the broad distribution of antitumor activity in the plant kingdom. Table II lists the names of some 400 plant species in 315 genera and 97 families reported in the literature to be appreciably active as tumor inhibitors. A great variety of chemical classes and structure types are also represented in the known active plant principles, *i.e.*, alkaloids (vincaleukoblastine, leurocristine, tylocrebrine, lochnerinine, cissampareine, monocrotaline), cardenolides (apocannoside, caloptropin, cymarin), lignans (podophyllotoxin), flavonoids (eupatorin), tannins (*Rumex hymenosepalus*), proteins (*Gutierrezia sarothrae*), sesquiterpene lactones (gallardin), tetracyclic triterpenes (elatericin A, elatericin B, elaterin), as well as others (Fig. 1).

If one examines negative as well as positive screening data from plant extracts, it becomes apparent that active antineoplastic compounds are not usually distributed throughout related taxa. The problem of screening plant extracts is further compounded through a knowledge that active inhibitors are often localized in one plant organ and not distributed throughout the plant. Thus, the fruits or seeds of one species could well contain active material, yet evaluation of the whole plant, containing few if any fruits or seeds, would probably give negative results. Seasonal variation has been shown to affect the amount of active material present in certain species (148). Also, the selection of a proper solvent for preparation of extracts to be evaluated could be an important factor. Furthermore, most of the active compounds isolated from higher plants to date, as well as the extracts themselves, are often quite specific in their action for a given tumor or set of test conditions. Additionally, correlation of *in vitro* and *in vivo* testing results, with those of the clinical effects of antitumor agents, remains to be established.

It appears, however, in the light of present knowledge and experience, that a random selection and testing of plants selected from a broad cross section of families and genera will prove of greatest value in attempts to discover new entities for the treatment of clinical malignancies. It is interesting to note that the efforts to arrive at new antitumor agents by chemical synthesis appear to be no more productive than those of the natural product researcher. Even though the net result of these tedious procedures involving the search, collection, identification, processing, and biological evaluation of the flora of the earth does not yield additional compounds of clinical value, the new structures evolving from these efforts should prove of use to the chemist for his work involving structural modifications.

Recent findings (140, 142, 145, 149) that cytotoxicity is associated with the unsaturated lactone of cardenolides and related compounds, attached either to position 17 of the nucleus by a C—C bond or fused to ring D across the 16, 17-position, offer insight to the minimum qualifications for cytotoxicity. Medawar *et al.* (150) were the first to suggest that unsaturated lactones exert a specific inhibitory effect on the growth of tissue. Subsequent investigators confirmed this interesting relationship (151–152). Pike *et al.* (153) synthesized 150

TABLE II.—DISTRIBUTION OF ANTINEOPLASTIC ACTIVITY IN PLANTS[a]

Plant Name[b]	Neoplasm[c]	Ref.
Acanthaceae		
Andrographis paniculata	S-4	(176)
Jacobinia coccinea	C-1, S-4	(166, 176)
Aceraceae		
Acer negundo	C-5	(175)
Acer rubrum	C-1	(161)
Amaryllidaceae		
Agave americana	H-2, S-1	(160)
Agave expansa (A. americana)	C-1	(165)
Agave micracantha	S-1	(160)
Cooperia pedunculata	C-1, S-7	(148, 163, 167)
Galanthus nivalis	S-1	(160)
Haemanthus puniceus	S-1	(160)
Hymenocallis sp.	C-2	(376)
Hymenocallis caribaea	S-1	(160)
Hymenocallis occidentalis	S-1	(160)
Leucojum aestivum	S-1	(160)
Lycoris incarnata	S-1	(160)
Lycoris sprengeri	S-1	(160)
Lycoris squamigera	S-1	(160)
Manfreda maculata	C-1	(165)
Narcissus poetaz var. *geranium*	S-1, S-2	(160)
Narcissus tazetta	C-5, S-13	(171)
Polianthes tuberosa	S-1	(160)
Rhodophiala chilensis	S-1	(160)
Zephyranthes carinata	C-5, S-4, S-10, S-13	(171)
Zephyranthes texana	C-1, S-12	(148, 164, 167)
Anacardiaceae		
Anacardium sp.	C-1	(165)
Loxopterygium huassango	C-1	(158)
Melanorrhoea woodsiana	S-4	(176)
Pistacia chinensis	C-1	(158, 162)
Pistacia lentiscus	C-1	(158, 161, 162, 165, 166)
Pistacia vera	C-1	(161)
Rhus glabra	C-1, C-2	(164)
Rhus javanica	C-7	(171)
Rhus toxicodendron	C-1, C-2	(148, 163, 167)
Rhus typhina	C-2	(166)
Annonaceae		
Annona cherimola	C-1	(161)
Artabotrys suaveolens	S-4	(176)
Apocynaceae		
Apocynum androsaemifolium	S-1	(155)
Apocynum cannabinum	C-1, E-1	(140, 161)
Catharanthus lanceus	C-2, E-1, L-2	(128, 377)
Catharanthus pusillus	E-1	(341)
Catharanthus roseus	L-2	(133, 134)
Ervatamia dichotoma	E-1	(378)
Funtumia sp.	C-1	(158, 165)
Nerium oleander	C-1	(167)
Plumeria obtusa	S-4	(176)
Strophanthus hispidus	C-1	(376)
Trachelospermum asiaticum	C-5	(170, 173)
Aquifoliaceae		
Ilex coriacea	C-1	(161)
Araceae		
Symplocarpus foetidus	S-1	(157)
Araliaceae		
Eleutherococcus senticosus	S-9, S-10	(380)
Panax ginseng	S-9, S-10	(380)
Panax repens	C-5	(173)
Tetrapanax papyriferum	C-5	(172)
Aristolochiaceae		
Aristolochia sp.	C-1	(161)
Aristolochia indica	C-4	(137)
Asarum canadense	C-1	(161)
Asarum reflexum	C-1	(161)
Asclepiadaceae		
Asclepias curassavica	E-1	(142)
Asclepias decumbens	C-1	(148)
Marsdenia cundurango	C-1	(157)
Tylophora crebriflora	L-1	(381)
Berberidaceae		
Berberis aquifolium	C-2	(167)
Podophyllum emodi	S-1	(383)
Podophyllum peltatum	S-1	(382, 383)
Betulaceae		
Alnus serrulata	C-1	(166)
Carpinus caroliniana	C-1	(166)
Corylus americana	C-1	(161)
Ostrya virginiana	C-1	(161, 166)
Bixaceae		
Bixa orellana	C-2	(161)
Boraginaceae		
Alkanna sp.	C-5	(384)
Lithospermum arvense	C-1	(161)
Symphytum officinale	C-1, C-2, C-3	(328, 385)
Bromeliaceae		
Bromelia pinguin	C-1	(166)
Pitcairnia corallina	C-1	(166)
Tillandsia usneoides	C-2, S-2	(167)
Cactaceae		
Opuntia sp.	C-1	(167)
Opuntia maxima	CIT	(386, 387)
Cannabinaceae		
Cannabis sativa	C-1	(164)
Humulus japonicus	C-1	(158)
Capparidaceae		
Forchhammeria watsoni	C-1	(376)
Caprifoliaceae		
Sambucus simpsonii	C-1	(161)
Viburnum acerifolium	C-1	(158)
Viburnum macrocephalum	C-1	(376)
Viburnum prunifolium	C-1	(161)
Caryophyllaceae		
Arenaria caroliniana	C-2	(161)
Celastraceae		
Celastrus scandens	C-4, C-6	(388)
Chenopodiaceae		
Beta vulgaris	C-1, C-2	(167)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Beta vulgaris var. *rubra*	C-5	(389)
Chenopodium ambrosioides var. *anthelminticum*	S-1	(156)
Cistaceae		
Lechea villosa	C-1	(161)
Combretaceae		
Conocarpus erectus	C-1, C-2	(158, 165, 166)
Terminalia bellerica	C-2	(166)
Terminalia chebula	C-5	(390)
Compositae		
Acroptilon picris (*Centaurea picris*)	C-5	(175)
Arnica montana	S-1	(157)
Artemisia tournefortiana	C-5	(175)
Aster pilosus var. *demotus*	C-2	(166)
Bidens pilosa	C-1	(158, 165)
Bigelowia nudata	C-1	(158, 165)
Boltonia diffusa	C-1	(158, 165)
Brauneria pallida (*Echinacea pallida*)	S-1	(157)
Cacalia atriplicifolia	C-1	(165)
Calendula officinalis	C-5	(384)
Centaurea balsamita	C-5	(175)
Chrysopsis tricophylla	C-1, C-2	(161)
Cirsium altissimum	C-1	(158)
Cirsium arvense	C-2	(166)
Coreopsis lanceolata	C-2	(166)
Echinacea pallida	C-2	(166)
Eupatorium semiserratum	E-1	(144)
Gaillardia pulchella	C-1, E-1	(145, 158)
Gutierrezia sarothrae (*G. enthamia*)	S-2	(147)
Inula helenium	C-5	(172)
Lactuca canadensis	C-1	(161)
Liatris punctata	C-1, C-2, S-12	(163, 164, 167)
Mikania scandens	C-1	(158)
Onopordon acanthium	C-5	(384)
Parthenium hysterophorus	C-1, C-2, S-12	(164)
Ratibida pinnata	C-2	(166)
Saussurea sp.	C-5	(175)
Silphium compositum	C-1	(158)
Solidago fistulosa	C-2	(166)
Solidago gigantea	C-2	(166)
Sonchus oleraceus	S-1	(154)
Spilanthes americana (*S. mutisii*)	C-1	(158, 165)
Tanacetum vulgare	C-2	(166)
Tessaria integrifolia	C-1	(161)
Trilisa paniculata	C-2	(161)
Verbesina aristata (*V. nudicaulis*)	C-1, C-2	(161)
Xanthium sp.	C-1	(161)
Convolvulaceae		
Ipomoea orizabensis	S-1	(154)
Cornaceae		
Aucuba japonica	C-5	(170, 173)
Cornus florida	C-2	(161)
Cornus officinalis	C-5	(172)
Cruciferae		
Capsella bursa-pastoris	S-1	(155)
Descurainia pinnatum (*Sisymbrium pinnatum*)	C1-12	(161)
Cucurbitaceae		
Bryonia alba (*B. dioica*)	S-1	(154)
Citrullus colocynthis	C-5, S-1, S-2, S-5	(154, 391, 392)
Cucumis melo	C-1, C-2, C-5	(163, 167, 175)
Cucumis melo var. *cantalupensis*	S-1	(157)
Cucumis myriocarpus	S-1	(157)
Cucumis sativus	C-1	(167)
Cucurbita foetidissima	C-1, C-2, S-12	(163, 164, 167)
Cucurbita pepo	C-1, C-2	(167)
Ecballium elaterium	C-4, C-5, C-6, S-1, S-2, S-5	(154, 388, 392)
Datiscaceae		
Datisca cannabina	C-5	(175)
Dioscoreaceae		
Dioscorea villosa	S-1	(155)
Dipsacaceae		
Dipsacus sylvestris	C-1	(161)
Ebenaceae		
Diospyros discolor	S-4	(176)
Diospyros kaki	C-1	(158, 165)
Elaeagnaceae		
Elaeagnus philippensis	C-1	(166)
Hippophae rhamnoides	C-5, S-2, X	(174, 175, 393)
Hippophae salicifolia	S-6	(394)
Equisetaceae		
Equisetum arvense	S-1	(155)
Equisetum hellocharis	C-5	(175)
Equisetum hyemale	S-1	(155)
Ericaceae		
Arctostaphylos uva ursi	C-5	(173)
Chaemaedaphne calyculata	C-2	(166)
Gaultheria procumbens	S-1	(157)
Lyonia mariana	C-1	(161)
Menziesia pilosa	C-2	(166)
Oxydendrum arboreum	C-1, S-1	(155, 161)
Euphorbiaceae		
Acalypha phleoides	C-1	(164, 167)
Bridelia ovata	S-4	(176)
Croton monanthogynus	C-2, S-7	(163, 167)
Emblica officinalis	S-4	(176)
Euphorbia amygdaloides	C-5, S-2	(395)
Euphorbia drummondii	S-1	(154)
Euphorbia marginata	C-1	(148)
Euphorbia pilulifera	S-1	(154)
Euphorbia resinifera	S-1	(154)
Excoecaria agallocha	S-4	(176)
Macaranga triloba	S-4	(176)
Mallotus philippensis	S-1	(156)
Piscaria sp.	S-12	(164)
Poinsettia sp.	S-2	(396)
Ricinus communis	C-1, S-12	(148, 164)
Sapium sebiferum	C-1	(158, 165)
Stillingia sylvatica	C-2	(161)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Fagaceae		
Quercus virginiana	C-1	(161)
Gentianaceae		
Gentiana lutea	C-5	(173)
Gentiana scabra var. *buergeri*	S-4	(397)
Gnetaceae		
Ephedra trifurca	C-2	(161)
Gnetum latifolium	S-4	(176)
Gramineae		
Anthoxanthum odoratum	C-1	(376)
Arundo donax	C-1, C-2	(163, 167, 376)
Arundo plinii	C-1	(376)
Coix lachryma-jobi	S-11	(398)
Digitaria sanguinale	C-1	(161)
Sasa albomarginata	C-5, S-2	(399)
Spartina synosuroides	C-1	(161)
Tripsacum laxum	C-1	(376)
Uniola paniculata	C-1	(161)
Zea mays	C-2	(167)
Guttiferae		
Garcinia hanburyi	S-1	(154)
Haemodoraceae		
Haemodorus corymbosum	C-4, C-6	(388)
Hamamelidaceae		
Hamamelis virginiana	C-2	(161)
Liquidambar styraciflua	C-1	(161)
Hippocrateaceae		
Pristimera indica (*Hippocratea indica*)	C-4, C-6	(388)
Hydrocaryaceae		
Trapa natans	C-5	(173, 390)
Iridaceae		
Iris japonica	X	(171)
Julianiaceae		
Amphiteryngium adstringens	C-1	(164)
Juncaceae		
Juncus biflorus	C-1	(161)
Juncus repens	C-1	(161)
Labiatae		
Marrubium vulgare	S-1	(157)
Mentha piperita	S-7	(167)
Mentha spicata	C-1, C-2	(167)
Nepeta cataria	C-1	(161)
Plectranthus rugosus	C-5, S-2	(400)
Plectranthus trichocarpus	C-5, S-2	(400)
Salvia sp.	C-1	(164)
Salvia greggii	C-1	(164)
Thymus serpyllum	S-1	(157)
Teucrium canadense	C-1	(161)
Lauraceae		
Persea pubescens (*P. carolinensis*)	C-1	(161)
Leguminosae		
Abrus precatorius	C-5	(172)
Acacia auriculaeformis	S-4	(176)
Acacia spadicigera	C-1	(158, 165)
Adenanthera microsperma	C-1	(158)
Albizza julibrissin	C-1, C-2, S-7	(163, 167)
Albizza sassa	C-1	(161)
Andira surinamensis	S-4	(176)
Arachis hypogaea	C-1, C-2	(167)
Astragalus wootoni (*A. subcinereus*)	C-1, C-2	(164, 167)
Bauhinia japonica	S-10, S-13	(171)
Cassia alata	S-1	(154)
Crotalaria spectabilis	C-4	(139)
Delonix regia	S-4	(176)
Erythrina senegalensis	C-1	(161)
Euchresta japonica	H-1, S-4	(401)
Gleditschia triacanthos	C-1, C-5, S-2	(158, 165, 175, 402)
Indigofera hirsuta	S-4	(176)
Leucaena glauca	C-1	(158)
Lupinus texensis	C-1	(167)
Mimosa sepiaria	S-4	(176)
Parkinsonia aculeata	C-2	(164)
Peltophorum vogelianum	C-1	(376)
Piscidia mollis	C-1	(158, 165)
Prosopis glandulosa	C-1, C-2, S-12	(163, 164, 167)
Robinia nana	C-1	(161)
Robinia pseud-acacia	C-1	(161)
Sophora angustifolia	S-3	(171)
Sophora subprostrata	S-3	(403)
Tamarindus indica	C-1	(376)
Tephrosia virginiana	C-1	(161)
Trifolium pratense	S-1	(157)
Vigna sinensis	C-1	(165)
Wistaria chinensis	C-5	(390)
Liliaceae		
Aletris aurea	C-1	(161)
Allium halleri	C-2	(164, 167)
Allium sativum	C-5, S-2	(173, 404)
Aloe perryi	S-1	(154)
Asparagus chochinchinensis	C-5	(172)
Asparagus davuricus	C-1	(165)
Asparagus officinalis	S-1	(155)
Aspidistra elatior	S-3, S-10	(171)
Hosta sieboldiana	S-3, S-10, S-13	(171)
Ornithogalum umbellatum	E-1	(149)
Smilax spinosa	C-1	(376)
Trillium apetalon	C-5, S-3, S-10, S-13	(171)
Yucca arkansana	C-1, S-12	(148, 163, 164)
Yucca gloriosa	C-1	(376)
Yucca pallida	C-1	(376)
Loranthaceae		
Arceuthobium vaginatum	C-1	(148)
Phoradendron flavescens	C-1, C-2, S-12	(163, 164, 167)
Viscum album	M-1, S-2	(405–407)
Lythraceae		
Lagerstroemia speciosa	S-4	(176)
Lawsonia inermis	S-4	(176)
Magnoliaceae		
Kadsura japonica	C-5	(172)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Liriodendron tulipifera	S-1	(157)
Malpighiaceae		
Galphimia glauca	S-4	(176)
Malvaceae		
Hibiscus cannabinus	C-1	(165)
Gossypium sp.	C-2	(164)
Melastomataceae		
Tetrazygia bicolor	C-1	(166)
Meliaceae		
Carapa guianensis	S-4	(176)
Melia azedarach	C-2, S-12	(164)
Melia azedarach var. *japonica*	C-5, C-7, S-13	(171)
Swietenia macrophylla	S-4	(176)
Trichilia hirta	C-1	(166)
Menispermaceae		
Cissampelos pareira variegata	E-1	(143)
Fibraurea chloroleuca	S-4	(176)
Moraceae		
Ficus aurea	C-1	(166)
Ficus elastica	S-2	(396)
Maclura pomifera	C-1, C-2	(167, 376)
Myristicaceae		
Knema hookeriana (*Myristica hookeriana*)	S-4	(176)
Myrsinaceae		
Ardisia elliptica	S-4	(176)
Maesa ramentacea	S-4	(176)
Myrsine capitellata	S-4	(176)
Myrtaceae		
Eucalyptus triantha	C-1	(165)
Eugenia jambos	C-2	(166)
Eugenia javanica	S-4	(176)
Nepenthaceae		
Nepenthes arbomarginata	S-4	(176)
Nymphaeaceae		
Nuphar luteum	C-5	(175)
Oleaceae		
Chionanthus retusa	C-1, C-2	(376)
Forsythia sp.	C-1	(158, 165)
Fraxinus oregona	C-1	(376)
Palmae		
Acoelorrhaphe arborescens	C-2	(166)
Aeria attenuata	C-2	(166)
Butia nehrlingiana	C-1	(165)
Latania commersonii	C-1	(165)
Phoenix pusilla	C-1	(165)
Phoenix roebelenii (*P. humilis*)	C-1	(158)
Pseudophoenix vinifera	C-1	(158, 165)
Seaforthia elegans	C-1	(165)
Papaveraceae		
Chelidonium majus	C-3	(408)
Papaver orientale	C-5, S-4, S-8	(409)
Papaver rhoeas	C-5, S-4, S-8	(409)
Passifloraceae		
Passiflora incarnata	C-2	(167)
Phytolaccaceae		
Limeum aethiopicum	C-1	(165)
Pinaceae		
Callitris quadrivalvis	C-1, C-2	(158, 165, 166)
Juniperus chinensis	C-5, C-7, S-3, S-10, S-13	(171)
Juniperus communis	S-1	(155)
Juniperus lucayana	S-2	(159)
Juniperus sabina	S-2	(159)
Juniperus sabina var. *tamariscifolia*	S-2	(159)
Juniperus scopulorum	S-2	(159)
Juniperus silicicola	S-2	(159)
Juniperus virginiana	E-1, S-2	(141, 159)
Polygalaceae		
Polygala senega	S-1	(157)
Polygonaceae		
Rheum sp.	C-2	(164)
Rheum japonicum	C-5	(172)
Rheum officinale	S-1	(154)
Rheum palmatum	C-5	(172)
Rumex crispus	S-1	(154)
Rumex hymenosepalus	S-2, S-10	(146)
Polypodiaceae		
Dryopteris marginalis	S-1	(156)
Dryopteris filix-mas	S-1	(156)
Polyporaceae		
Fomes igniarius (*Polyporus igniarius*)	S-3	(410)
Primulaceae		
Anagallis sp.	C-5	(175)
Pyrolaceae		
Chimaphila maculata	C-1	(376)
Ranunculaceae		
Aconitum napellus	S-1	(157)
Actaea spicata	C-5	(411)
Anemone decapetala	C-1, C-2	(163, 164, 167)
Clematis chinensis	C-5	(172)
Coptis japonica	C-5	(173)
Delphinium sp.	C-2	(164)
Delphinium ajacis	C-2	(167)
Delphinium staphisagria	S-1	(156)
Helleborus odorus	S-6	(384)
Paeonia albiflora	C-5	(173)
Paeonia suffruticosa	C-5	(172, 173)
Ranunculus glaber (*R. ternatus* var. *glaber*)	C-5	(170, 173)
Rhamnaceae		
Rhamnus cathartica	S-1	(154)
Rhamnus japonica	C-5, C-7, S-4, C-13	(171)
Rhamnus purshiana	C-7	(167)
Rosaceae		
Agrimonia gryposepala	C-1	(158, 165)
Brayera anthelmintica	S-1	(156)
Crataegus cuneata	C-5	(172)
Duchesnea indica (*Fagaria indica*)	C-1	(158)
Geum aleppicum var. *strictum*	C-2	(166)
Gillenia stipulata	S-1	(157)
Padus racemosus	C-5	(175)
Physocarpus opulifolius	C-2	(166)

(*Continued on next page.*)

TABLE II.—(*Continued*)

Plant Name[b]	Neoplasm[c]	Ref.
Prunus americana	S-1	(157)
Spiraea ulmaria	S-1	(155)
Rubiaceae		
Cephaelis acuminata	S-1	(157)
Cinchona succirubra	C-5	(173)
Gardenia jasminoides	C-5	(173)
Genipa americana	S-4	(176)
Mussaenda glabra	S-4	(176)
Randia densiflora	S-4	(176)
Randia spinosa	S-4	(176)
Timonius wallichianus	S-4	(176)
Uncaria longifolia	S-4	(176)
Uncaria pteropoda	S-4	(176)
Uncaria roxburghiana	S-4	(176)
Rutaceae		
Atalantia citroides	C-1	(158, 165)
Citrus amblycarpa	C-2	(166)
Evodia roxburghiana	S-4	(176)
Evodia rutaecarpa	C-5, S-10, S-13	(171)
Phellodendron sp.	C-1	(165)
Phellodendron amurense	C-5, II-1, S-4	(173, 412)
Sapindaceae		
Alectryon subcinereum	C-1	(165)
Dodonaea viscosa	C-1	(165)
Nephelium longana	C-5	(172)
Sapindus senegalensis	C-2	(166)
Sapindus utilis	C-1	(158, 165)
Sapotaceae		
Achras sapota	S-4	(176)
Madhuca indica (*Bassia latifolia*)	C-1	(165)
Saxifragaceae		
Dichroa febrifuga	X	(413)
Hydrangea arborescens	S-1	(155)
Scrophulariaceae		
Veronica virginica	S-1	(154)
Simaroubaceae		
Castela nicholsoni texana	S-1	(156)
Simaruba amara	S-1	(156)
Solanaceae		
Cestrum parqui	C-1, C-2	(158, 166)
Datura sp.	C-1	(167)
Datura stramonium	C-1	(167)
Dunalia campanulata	C-2	(166)
Hyoscyamus niger	C-1, C-2	(167)
Lycium halimifolium (*L. vulgare*)	C-2	(166)
Solanum rostratum	C-1	(148, 161, 167)
Solanum torvum	S-4	(176)
Sterculiaceae		
Cola nitida (*C. acuminata*)	S-4	(176)
Thymelaeaceae		
Daphne mezereum	S-1	(157)
Umbelliferae		
Aletes acaulis	C-2	(163)
Angelica brevicaulis	C-5	(175)
Anthriscus neglecta (*A. vulgaris*)	L-1	(414)
Conium maculatum	C-1, C-2	(163, 164, 167)
Pimpinella saxifraga (*P. magna*)	S-1	(157)
Urticaceae		
Parietaria officinalis	S-1	(155)
Vacciniaceae		
Polycodium floridanum (*Vaccinium floridanum*)	C-2	(166)
Vaccinium bracteatum	C-5	(169)
Verbenaceae		
Verbena bipinnatifida	C-2	(167)
Vitaceae		
Vitis candicans	C-2, S-12	(164)
Zingiberaceae		
Zingiber officinale	C-5	(173)
Zygophyllaceae		
Peganum harmala	C-5	(175)

[a] All plants listed were considered active in the original reference against the tumor(s) indicated. Criteria for activity vary and undoubtedly many of the plants listed in this table exert only marginal, if any, real activity. For plant parts used and type extract evaluated, see original reference. [b] Names of plants are entered as found in the original reference except for corrections in spelling. Classification is according to the "Index Kewensis" and its supplements (375). Plant names in parentheses are preferred according to the "Index Kewensis." [c] Key for neoplasms (see reference for host): C-1, carcinoma, C_3H, mammary; C-2, carcinoma, RC, mammary; C-3, carcinoma, mammary, spontaneous; C-4, adenocarcinoma 755; C-5, carcinoma, Ehrlich; C-6, carcinoma, renal; C-7, carcinoma, Bashford, 63; E-1, 9 KB cell culture; L-1, leukemia, L-1210; L-2, leukemia, P-1534; S-1, sarcoma 37; S-2, sarcoma 180; S-3, sarcoma, Crocker; S-4, sarcoma, Yoshida; S-5, sarcoma, Black, SBL-1; S-6, fibrosarcoma; S-7, lymphosarcoma, unspecified; S-8, sarcoma, genital; S-9, sarcoma, DBA; S-10, sarcoma, Walker; S-11, sarcoma, Ehrlich; S-12, sarcoma, DBA; S-13, sarcoma, NF; H-1, hepatoma, ascitic; H-2, lymphoma No. 1; M-1, mastocytoma, P-815; CIT, chemically induced tumor; X, active against several neoplasms (see reference).

steroids and found that 25 were cytotoxic. All of the 25 cytotoxic steroids contained an α-β-unsaturated lactone ring, whereas this moiety was absent in the steroids devoid of cytotoxicity. An inspection of the heterogeneous group of structures making up some of the known antineoplastic or cytotoxic plant constituents suggests that factors other than the α-β-unsaturated lactone ring are involved with cytotoxicity (Fig. 1).

Several extensive surveys for antineoplastic activity in plant extracts have been published. A majority of the studies have been carried out with plants collected in the U. S. (154–167), Japan (168–173), the U.S.S.R. (174, 175), and most recently in Malaya (176). Correlation of data derived from these surveys is difficult because of inconsistencies in the processing of plant extracts, in the test procedures, in the tumor systems utilized, and in the validity of data interpretation.

Antimicrobial Activity.— References concerning the biological screening of plant extracts are encountered most frequently in connection with the determination of antimicrobial activity.

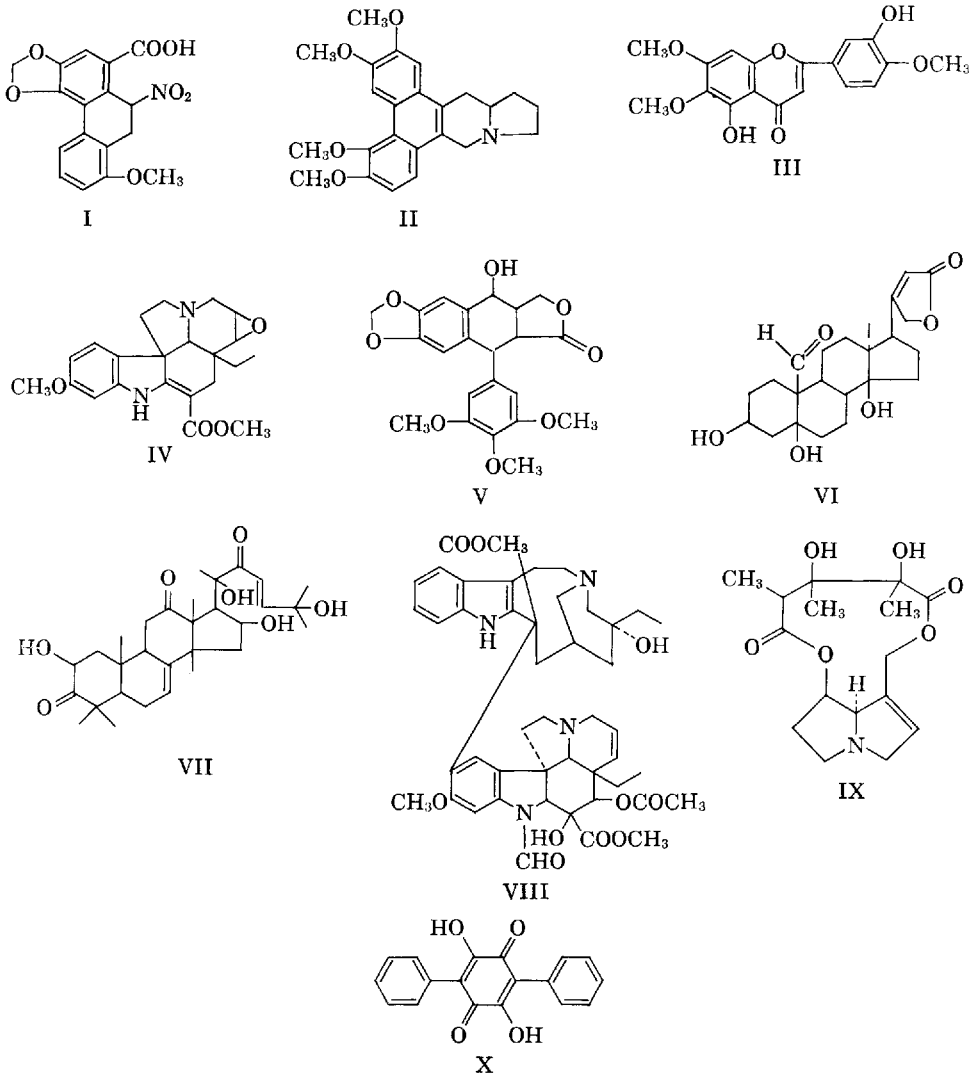
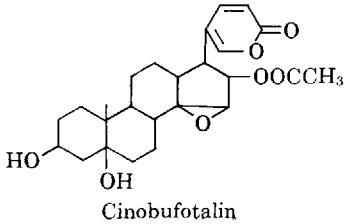
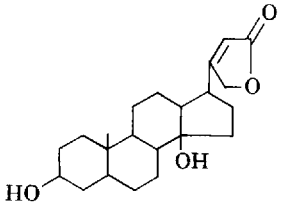


Fig. 1.—Structures of natural products known to have either antineoplastic or cytotoxic properties. Key: I, aristolochic acid (adenocarcinoma 755) (137); II, tylocrebrine (L-1210 leukemia) (381); III, eupatorin (9 KB cell culture) (144); IV, lochnerinine (9 KB cell culture) (341); V, podophyllotoxin (sarcoma 37, 9 KB cell culture) (141, 383); VI, strophanthidin (9 KB cell culture) (149); VII, elatericin A (sarcoma 180) (392); VIII, leurocristine (P-1534 leukemia) (133); IX, monocrotaline (adenocarcinoma 755) (139); X, polyporic acid (L-1210 leukemia) (379).

These evaluations are usually carried out by means of standard *in vitro* assays (disk, cup, cylinder, diffusion) utilizing a broad selection of pathogenic as well as nonpathogenic microbes. In most cases, a minimum of 1 Gram-positive and 1 Gram-negative organism, usually *Staphylo-*



Cinobufotalin



Digitoxigenin

Cytotoxic Unsaturated Lactones

coccus aureus and *Escherichia coli*, are included for initial screening. However, filamentous fungi, yeasts, and acid-fast organisms are often included. The studies of Lucas, Gottshall, Frisbey *et al.* are typical surveys of plants for antimicrobial activity (177–184). These investigators screened hundreds of extracts for inhibitory activity against *S. aureus*, *Salmonella typhimurium*, and *Mycobacterium tuberculosis*, and subsequent studies on the most promising plants by this group led to the isolation of many active principles (185–188). Other surveys for antimicrobial activity in plants native to the U.S. have been conducted using plants collected in Southern California (189, 190), Pennsylvania (119), Florida (191, 192), Indiana (193), Ohio and Michigan (194–197), Vermont (198), and Hawaii (199). The study on Hawaiian plants was based on a selection of those used in that area as home remedies. Tucakov (200), Kliewe and Hathmacher (201), Maruzzella and Henry (202), and Okazaki and Oshima (203, 204) studied the antimicrobial effects of a number of essential oils, a group of compounds in which antifungal activity is predominant. Burlage *et al.* investigated several plants reported to be toxic (205, 206). Studies involving fewer numbers of plants, plant parts, or extractives have also been reported (207–217).

Osborn (218) has surveyed more than 2300 different species of plants collected in England, while Australian plants have been extensively examined by Atkinson *et al.* (219–221). The Australian group also surveyed a number of higher fungi for antimicrobial activity (222). Similar surveys have been conducted on the flora of Nova Scotia (223–225), Brazil (226–228), Mexico (228–230), India (231–234), Japan (168, 235–243), China (244), the Philippines (245), as well as the U.S.S.R. (246–255) and other scattered areas (256–260). The antimicrobial substances from algae (261) and mosses have recently been reviewed (262), and their presence was demonstrated in a number of lichens (263, 264). Active substances in seaweed extracts have also been reported (265, 266).

Nickell (267), in a review on this area of investigation, has tabulated results of studies through 1959 in which vascular plants were demonstrated to elicit inhibitory action on microbes. Included in his tables are the plant name, family, class of organism(s) inhibited, type of extract utilized, and the plant part(s) tested. At about the same time, reviews on antibiotics from higher plants were published by Arnold (268), Virtanen (269), and Drobot'ko *et al.* (270) which were preceded by an earlier review by Freerksen (271). Antifungal substances from higher plants similarly were reviewed in 1961 by Sehgal (272).

Of particular interest is a study by Winter (273) in which he compared the antimicrobial properties of 2 groups of plants. One group included randomly collected native plants, whereas the second group comprised plants mentioned in a 300-year-old herbal which suggested that they were useful for the treatment of infections. Only 29.5% of plants from the randomly selected group exhibited antimicrobial activity, while 65% of plants selected because of their mention in the herbal were found to be active.

Karel and Roach (274) and Baron (275) have compiled lists of antibiotic substances isolated from higher plants as well as from microbes and other sources.

Because of the presence in crude plant extracts of substances which could exert antagonistic effects during the testing procedures, or which contain substances that stimulate the growth of the test organism and hence negate the effect of inhibitory substances present, new procedures should be developed. It would seem reasonable to utilize the new technique of thin-layer chromatography for these studies, since a resolution of components in the plant extracts could be expected, thus separating antagonistic and/or microbial stimulant constituents from those which might be active. Developed chromatograms then could be evaluated by means of bioautography, as suggested by either Kline and Golab (276) or Meyers and Smith (277).

Antiviral Activity.—Until the recent introduction of 5-iodo-2′-deoxyuridine (IDU), antiviral therapy in humans was nonexistent. Today, IDU is useful for the treatment of acute ophthalmic herpes simplex infections and is the only drug licensed by the U. S. Food and Drug Administration for the treatment of viral infection. Experimentally, IDU appears to be useful in the treatment of human vaccinia as well as smallpox infections (278). Aside from IDU, other synthetic compounds that appear interesting as antiviral agents are hydroxybenzylbenzimidazole, guanidine, and *N*-methyl-isatin-β-thiosemicarbazole. These compounds, as well as the problems associated with virus chemotherapy, have been reviewed recently by Kaufman (278).

Herrmann (279) has suggested that natural products should be preferable over synthetics as a source for new antiviral agents because naturally occurring mixtures, whether they be plant extracts or antibiotic filtrates, provide both a mixture of compounds, any one of which could be

active, and a diversity of chemical structures not easily obtained from chemical syntheses (279). However, little has been published on attempts to detect antiviral activity in plant extracts. Cochran and Lucas (280) investigated extracts from 46 members of the *Orchidaceae*, 24 species of other higher plants, and 6 species of mushrooms for their ability to protect mice against the polio virus. The orchids were selected because natives of New Guinea were said to use the flowers of certain of these species as drugs for the treatment of contagious diseases. Holobasidiomycetes were included in the study because of previous reports that extracts from selected members of this group, *i.e.*, *Boletus edulis* (281) and *Calvatia* species (280), were active tumor inhibitors. Most of the remaining higher plants tested were selected on the basis of suggested antimicrobial activities from the folklore. It was determined that 21 of 46 strains or species of orchids, 6 species of mushrooms, and *Hypericum perforatum*, *H. prolificum*, *Allium ampeloprasum*, *Kalmia latifolia*, *Maclura pomifera*, *Phellodendron amurense* *Medicago sativa*, and *Ribes hirtellum* exerted varying degrees of protection against poliomyelitis in the infected mice. Goulet *et al.* (282) studied the effects of extracts from 12 species of higher plants and basidiomycetes on 13 ECHO viruses, finding that many of the species were active, but only against specific viruses.

Taylor *et al.* (283), utilizing yolk-sac adapted vaccinia, influenza, eastern equine encephalomyelitis, and ornithosis viruses, evaluated extracts from 44 different species of plants. Extracts from 10 plants were effective in prolonging the survival time of vaccinia-infected chick embryos 20% or more. Four plants were effective against encephalitis, 6 against ornithosis, and 10 against influenza. Only 3 of the plants (*Allium halleri*, *Ambrosia apters*, unidentified native plant) produced 20% or greater survival against one or more viruses, thus substantiating the specificity pointed out by Goulet *et al.* (282). Twenty-two of the 44 species tested were active against one or more of the viruses.

More recently, a study in our laboratories involved the testing of extracts from 200 native plant species against vaccinia, polio type III, and pseudorabies viruses (119). It was found that 6 of the 200 species evaluated were found to have activity against one or more of the test viruses (*Apocynum* sp., *Asclepias incarnata*, *Artemisia* sp., *Aster divaricatus*, *Xanthium* sp., *Sium suave*). Two hundred types of crude drug plants were studied by Goro *et al.* (168) in a broad screening program in which extracts were evaluated for antitumor, antimicrobial, and antiviral activity. Influenza virus was used in this study.

With only a limited number of studies to evaluate the screening of plant extracts for antiviral activity, it appears that there is justification for more extensive studies in this area. As with the distribution of antineoplastic activity in the plant kingdom, antiviral inhibitory effects have been observed in diverse taxa. However, from preliminary observations, it can be noted that at least 13 genera of higher plants distributed in 10 families have been shown to elicit antiviral activity (*Artemisia*, *Ambrosia*, *Aster*, *Xanthium*, *Asclepias*, *Apocynum*, *Kalmia*, *Hypericum*, *Phellodendron*, *Medicago*, *Ribes*, *Maclura*, *Allium*). An inspection of the distribution of antineoplastic activity in the plant kingdom (Table II) discloses that only 5 of the above 13 genera have not given rise to at least 1 active antineoplastic species (*Ambrosia*, *Kalmia*, *Hypericum*, *Medicago*, *Ribes*). This observation, although admittedly based on insignificant numbers, especially those representing antiviral activities, suggests a relationship between antiviral and antineoplastic-cytotoxic activity. Only time and continued research will provide enough evidence to confirm this correlation.

Antimalarial Activity.—Whereas a number of isolated examples can be found in the folklore concerning plants alleged useful for the treatment of malarial infections, "fevers," or for use as "antiperiodics," little experimental evidence has been put forth to substantiate these claims. The most extensive survey of plants intended to detect substances having potential antimalarial activity was that reported in 1947 by Spencer *et al.* (284). They studied the effects of several solvent extracts, from more than 600 species distributed within 126 plant families, on chicks and ducklings infected with *Plasmodium gallinaceum*, *P. cathemerium*, and *P. lophurae*. Plants judged most active as a result of this evaluation were *Hymenocallis caribaea* and *Cooperia pedunculata* (*Amaryllidaceae*); *Castela tortuosa*, *Simaba cedron*, and *Simaruba amara* (*Simaroubaceae*); *Cornus florida* (*Cornaceae*); *Dichroa febrifuga* (*Saxifragaceae*); *Gentiana* sp. (*Gentianaceae*); *Croton* sp. (*Euphorbiaceae*); *Cissampelos pareira* (*Menispermaceae*); *Aristolochia* sp. (*Aristolochiaceae*); *Datisca glomerata* (*Datiscaceae*); and *Eryngium foetidum* (*Umbelliferae*). Eleven of these 13 species have been shown to elicit varying degrees of antineoplastic and/or cytotoxic activity (Table II). Only *Simaba cedron* and *Eryngium foetidum*, of the 13 plants indicated, have not been reported to elicit antineoplastic activity. *Castela tortuosa* also has not been reported, but the related *C.*

nicholsoni is active against sarcoma 37. Kupchan *et al.* have shown that cissampareine is the tumor inhibitor of *Cissampelos pareira* (143) and that aristolochic acid is the antitumor agent in *Aristolochia indica* (137). *Datisca hirta* is a hybrid of *Rhus glabra* and *Rhus typhina*, and while it has not been evaluated for antineoplastic activity *per se*, both of the hybridizing species have been shown to be active against carcinomas (Table II).

Malaria is still a disease of importance in many parts of the world today, and it is necessary to insure the availability of safe and effective drugs for the treatment of this condition. Recent reports indicate that the malarial parasite may well be developing a degree of resistance to the synthetic antimalarial drugs which may return the quinine alkaloids to their position of former importance as therapeutic agents. However, at least at the present time, a shortage of these alkaloids exists (285). In any event, future needs may require a continuation of surveys such as the one initiated by Spencer *et al.* (284), and to a lesser degree by Carlson *et al.* (195), as well as a study of the leads that have evolved from these screening reports.

Insecticide Activity.—Rotenone, nicotine, and the pyrethrins are important naturally derived insecticidal materials. Because of the economic value of insecticides in general, and particularly with regard to these 3 substances, a great deal of interest has been generated over the years to find new naturally occurring as well as synthetic substitutes. Included as part of this interest has been the initiation of several plant collection and screening programs, in addition to subsequent phytochemical investigations on the most promising plants discovered in this manner. McIndoo (286) has compiled all available literature reports on plants containing potential insecticidal substances up to 1941, and Jacobson (287) has extended these compilations through 1953. Since 1953, reviews have appeared by Sobotka in 1956 (288), by Ts'e in 1958 (289), by Hsiung (290) and Liu (291) in 1959, and most recently by Lipa in 1962 (292).

Hypoglycemic Activity.—Recent evidence indicates that orally effective hypoglycemic agents can be obtained from plant sources. Svoboda *et al.* (293) have demonstrated that 6 alkaloids from *Catharanthus roseus* (leurosine sulfate, lochnerine, vindoline, vindolinine dihydrochloride, catharanthine hydrochloride, and tetrahydroalstonine) are at least equal to tolbutamide in hypoglycemic action when administered orally to rats. It is interesting to note that the crude extracts from which the alkaloids were derived failed to elicit a hypoglycemic response (293). Also, the predominant folkloric use for *C. roseus* has been as an oral substitute for insulin; however, several studies have failed to confirm this action. These reports have been summarized by Farnsworth (294). Examples such as this, where biologically active substances can occur in a crude plant extract which apparently is devoid of activity, serve to point out the frustrations encountered in natural product biological evaluations.

Garcia has investigated a number of Philippine plants for which oral hypoglycemic activity is alleged through reports of their use in native medicine for this purpose (295–300). An extract from some of these plants, referred to as "plantisul," has been used with some degree of success as an insulin substitute according to reports in the literature (296, 298–300). *Tecoma* species of plants have been used orally by the natives of Mexico as antidiabetic remedies (301, 302), and a recent report by Hammouda *et al.* (303) claims that tecomine and tecostanine, 2 alkaloids from *Tecoma stans*, are potent hypoglycemic agents. Unfortunately, this report compares the activity of the 2 alkaloids after i.v. administration with tolbutamide administered orally. Similarly, vincamine, the major alkaloid of *Vinca minor*, has been reported to lower blood sugar when administered i.v. (304, 305). A number of common edible plants have been screened for hypoglycemic activity because of previous reports that each had been used for this effect (306, 307), and Mukerji (308) has reviewed the indigenous plants of India alleged to exert hypoglycemic effects. Other plants having potential oral hypoglycemic activity have been pointed out by Aliev and Rachimova (309).

The literature on plants claiming antidiabetic properties is voluminous. However, the validity of data interpretations in many such reports must be considered equivocal. Surely there is a need to evaluate plants which have been widely reported of value when used orally for the treatment of diabetes.

Although many compounds that show promise as hypoglycemic agents (leurosine, lochnerine, vindoline, vindolinine, catharanthine, tetrahydroalstonine, tecomine, tecostanine, vincamine, and others) have been isolated from plants, further studies could well prove them to be undesirable due to toxicity or side effects. In any event, these compounds represent new structures which could serve as models in the synthesis of active and safe hypoglycemic agents.

Cardiotonic Activity.—Hoch (310) has surveyed the literature up to 1961 concerning the

occurrence of cardiac glycosides and genins. His listings, which include only compounds that have been isolated in a pure state, indicate that these phytoconstituents are distributed in 39 genera of 14 plant familes. The importance of the cardiotonic glycosides in medicine has led to several extensive surveys of plants for their presence. Only a few surveys, however, have utilized biological testing exclusively to detect typical cardiotonic activity. Thorp and Watson (311), utilizing anesthetized guinea pigs, evaluated extracts from all of the available apocynaceous and asclepiadaceous plants of Australia. Evidence for the presence of cardiotonic activity was considered positive, following intravenous administration of a hydroalcoholic plant extract, when the heart slowed and contractions became more forceful. Typical cardiotonic activity was observed with extracts of *Carissa*, *Cerbera*, *Thevetia* (*Apocynaceae*), and *Gomphocarpus* (*Asclepiadaceae*) species. A total of 26 species was evaluated. More recently, Patel and Rowson (312) evaluated 33 species of Nigerian plants alleged to be useful as drugs. They utilized the isolated toad heart for detection of cardiotonic activity and found that species of *Allamanda*, *Callichilia*, *Hedranthera* (*Apocynaceae*); *Calotropis*, *Marsdenia*, *Pergularia* (*Asclepiadaceae*); *Vernonia* (*Compositae*); *Urginea* (*Liliaceae*); *Antiaris* (*Moraceae*); *Nauclea* (*Rubiaceae*); *Schwenkia* (*Solanaceae*), and *Mansonia* (*Sterculiaceae*) were active. However, subsequent chemical tests for the typical cardenolide nucleus were negative in all of the apocynaceous plants tested, as well as for the species of *Nauclea* (*Rubiaceae*) and *Schwenkia* (*Solanaceae*). Krider *et al.* (313) evaluated a number of plant extracts first chemically for the presence of typical cardenolides and then, for confirmation, tested all positive results by biological assay using the frog heart. A number of additional surveys of plants for cardiotonic activity have been reported; however, these involve only chemical tests which were designed to detect either the presence of 2-deoxy sugars or the unsaturated lactone, moieties considered characteristic for cardio-active substances. These surveys will be considered under *Phytochemical Screening Approaches*.

Androgenic, Estrogenic, and Related Activities.—The majority of literature reports involving a search for plant androgens, estrogens, and related materials have involved studies on specific folkloric remedies, those which have been alleged useful either as oral contraceptives, as ecbolics, or as emmenagogues. Undoubtedly, the majority of the emmenagogue and ecbolic plants derive their activity from an irritant mechanism such as that induced by the well-known plant purgatives and vesicant oils. Casey (314) has tabulated some 298 Indian plants used for these purposes in native medicine, and therefore the title "298 Alleged Anti-Fertility Plants of India" is misleading. Saha *et al.* (315) have also contributed a list of 277 Indian plants used for these purposes, many of which duplicate Casey's tabulations. The work by Saha and co-workers (315, 316) also included some experimental studies on a few of the most promising folkloric ecbolic plants found on their list. They evaluated extracts from these plants *in vitro* on excised guinea pig uteri and found several to have marked uterine stimulant activity (315, 316). A similar study, comprising 18 species of Indian ecbolic plants, showed 10 to exert varying degrees of stimulant activity on uterine tissue *in vitro* (317).

A search for effective oral contraceptives has been accelerated due to the alarm generated by the rapid increase in world population. Most studies have involved the synthesis of anovulatory compounds, several of which have been made available and apparently are widely used. Administration of antispermatogenic compounds to the male does not appear to be a popular approach to the problem of population control; however, there is some evidence that this may be effective as a means to prevent conception (318).

Jackson (319), in a review on antifertility substances, has pointed out a few alleged antifertility plants, as have Henshaw (320) and Jochle (321) in similar general reviews. DeLaszlo and Henshaw (322), on the other hand, have compiled a list of 60 antifertility plants used by primitive people.

Lithospermum ruderale, first reported by Train *et al.* (61) to be used by squaws of the Nevada Indian tribes as an oral contraceptive, has received considerable attention in laboratory studies. Several phytochemical investigations (323–325) have failed to yield the active antifertility compound generally acknowledged to be present in this plant (319, 325, 326). Gassner *et al.* (325), however, point out that certain extraction conditions used when processing this plant can alter its biological activity. Similar antifertility effects have been noted for *L. officinale* (327, 328), but attempts to isolate the active component(s) have not, as yet, been successful (327, 329). Other plants alleged to have antifertility effects in humans, and for which some

validating evidence in animals has been reported, are *Mallotus philippinensis* (330, 331), *Polygonum hydropiper* (332), *Psoralea corylifera* (333), *Capsella bursa pastoris* (334), *Sanguisorba officinalis* (335), *Withania somnifera* (336), *Punicum granatum* (331), and *Jatropha curcas* (337), as well as others (338).

Although no antifertility screening programs involving large numbers of plants have been reported in the literature, scattered laboratory evidence exists suggesting this area as a fertile one for development. The discovery of an orally effective, nonsynthetic antifertility agent, occurring free in nature could possibily be an acceptable answer to the question of population control.

More specifically, several examples of estrogenic and androgenic activity in plant extracts have been reported. Androgenic activity has been associated with cinchona bark (339) and with *Rhynchosia pyramidalis* (340), but these reports lack confirmation. In the latter instance, we have found no evidence of activity in *R. pyramidalis* using the standard androgen assay with castrate rats, following either oral or i.p. administration of extracts (341). No example of a steroid estrogen isolated from plants thus far has been reported, the majority of the estrogenic activities being attributed to isoflavones or related structures. These compounds, (isoflavones), probably because of their structural relationship to stilbestrol, are generally acknowledged to be weakly estrogenic, *i.e.*, genistin, genistein, biochanin A, prunetin, coumestrol (342), phloretin (343), and the plant stilbenes (342). This area has been reviewed extensively by Biggers (342), Bradbury and White (344), and by Cheng *et al.* (345).

Although estriol (346) and estrone (347) have been reported isolated in crystalline form from female willow catkins and palm kernel residues, respectively, and represent the only apparently clear cut examples for the isolation of steroid estrogens from plants, these reports have recently been challenged by Jacobsohn *et al.* (348). Jacobsohn and co-workers were unsuccessful in their attempts to duplicate the isolation of estrone from palm kernels, even though the methods used must be considered highly sensitive and usually effective for this type of work. There is little doubt that Butenandt and Jacobi (347) did, in fact, isolate authentic estrone (18 mg. from 50 Kg.) of palm kernel extracts. However, the extract from which the isolated estrone was derived had been previously prepared and supplied by the Schering-Kahlbaum, AG, Berlin (348), a fact that does not preclude the possibility of estrone contamination of the palm kernel residues during processing (348). Skarzynski's (346) isolation of estriol from willow catkins, on the other hand, was not so clearly established. He isolated 7.5 mg. of crystals from 65 Kg. of starting material and found them identical with estriol on the basis of comparision of microscopic appearance, solubility in several solvents, ultraviolet spectrum, melting point of acetyl derivatives (126°), and, in addition, the mixed melting point of the isolate and authentic estriol was depressed only 1°. However, the major difference was that the estrogenic activity of the isolate was only one-fourth that of estriol (346–348). From these data, and on the basis of subsequent negative efforts by Jacobsohn *et al.* (347), it appears that the presence of estrone and estriol in higher plants, as well as steroid estrogens in general, remains to be demonstrated unequivocally.

Conversely, a potent estrogenic activity from *Butea superba*, assaying at about 900,000 mouse units/Kg. has been reported (349). Physical data presented for the compound responsible for this activity are insufficient for its characterization; however, from all available data, it appears to lack the qualifications of a steroid (349). Much of the controversy that has developed over the relative estrogenic potency of plant extracts resides, no doubt, in the selection of an appropriate biological assay. Biggers (342) has critically reviewed methods that have been applied to the detection of estrogenic activity in plant extracts, while Cheng *et al.* (345) have specifically surveyed the literature on the estrogenic activity of naturally occurring isoflavones. Also, a broad review on plant estrogens, encompassing all areas of the subject, has been prepared by Bradbury and White (344). In the latter publication, a list of 55 species of estrogenic plants, representing 49 genera and 28 families, has been tabulated.

Further exploitation of the plant kingdom for this interesting and potentially useful group of phytoconstituents, through a broad biological screening program, could very well be rewarding.

General Pharmacological Screening.—Malone and Robichaud have remarked on general screening as follows:

"The basic premise of pharmacologic screening is not to allow true biologic activity to go undetected even though the activity may be new, unexpected and unique. The initial screening procedure must unequivocally establish this activity as well as its probable nature in order to indicate the course of further, more specific pharmacologic evaluation." (98).

These workers have proposed a "Hippocratic Screen," utilizing normal unanesthetized rats, for the detection of biological activities in crude plant extracts. Using a modification of this procedure, which we refer to as the "Mouse Behavior Screen," extracts from 200 native plants have been evaluated for biological activity with the results being categorized as follows: (*a*) CNS depressant (weak, toxic, etc.), (*b*) CNS stimulant (weak, toxic, etc.), (*c*) autonomic (weak, toxic, etc.), (*d*) mixed CNS stimulant and CNS depressant, (*e*) other types of activity (119). It is our intent to continue this type of evaluation and attempt to correlate characteristic biological effects of plants with their respective taxonomic position and thus develop an area of so-called "Biotaxonomy."

Similar evaluations of plant extracts have been made on 163 West Indian medicinal plants (95–97). In addition, these workers have included *in vitro* or *in vivo* studies of 55 extracts (95) on guinea pig ileum, rat uterus, rabbit duodenum, isolated rabbit heart, and also on dog respiration and blood pressure, the rat stomach fundus (5-HT activity), and the rat diaphragm phrenic nerve preparation. The remaining 108 plants were evaluated in a similar manner, although not so completely (96, 97).

Train *et al.* (61), using plants alleged to be useful in Nevada Indian folk medicine, evaluated some 100 species of plants for their effect on rabbit ileum and for effects on dog blood pressure and respiration.

If an investigator in search of biologically active plant materials has no prior ethnobotanic or biosystematic knowledge of his investigational material, studies such as these are invaluable to supply a justification for continued and detailed phytochemical analysis.

Miscellaneous Phytopharmacological Surveys.—In addition to the more extensive screening programs described above, a number of additional efforts have been carried out, usually on fewer specimens of plants, involving the search for biologically active compounds. Borchers and Ackerson (350), in a search for trypsin inhibitors, examined the seeds of 38 species of plants. Similar substances which inhibited the growth of rats and chicks had been reported in legume seeds (350). The inhibitor was found in all *Leguminosae* seed samples analyzed, but it was absent from 8 seed samples representing other plant families.

In a search for new human plasma cholinesterase inhibitors, Orgell has studied extracts from a large number of plant species (351–353), in addition to several alkaloids (354) and other purified natural substances (354). Inhibitors have been found in extracts from plants in 11 families, and among the inhibitory compounds are riboflavin, bishydroxycoumarin, aloin, naringenin, and some 26 different alkaloids, the majority of which are indoles (354). Orgell has suggested that cholinesterase inhibition techniques may be useful for the detection of certain alkaloids and glycosides in plant extracts or crude drugs (354).

Srivastava *et al.* (355) screened 11 species of Indian plants for fibrinolytic and anticoagulant activity using rather simple *in vitro* methods. Recently, a simple test tube arrangement designed for the rapid evaluation of compounds for fibrinolytic activity has been described that could be adapted for use in the rapid screening of large numbers of plant samples (356).

Several extensive surveys of plant material have been reported in the literature with the intent to discover new hemagglutinins (phytohemagglutinins) that might serve as useful blood-typing reagents (357–370). The most recent of these surveys included the evaluation of seed extracts from 311 species of plants, representing 42 families, against 24 different hemagglutinating antigens (368). Specific positive reactions were given by 17 species in 12 families, exclusive of the *Leguminosae*, whereas 32 of 45 *Leguminosae* species gave similar specific results. A number of additional plants from both groups gave nonspecific hemagglutination reactions. Schertz and co-workers have drawn attention to the need for a continuation of similar screening programs and for the subsequent investigation of the more promising plants which give specific reactions (368).

Although remarks in this section of the review have been directed principally to problems and approaches involved in the detection of biologically active substances in higher plants, other fertile natural product areas remain essentially untapped. The biological activities of algae (371, 372), plankton (371), and marine biotoxins (373, 374) point to this fact, and the prospect of discovering new antibiotic producing organisms in the sea should stimulate the fins and gills in many a natural product investigator.

PHYTOCHEMICAL SCREENING APPROACHES

Ultimately, the goal in surveying plants for biologically active or medicinally useful compounds should be to isolate the one or more constituents responsible for a particular activity.

Hence, with the selection of a specific plant for phytochemical investigation, either on the basis of one or more approaches set forth under *Phytopharmacologic Approaches*, or through some other avenue, phytochemical screening techniques can be a valuable aid.

Certain investigators feel that an initial selection of investigational plants should be made, not on evidence that extracts elicit a particular and interesting biological activity, but rather on the basis that certain chemicals are present in the plant, relatives of which can usually be associated with biological activity. Thus, some investigators will select initially only alkaloid-containing plants for study on the premises that (*a*) alkaloids normally exert some type of pharmacologic activity, usually on the central nervous system, but not always so; (*b*) the greatest majority of natural products used in medicine today are alkaloidal in nature; (*c*) tests for the presence of these compounds in plants are simple, can be conducted rapidly, and are reasonably reliable, and (*d*) because of their chemical nature, alkaloids are more easily manipulated making extraction and isolation less of a problem. In addition, economics, as well as other factors associated with biological testing, often force the investigator to pursue a phytochemical approach. However, should a phytochemical group other than alkaloids be selected for investigation, say the flavonols, the diversity of expected biological activities can be enormous. Willaman has surveyed the literature and has found that at least 137 natural flavonoids are known, occurring in some 62 families, 153 genera, and 277 species of plants (415). Also, some 33 different pharmacologic or biological activities have been reported for one or more of 30 flavonoids (415). More recently, Horhammer and Wagner have reviewed the same area, and these numbers are therefore to be increased (416, 417). Also, Orzechowski has considered the role of flavonoids as therapeutic agents (418). Along similar lines, the coumarins have been reported to exert some 31 different biological effects, and according to Soine, their full range of pharmacologic activities is not appreciated by most investigators (419). Other examples pointing out the complexity of expected biological effects for any one category of phytoconstituents could, of course, be made. In any event, publications representing the phytochemical screening approach far outweigh those following phytopharmacologic avenues, not only in numbers of reports, but in representation of total plants examined.

Since the number of chemical categories of plant constituents is great, and each is capable of eliciting biological activity, no attempt will be made in this review to be all inclusive. This section of the review will be restricted to some general considerations of phytochemical screening methodology, followed by discussions of those categories of phytoconstituents which have been represented in major published surveys of screening programs. These will include: alkaloids, glycosides as a general class (heterosides), saponins (steroid and triterpenoid), sterols, cardiac glycosides, cyanogenetic glycosides, *iso*thiocyanate glycosides, anthraquinones, flavonoids and related compounds, tannins, and coumarins and related compounds. Surveys which have been conducted for each of these categories will be discussed along with the general methodology involved. The examples to be cited are intended to be representative of each class and are not meant to include all available published data.

General Considerations

A method for use in phytochemical screening should be (*a*) simple, (*b*) rapid, (*c*) designed for a minimum of equipment, (*d*) reasonably selective for the class of compounds under study, (*e*) quantitative in so far as having a knowledge of the lower limit of detection is concerned, and if possible, (*f*) should give additional information as to the presence or absence of specific members of the group being evaluated. Most published procedures adhere to criteria (*a*) through (*d*), but few are designed to provide the information included in (*e*) and (*f*). In fact, certain procedures cannot be duplicated because of insufficient details included in some reports. For example, Arthur and Cheung (420), in a phytochemical survey of Hong Kong plants, screened 332 species for alkaloids. They equated the precipitates observed following the addition of standard alkaloid precipitating reagents to results obtained by adding the same reagents to standard solutions of 1:100, 1:500, 1:2500, and 1:10,000 quinine sulfate. It is implied that water was the solvent. However, the solubility of quinine sulfate is stated to be 1 Gm. in 810 ml. of water (421). Along similar lines, Wall *et al.* (422) have used the cyanidin test for the detection of the γ-benzopyrone nucleus as indicative of the presence of flavonoids. They compare a test result color with a similar color produced by a 0.1% solution of rutin and equate it as a (+) reaction. Their extraction solvent is 95% ethanol (but fresh plant material was often extracted which would decrease this percentage considerably), and rutin is stated to be only slightly soluble in ethanol and soluble about 1

Gm. in 8 L. of water (421). We find that the maximum solubility of rutin at room temperature is about 0.02% for both 80 and 95% ethanol.

Webb, using a field method, estimated alkaloid precipitates with reagents on a + to ++++ basis but used no reference for comparison (423, 424). He also states, ". . . On the other hand, while the method may yield a percentage of 'false positives,' it has never failed to detect species with alkaloids" (424). If the initial field test did indeed fail to detect alkaloids, perhaps because of a low concentration in the plant, how could it be determined that the test was infallible when only field test positive species were collected for more specific laboratory examination?

One of the most important and fundamental considerations in designing a phytochemical screening procedure is the selection of a proper extraction solvent. It is often difficult to follow general or expected solubility rules for a given class of phytoconstituents since there are often substances of unknown character present in crude plant extracts that affect solubility. For example, Woo (425) has reported the effect of saponin in plant extracts on the solubility of certain normally insoluble compounds using selected solvents. Apparently saponin acts as a wetting agent to enhance the formation of micelles; thus, an increase in solubility of certain constituents is effected. This phenomenon has been noted through the use of synthetic detergents to enhance the solubility, and thus extractability, of alkaloids from Cinchona (426). Since saponins, or other similar surface-active agents, do not occur universally in plants, prediction of general solubilities for a class of phytoconstituents precipitates a major problem. In our laboratory *n*-hexane-soluble extractives from *Catharanthus lanceus* were found to be rich in alkaloids. Subsequent isolation of individual akaloids from the crude mixture proved them to be totally insoluble in *n*-hexane. Presumably the alkaloids occur in the plant, at least in this instance, dissolved in some lipid material, the latter being soluble in *n*-hexane.

No solution is offered for these problems involving solubility except to say that extract residues should always be examined with a variety of solvents to determine whether abnormal solubility phenomena have occurred.

Even though a great many problems are presented by the diverse methodology utilized by investigators in phytochemical screening, much useful information can be derived from published studies. Positive test results are usually clear cut and only the possibility of false-positive results need be further explored. Negative results, on the other hand, must be carefully weighed in terms of being due to a real absence of the test material in the sample being evaluated, or to the methodology employed.

Alkaloid Screening

Prior to a consideration of screening plant material for alkaloids, it would seem in order to define the term "alkaloid" as used in this review; however, the nature of the word itself precludes anything more than a vague definition. Anyone familiar with alkaloids surely has a knowledge of their character, but seldom can one give an acceptable definition. Most authorities agree that chemical, botanical, and pharmacologic implications must be reflected in an acceptable definition. Hegnauer's (427) suggestion that:

> "Alkaloids are more or less toxic substances which act primarily on the central nervous system, have a basic character, contain heterocyclic nitrogen, and are synthesized in plants from amino acids or their immediate derivatives. In most cases they are of limited distribution in the plant kingdom."

seems as acceptable as any. For purposes of this discussion we will utilize Hegnauer's concept except, of course, we cannot be concerned with the site or mechanism of synthesis. Thus, compounds such as aliphatic nitrogenous bases (ephedrine), amides (colchicine), and the amino acids (thiamine) themselves will not be considered as alkaloids.

Estimates for the distribution of alkaloids in vascular plants have been placed as high as 15–20% (427), although this figure appears somewhat high with respect to data derived from several extensive phytochemical screening programs. Wall *et al.* (423, 428–435) have screened more than 4000 species of plants and report a distribution of about 10% alkaloids. Webb (424) in his experience with some 1700 species indicates alkaloid occurrence to be about 14%, whereas the Smith Kline & French survey found that about 10% of 25,000 species screened were positive for alkaloids (436). Since a few of these undoubtedly will be determined through future studies to be false-positive alkaloid containing species, 9–10% seems to be the more logical estimate representing alkaloid-yielding plant species.

Alkaloids are widely distributed in the plant kingdom, although certain groups have been shown to be characteristically devoid of them. Excellent essays on this subject have been published by Willaman and Schubert (93, 94) and by Webb (424). The handbook of alkaloid-bearing plants by Willaman and Schubert is also

useful to establish this relationship among plant taxa (437).

Since alkaloids usually occur in plants as their water-soluble salts, some workers believe that extraction with acidulated water can result in a crude extract which can be tested directly with one or more standard alkaloid precipitating reagents. Other workers feel that the presence in such an extract of materials that are capable of giving false-positive alkaloid tests necessitate a purification procedure before valid results can be obtained. This is usually accomplished by the addition of base and subsequent extraction with a water-immiscible organic solvent. The organic extract can then be tested by application to filter paper, drying, and dipping or spraying with an alkaloid detecting reagent that gives a chromogenic response with alkaloids. If the latter method is not preferred, the organic solution can be re-extracted with dilute acid and the usual alkaloid precipitating reagents added to separate portions of this acid extract.

Another method of removing impurities that are capable of giving false-positive tests (*i.e.*, proteins) from an initial aqueous acidic extract is to "salt out" these materials by the addition of powdered sodium chloride. An additional procedure for alkaloid detection could be based on the addition of alkali directly to the powdered plant sample, followed by extraction with an appropriate organic solvent. This extract could then be purified by partition as described above, or be tested directly.

With respect to these general methods, certain anomalies have been reported in the literature which should be pointed out. There is no implication that these examples are frequently encountered in alkaloid screening; however, one should be aware that they do exist. Certain plants (*i.e.*, *Saussurea lappa*) are known to contain labile nonbasic constituents and may yield nitrogenous materials (pseudoalkaloids) on extraction with ammoniacal solvents (438), while others contain alkaloids that are susceptible to modification by acidic reagents (438). That proteins, which may be present in aqueous or acidic aqueous plant extracts, can precipitate on the addition of heavy metal alkaloid precipitating reagents and thus yield false-positive tests, is well established (438–444). Such proteins can be removed by treatment of the extract with sodium chloride prior to the use of the heavy metal reagent, a procedure which usually salts out the protein (438). However, alkaloids such as alstonine may be quantitatively precipitated as hydrochloride under these conditions (438). In the treatment of a crude plant extract to remove impurities by the acid–base–organic solvent –acid procedure, it is quite possible that plants containing water-soluble alkaloid bases will go undetected. Quaternary bases, amine oxides, betaines, and choline would fall into this category (438).

Variability of results in alkaloid testing of plant material can be induced by a number of factors such as age, climate, habitat, plant part tested, season, time of harvest, chemical races of plants, sensitivity of alkaloid type to reagents, etc. A few examples regarding these factors should serve to point out their importance. *Geijera salicifolia* was found by Webb to give consistently better alkaloid tests as the broad leaf form, than the narrow leaf form, even when the 2 were growing side by side in the field (424). In certain groups of plants (*i.e.*, *Compositae*), alkaloids often are found only in or near the flower tops (438), and in the *Apocynaceae*, alkaloids generally tend to concentrate in the root or bark, often to the exclusion of other parts of the plant (438); thus, the proper selection of plant parts for testing is quite important. To obtain equivalent results, quantitation of precipitates obtained with alkaloid reagents is not always possible, especially when comparing different genera or families. This is exemplified through knowledge that *Galbulimima baccata* (*Himantandraceae*) was found to be rated a ++++ in field tests and subsequent analysis resulted in a yield of 0.01–0.05% of 4 alkaloids. A ++++ rating for *Daphnandra aromatica* (*Monimiaceae*) was determined in the field and subsequent analysis in the laboratory yielded 6+% of crude alkaloids (424). *Antireha putaminosa* (*Rubiaceae*) loses 50% of its alkaloid after 2 months' storage, and high alkaloid decomposition rates have also been noted for *A. tennuifolia*, *Randia racemosa*, and *Gardenia vilhelmii*, 3 additional rubiaceous plants (424). Silica gel drying of *Antirhea tennuifolia* for 1 month resulted in material that gave a ++++ alkaloid test, whereas this same plant dried in the shade for 1 month gave a negative alkaloid test (424). *Acronychia baueri*, on the other hand, gave strong alkaloid positive tests when 124-year-old herbarium specimens were evaluated (424, 445). Along similar lines, Raffauf and Morris have reported that a plant sample identified as *Nicotiana attenuata* (*Solanaceae*), and estimated to be some 1300 years old, gave positive alkaloid tests (446). *Duboisia myoporoides* yielded 3% of hyoscyamine when harvested in October, but when harvested in April of the same year, 3% hyoscine was isolated (424). Examples of alkaloid decomposition as a result of milling

TABLE III.—SOME USEFUL ALKALOID PRECIPITATING REAGENTS

Name	Comp.	Ref.
Bouchardat	Iodine–potassium iodide	(493, 504)
Dragendorff	Bismuth potassium iodide	(447, 496, 504, 632)
Ecolle	Silicotungstic acid	(447, 732)
Gold chloride	Chlorauric acid	(493)
Hager	Picric acid	(448)
Kraut	Iodine–zinc chloriodide	(447)
Marme	Cadmium potassium iodide	(447)
Mayer	Potassium mercuric iodide	(447, 448, 452, 453)
Platinum chloride	Chloroplatinic acid	(448, 493, 781)
Scheibler	Phosphotungstic acid	(448)
Sonnenschein	Ammonium phosphomolybdate	(496)
Valser	Potassium mercuric iodide	(452)
Wagner	Iodine–potassium iodide	(496)
	Bismuth antimony iodide	(782)
	Bromauric acid	(783)
	Bromoplatinic acid	(497)
	Bromothallic acid	(784)
	Picrolonic acid	(785)
	Sodium tetraphenylboron	(786)
	Trinitroresorcinol	(787)

dried plant material have also been cited (424). These examples should suffice to point out just a few of the problems encountered by the natural product investigator who is interested in the detection and isolation of biologically active alkaloids.

Alkaloid Detecting Reagents

For detecting alkaloids in phytochemical screening, two types of reagents are available, *i.e.*, alkaloidal precipitants and spray or dip reagents. Table III lists 20 precipitating reagents commonly used for the detection of alkaloids, whereas Table IV presents 15 reagents that were used in 45 recent phytochemical surveys for alkaloids. At least 2 reagents were used in 38 of the surveys, while 7 surveys depended solely on 1 reagent to establish the presence of alkaloids. Because of the variable sensitivities of these reagents and because of their nonspecificity for alkaloids, many investigators utilize 4 or 5 reagents in their screening of plant extracts, and only samples yielding precipitates with all reagents are considered to contain alkaloids. Fulton (447) has tabulated some 200 of these reagents and presents a great deal of information concerning their specificity and sensitivity. A series of papers by Munch *et al.* (448–451) is concerned with the effect of 17 different alkaloid detecting reagents on several classes of nitrogenous bases. Travell (452) has studied the sensitivity of Mayer's and Valser's reagents, both solutions of potassium mercuric iodide, with the former prepared from mercuric chloride and potassium iodide and the latter from mercuric iodide and potassium iodide.

TABLE IV.—ALKALOID DETECTING REAGENTS EMPLOYED IN SCREENING PROGRAMS

Reagent	Surveys Used in,[a] No.
Mayer's reagent	39
Silicotungstic acid reagent	23
Dragendorff's drop reagent	19
Wagner's reagent	11
Dragendorff's spray reagent	10
Sonnenschein's reagent	9
Hager's reagent	7
Bouchardat's reagent	3
Phosphotungstic acid	2
Valser's reagent	1
Chloroplatinic acid reagent	1
Chlorauric acid reagent	1
Sodium tetraphenylboron reagent	1
Ammonium reineckate reagent	1
Tannic acid reagent	1

[a] Two or more reagents were used in 38 randomly selected surveys; 1 reagent only was employed in 7 surveys.

The reagent used by most investigators for phytochemical screening is essentially the same formula that Mayer originally introduced in 1862. Several investigators have demonstrated, however, that the original formula is perhaps the least sensitive for alkaloid detection, in comparison with many proposed modifications (452–454), and Travell (452) has conclusively demonstrated the superiority of Valser's over Mayer's reagent. In our laboratories we have compared the sensitivity of several common alkaloid precipitating reagents using 40 different alkaloids and representing several different chemical types (454). The reagents tested were Mayer's (3 formulas), Valser's, Wagner's (2 formulas), Bouchardat's, Hager's, Scheibler's, silicotungstic acid, Dragendorff's, Marme's, gold chloride, and Sonnenschein's. It was demonstrated in this study that the various reagents exhibit wide differences in sensitivity for structurally dissimilar

alkaloids. None of the reagents would detect ephedrine at a concentration of 0.1%, but Wagner's, Bouchardat's, Dragendorff's, and Scheibler's each detected all of the other alkaloids at concentrations ranging from 0.001 to 0.1%. Hager's, Marme's, and gold chloride reagents were by far the least effective detecting reagents, failing to react with 13, 12, and 10, respectively, of the 40 test alkaloids. All 3 of the Mayer's formulations were inferior to Valser's reagent with respect to sensitivity and specificity of alkaloid detection (454). It should be pointed out that the majority of these precipitating reagents must be used to detect alkaloids only in acid solution, and furthermore, that a large number of naturally occurring non-nitrogenous plant principles will react to give false-positive tests. These will be discussed subsequently.

Investigators who prefer to use spot tests, or those who prefer to chromatograph concentrated plant extracts for the detection of alkaloids, have a variety of available reagents. The most widely utilized, however, are modifications of the original Dragendorff drop test reagent, which produce orange to red colors with most alkaloids. Although a number of modified formulas have been proposed, each reported to have advantages over the others, the 2 most frequently utilized in phytochemical studies are the 1951 Munier and Macheboeuf (455) and the Thies and Reuther (456) modifications. A literature search has revealed the availability of at least 15 modifications of the Dragendorff spray reagents (457–471). We were prompted to study the stability and sensitivity of one of these modified reagents since a number of published reports had commented on the need for their storage under refrigeration with concomitant protection from light. It was determined that prepared concentrates of the 1951 Munier-Macheboeuf Dragendorff's reagent required a storage period of about 1 week prior to its use in the preparation of the diluted reagent. Also, the diluted spray reagent should be stored for a minimum of 1 week prior to its use for alkaloid detection in order to obtain maximum sensitivity. The reagent maintained its stability and sensitivity for at least 6 months and no special storage conditions were found necessary (472).

Some alkaloid detecting reagents are available which on reaction with certain groups of alkaloids, or with specific functional groups, produce characteristic chromogenic responses. These can be of considerable value in screening work, but only after alkaloids have been determined in the sample being evaluated. A selected list of general as well as specific chromogenic reagents has been prepared and is presented in Table V.

False-Positive Alkaloid Reactions.—Mechanisms for the reaction between alkaloids and detecting reagents are dependent chiefly on the chemical character of the reagent. Fulton (447) classifies alkaloidal precipitants as (*a*) those which react with basic compounds (alkaloids) to form insoluble salts; examples are silicotungstic, phosphomolybdic, and phosphotungstic acids. (*b*) Those which react with alkaloids as loose complexes to form precipitates; examples are Wagner's and Bouchardat's reagents (iodine-potassium iodide). (*c*) Those which react to form insoluble addition products through the alkaloid nitrogen; examples are the complex heavy metal salt reagents, Mayer's, Valser's, Marme's, and Dragendorff's. And (*d*) those which react through the attraction of organic acids with basic alkaloids to form insoluble salts. An example of such a reagent would be Hager's (picric acid). Obviously, these are rather nonspecific reactions and a number of nonalkaloidal plant constituents should be expected to precipitate also from solution on the addition of these reagents to crude plant extracts. These false-positive reactions are most liable to occur when testing an extract that has not been treated by at least one acid-base–organic solvent purification.

The most frequent false-positive reactions have been attributed to the presence of proteins which precipitate on the addition of heavy metal containing reagents (423, 438–444). Included in this category are "albuminous substances" (452), peptones (466), and ptomaines (441, 447). At least one textbook has indicated that amino acids will also precipitate with the general alkaloid reagents (444). However, a study by Winek and Fitzgerald (473) appears to disprove this allegation. Among other substances reported in the literature as the cause of false-positive alkaloid reactions are certain glycosides (444, 474), and carbohydrates (474), betaine (423, 439, 474), choline (474), purines (439), methylated amines (439), tannins (423), and ammonium salts (439, 466). Recently, we were able to show that previous positive alkaloid tests reported for extracts of *Piper methysticum* were due to the α-pyrones: kawain, dihydrokawain, methysticin, dihydromethysticin, and yangonin (475). This prompted an investigation of the mechanism by which nonalkaloidal compounds are able to elicit a positive reaction with an alkaloid detecting reagent, in this case the modified Dragendorff reagent. It was de-

TABLE V.—GENERAL AND SPECIFIC ALKALOID REAGENTS FOR CHROMATOGRAPHY

Alkaloid Type	Reagent	Ref.
Simple amines	1-Fluoro-2,4-dinitrobenzene	(461, 788)
	Ninhydrin	(789, 790)
Free and phenolic-bound OH groups	Potassium ferricyanide–$FeCl_3$	(461)
Aryl amines	Chromosulfuric acid	(461)
	Glucose–H_3PO_4	(461)
Secondary aliphatic and alicyclic amines	Sodium nitroprusside	(461)
Methylene oxide group	Chromotropic acid	(791)
Piperidine–pyrrolidine	Isatin–acetic acid	(792, 793)
Pyridine	Konig's reagent	(461)
Purines	Bromine vapor	(794)
Morphine	Ehrlich's reagent	(795)
Steroid glycoalkaloids	Vanillin–H_3PO_4	(796)
Choline and related compounds	Chargraff's reagent	(461)
	Hydroxamic acid–$FeCl_3$	(461)
	Dragendorff's, modified	(797, 798)
	Dipicrylamine	(798)
Indoles	Aminopyramidine	(799)
	Ceric ammonium sulfate–H_3PO_4	(800–803)
	Ceric sulfate–H_2SO_4	(804–806)
	Cinnamic aldehyde–HCl	(461)
	Perchloric acid–$FeCl_3$	(461)
	2,6-Dichloroquinonechlorimide	(461)
	Van Urk's reagent	(807)
	Prochaska's reagent	(807)
	Keller's reagent	(808, 809)
Indoles, β-substituted	Van Urk's reagent	(808–810)
Indoles, 5,6-dihydroxy	Ehrlich's reagent	(811)
	Hopkin's-Cole reagent	(811)
	Salkowski reagent	(811)
	Gibb's reagent	(811)
	Folin and Ciocalteu's reagent	(811)
	p-Dimethylaminocinnamaldehyde	(811)
	Acidic oxidizing reagent	(811)
	Terephthalaldehyde	(811)
	Nitrose reagent	(811)
	Formaldehyde–HCl reagent	(811)
	Xanthydrol reagent	(811)
	Diazotized *p*-nitroaniline	(811)
	Sodium molybdate–HCl reagent	(811)
	$FeCl_3$–potassium ferricyanide	(811)
	Ferric chloride solution	(811)
	Ammoniacal silver nitrate	(811)
Indoles, ergot	Ehrlich's modified reagent	(461, 808, 812)
	Ehrlich's reagent	(808–810)
	Van Urk's reagent	(808–810)
	Alport-Cocking's reagent	(808)
	Glyoxalic acid	(808)
Indoles, yohimbine type	Modified Keller reaction	(813)
Miscellaneous (special applications)	Sodium tellurite–H_2SO_4	(814)
	Ceric sulfate–trichloracetic acid–sulfuric acid	(461)
	Nessler's reagent	(461)
	Sulfuric acid–methanol (fluorescence)	(461)
	Nitric acid–methanol (fluorescence)	(461)
	Levine-Chargraff reagent	(815)
	Sulfuric acid	(816)
	Dichromate–H_2SO_4	(817)
	Ninhydrin reagent	(818)
	Xanthydrol reagent	(784, 818)
	Hydroxylamine	(819)
	Hydroxylamine–$FeCl_3$	(820)
	Sodium nitroprusside	(821, 822)
General alkaloid reagents	Mayer's	(823)
	Potassium iodoplatinate	(461, 824–832)
	Iodine	(461)
	Dragendorff's reagent, modified	(457–471)

termined that any non-nitrogenous organic compound having conjugated carbonyl (ketone or aldehyde) or lactone functions would react in a manner typical of alkaloids (476). These minimum qualifications are quite prevalent in natural products and undoubtedly many false-positive alkaloid reactions are promulgated through this mechanism. Fortunately, the majority of com-

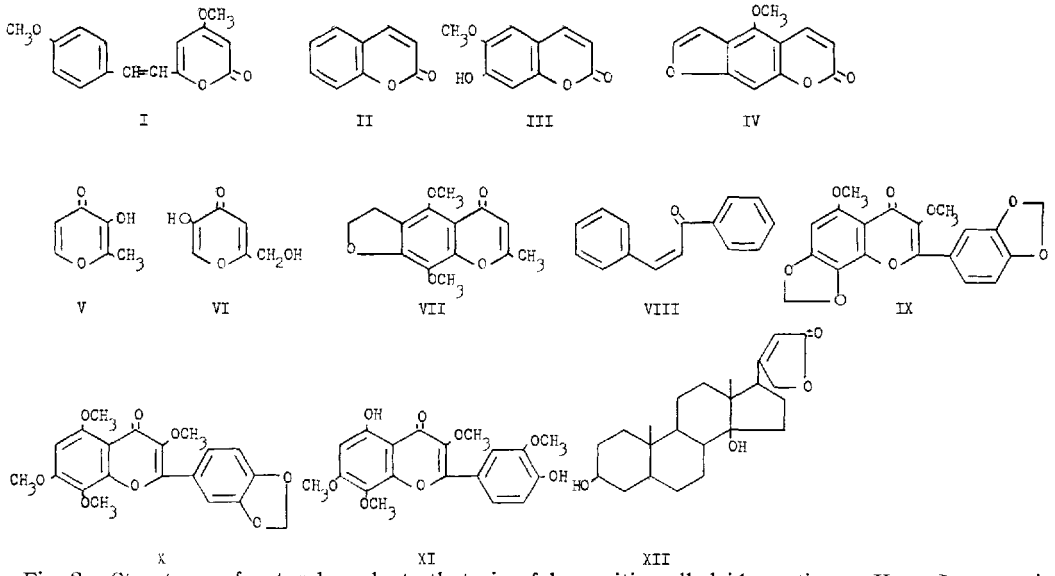
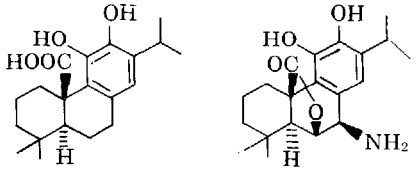


Fig. 2.—Structures of natural products that give false-positive alkaloid reactions. Key: I, yangonin (476); II, coumarin (476); III, scopoletin (476); IV, bergapten (476); V, maltol (476); VI, kojic acid (476); VII, khellin (476); VIII, chalcone (476); IX, meliternatin (477); X, meliternin (477); XI, ternatin (477); XII, digitoxigenin (476).

pounds with these functionalities can be separated from the alkaloids by treatment of the extract with base, followed by extraction with organic solvent which, in turn, is extracted with dilute aqueous acid. Another interesting report is that by Briggs and Locker (477), who in 1949 isolated 3 compounds from *Melicope ternata* which gave precipitates with the usual alkaloid reagents and crystalline salts with acids. However, these compounds contained no nitrogen and proved to be the completely alkylated hydroxyflavones, meliternatin, meliternin, and ternatin (Fig. 2). It would appear that, in the light of our work, the false-positive test for alkaloids was due to the presence of the conjugated carbonyl in each molecule rather than, as stated by the authors, the fact that each molecule was completely alkylated. Presumably other flavones would react similarly, as would most of the cardenolides and bufadienolides.

Householder and Camp (850) have recently pointed out that treatment of plant extracts with ammonium hydroxide and acetone can give rise to artifacts which give positive reactions with the standard qualitative alkaloid test reagents. These investigators were unable to identify the condensation products formed in this reaction but presented evidence to show that the rate of formation was affected by exposure to light and the atmosphere.

A recent report by Russian workers (851, 852) presented evidence for the isolation of an alkaloid named rosmaricine from *Rosmarinus officinalis* (*Labiatae*). This anomaly of an alkaloid from a member of the mint family prompted Wenkert and co-workers to investigate the validity of the Russian work (853). They found that rosmaricine was indeed not present in the plant prior to the addition of ammonia (used by the Russian workers in their isolation experiments), and that this "alkaloid" was undoubtedly formed as a result of the action of the base on the precursor carnosic acid (853).



Carnosic acid Rosmaricine

Other anomalous alkaloid reactions have been mentioned in the literature but as yet they cannot be explained. For example, *Samolus repens* (*Primulaceae*) extracts give a black color and precipitate with Dragendorff's reagent (478). We have observed this phenomenon frequently in the field testing of fresh plant material and have assumed the reaction to be one of free iodine in the reagent combining with starch to give a typical blue-black color. Extracts from *Plagianthus divaricatus* (*Malvaceae*) have been reported to give a pink color, but no precipitate, with Dragendorff's reagent (478).

Webb has pointed out that about 9% of species tested in the field for alkaloids were found to be false-positive reactions following subsequent detailed laboratory analysis (424). On the basis of experience resulting from tests on some 25,000 plant species, Douglas has estimated that not more than 5% of initial positive alkaloid tests have been found to be due to nonalkaloid entities (436).

False-Negative Alkaloid Reactions.—If one considers the nonheterocyclic nitrogen bases (protoalkaloids) as alkaloids, it will be noted that the greatest majority of these fail to react with the usual alkaloid precipitating reagents. Certain examples of this can be documented (454, 466). Also, unless certain precautions are observed in the test procedure, quaternary alkaloids and amine oxides (nupharidine, dilupine, trilupine) will not be detected (466). That is, if an acidic plant extract is treated with base and extracted with an immiscible organic solvent, both the aqueous basic layer and the organic layer must be tested for alkaloids. In most alkaloid screening procedures that have been reported, the basic layer has been neglected. Arguments for this omission have been based on the assumption that quaternary alkaloids or amine oxides would not be expected to occur in plants to the exclusion of tertiary bases which would be detected by this procedure. Raffauf points out that this may be an erroneous assumption (466).

Alkaloid Testing of Herbarium Specimens.—The validity of alkaloid tests on plant material derived from herbarium sheets is open to question. Often, for many and varied reasons, it is difficult to find certain plants in their native habitat at the time of collection of indigenous flora, and this alternative to collection has been used by several investigators (423, 424, 478–482) to survey a broad distribution of plant taxa. An obvious disadvantage in the use of such material is that usually only leaves and branches are available and instances are known wherein plants contain alkaloids in other organs, but their leaves and stems are relatively alkaloid-free (424). Also, herbarium material is often quite old, and a number of examples can be cited correlating alkaloid decomposition as a function of time (424). On the other hand, plants 1300 years old have been reported still to give alkaloid positive tests (445). It is common practice in some herbaria to treat specimens with formalin or mercuric chloride as preservatives. Formalin could very well decompose many alkaloids, and mercuric chloride reacts with certain alkaloidal precipitants to form abnormal precipitates. With Mayer's reagent this is evidenced by a bright orange precipitate with yellow streaks which eventually become red, and with Bouchardat's reagent a pale purplish brown precipitate is observed (424). In some herbaria, it is common to mark specimens in a manner that treatment of this type can be easily ascertained, while in others this practice is not carried out. Even with these problems, extensive herbarium specimen testing for alkaloids by Webb has allowed him to acknowledge this procedure as a valuable adjunct to the testing of fresh material (424). Cain *et al.* (479) have indicated that equivalent results were obtained in their studies with fresh plant material and dried herbarium specimens.

Field Tests for Alkaloids in Plants.—Investigators searching for new alkaloid bearing species in remote or distant areas of the world often find it difficult to return to make additional bulk plant collections for laboratory study. Therefore, simple field tests for alkaloids have been developed which are sufficiently reliable to distinguish alkaloid-containing plants, *i.e.*, those containing at least 0.01% of alkaloids, thus enabling bulk collections of these species initially and eliminating the need for a return expedition. These field tests can be classified in the following manner.

Organoleptic Evaluation.—It has been suggested by Webb that at least some of his collections of species for laboratory examination were made on the basis of taste, in conjunction with some knowledge of the botanical characteristics of the samples being evaluated (483). That is, he avoided tasting plants in such families as the *Anacardiaceae*, *Euphorbiaceae*, etc., but bitterness in a group such as the *Lauraceae*, particularly if a *Cryptocarya*, would suggest alkaloids. In Webb's opinion, differentiation of bitter alkaloids and saponins can be made on the basis of taste, but only after considerable experience (483). Also, bitterness in the inner bark of such groups as *Evodia*, *Acronychia*, and *Melicope* (*Rutaceae*), in conjunction with an observation of yellow pigmentation, is suggestive of the presence of alkaloids (acridones). While judgments such as these may be justified by an investigator who has had considerable experience, and whose botanical and chemical background are complementary, the average alkaloid hunter could hardly justify such an approach.

Spot Tests Using Alkaloid Test Paper.—Kraft (484) has developed a simple device for alkaloid detection in fresh plant material, a process which consists of impregnating filter paper with Dragendorff's reagent, followed by

TABLE VI.—PHYTOCHEMICAL SURVEYS FOR ALKALOIDS

Area	Author(s)	Yr.	Species Tested, No.	Ref.
Argentina	Barnes and Gilbert	1960	71	(492)
	Codoni	1947	17	(493)
Australia	Webb	1949	753	(423)
	Webb	1952	1040	(424)
China	Nikonov *et al.*	1961	35	(494)
Costa Rica	Saenz	1964	59	(495)
Hawaii	Swanholm *et al.*	1959	96	(496)
	Swanholm *et al.*	1960	29	(497)
	Scheuer *et al.*	1962	71	(498)
Hong Kong	Arthur	1953	116	(499)
	Arthur and Cheung	1960	332	(420)
	Arthur and Chan	1962	400	(500)
Japan	Kariyone *et al.*	1956	85	(501)
	Goto *et al.*	1959	220	(168)
Malaya	Amarasingham *et al.*	1964	542	(490)
	Douglas and Kiang	1957	[a]	(502)
	Kiang and Douglas	1957	214	(503)
	Kiang *et al.*	1961	708	(504)
	Nakanishi *et al.*	1965	89	(176)
Malgache	Meyer and Pernet	1957	[a]	(505)
	Pernet	1956	5	(506)
Mexico	Dominguez *et al.*	1960	8	(507)
New Zealand	Cain *et al.*	1961	21	(479)
	Cambie *et al.*	1961	697	(478)
	Cambie *et al.*	1961	74	(480)
	Cambie *et al.*	1961	251	(481)
	Cain *et al.*	1962	320	(482)
Nigeria	Patel and Rowson	1964	33	(312)
	Persinos *et al.*	1964	10	(99)
	Quimby and Persinos	1964	12	(100)
North Borneo	Arthur	1954	205	(491)
Papua—New Guinea	Webb	1955	295	(483)
Sweden	Hulton and Torssell	1965	191	(854)
Taiwan	Hsu	1957	51	(508)
	Huang *et al.*	1959	61	(509)
	Koo *et al.*	1965	1000	(510)
	Yeh *et al.*	1959	72	(511)
Thailand	Nilanidhi	1964	21	(512)
Tibet	Blinova and Stukkei	1960	113	(513)
U.S.S.R.	Aliev	1962	80	(514)
	Efros	1946	30	(515)
	Ismailov	1958	140	(516)
	Lazur'evskii and Saidykov	1939	259	(517)

(*Continued on next page.*)

drying. A plant part is incised with a razor blade and a small amount of juice is applied to the test paper which, if alkaloids are present, will give the characteristic orange color indicative of a positive test. This method has been applied to the field testing of about 1200 species of plants by Nikonov and Ban'kovskii (485), who found it to be acceptable with certain reservations. They found it unsuited for plants containing pigments in the sap which masked positive reactions, and it was further determined that protoalkaloids such as ephedrine were not detected. An important point that must be emphasized is that these workers stress that the typical color of an alkaloid-positive reaction must be observed within 30 sec. from the time that the sample was applied to the paper for a test to be considered valid (485). We have used paper similar to this in our laboratory for the detection of alkaloids in organic solvents during chromatographic separations and found that it was necessary to apply water to the paper, after the sample solvent had evaporated, in order for the reaction to take place.

Spot Tests on Paper Using Liquid Reagents.—The most extensive phytochemical survey for alkaloids in the plant kingdom is being conducted by scientists from the Natural Products Section, Smith Kline & French Laboratories, Philadelphia, Pa. This program was initiated in 1954, but extensive alkaloid testing did not begin until 1958. Briefly, their approach consists of a semirandom collection of plants from all parts of the world, with tests for alkaloids made in the field on all accessible parts of each species. Those found to be promising as a source of alkaloids are collected at the test site in sufficient quantity to enable laboratory extraction of the alkaloids for subsequent pharmacologic study. Extracts from

TABLE VI.—(*Continued*)

Area	Author(s)	Yr.	Species Tested, No.	Ref.
	Kurinnaya	1956	[a]	(518)
	Kuvayev and Blinora	1960	[a]	(519)
	Massagetov	1946	113	(520)
	Nikonov and Ban'kovskii	1959	1200	(485)
	Oparin and Chepurin	1953	[a]	(521)
	Orechoff	1934	368	(522)
	Sokolov	1956	[a]	(523)
	Soskov *et al.*	1963	[a]	(524)
	Stepanyan	1963	35	(525)
	Yakunina *et al.*	1961	55	(526)
	Zolotnitskaya	1954	231	(527)
World-wide	Wall *et al.*	1954	292+	(422, 428)
	Wall *et al.*	1954	598+	(429, 430)
	Wall *et al.*	1955	606+	(431, 432)
	Wall *et al.*	1957	432+	(433)
	Wall *et al.*	1959	921+	(434)
	Wall *et al.*	1961	1030+	(435)
Apocynaceae	Abisch and Reichstein	1960	31	(486)
	Abisch and Reichstein	1962	4	(487)
Asclepiadaceae	Abisch and Reichstein	1962	64	(488)
Campanulaceae	Gertig	1962	11	(528)
Caryophyllaceae	Naumenko	1957	[a]	(529)
Leguminosae	White	1943	145	(530)
	White	1944	3	(531)
	White	1951	53	(532)
	White	1951	55	(533)
	White	1957	54	(534)
Orchidaceae	Luning	1964	525	(535)
Periplocaceae	Abisch and Reichstein	1962	6	(488)
Pinaceae	Tallent *et al.*	1955	27	(536)
Ranunculaceae	Winek *et al.*	1964	10	(537)
Solanaceae	Scott *et al.*	1957	61	(538)
Seeds	Earle and Jones	1962	900	(5)
Seeds[b]	Kazimierz	1962	27	(539)
Misc. plants[c]	Paris and Moyse-Mignon	1956	73	(540)
Misc. plants	Stein and Kamienski	1957	220	(541)
Fungi	Worthen *et al.*	1965	37	(542)
	Worthen *et al.*	1962	3	(543)
	Tyler	1961	8	(544)
	Tyler and Stuntz	1962	160	(545)
	Tyler and Stuntz	1963	94	(546)

[a] Only an abstract of the paper was available; data not included. [b] Analyzed for choline. [c] Analyzed for choline and betaine.

all plants shown to contain alkaloids by this field test are screened for several types of pharmacologic activity (436).

The field test for alkaloids used by this group has been described (466) and is essentially the same as that utilized by Nikonov and Ban'kovskii (*vide supra*) with the exception of the special test paper. Instead, plant sap obtained by making an incision of the appropriate plant part, is applied to filter paper, dried, and a micro drop of specially prepared Dragendorff's reagent is added (466). Positive tests are evaluated as previously described. All positive field tests are confirmed by means of a laboratory alkaloid detection procedure (*vide infra*).

Of 25,000 species evaluted in this manner to date, about 10% have been recorded as alkaloid-positive (436). About 5% of the plants shown to contain alkaloids by the field test were not confirmed by the laboratory procedure (436).

Abisch and Reichstein (486–488) have also utilized this spot test technique for alkaloid detection in their study of plants of the *Apocynaceae*, *Asclepiadaceae*, and *Periplocaceae*. However, their extracts were prepared from dry plant material.

Test Tube Spot Tests.—Culvenor and Fitzgerald (489) have described a simple kit that can be taken into the field for use in testing samples of plant material for alkaloids. About 2–4 Gm. of fresh plant part is ground in a small mortar with sand and sufficient chloroform to make a slurry. Ammoniacal chloroform is added and the mixture stirred for 1 min. prior to filtration into a small test tube. Extraction of the alkaloids from the chloroform is accomplished by shaking the solution with 0.5 ml. of 2 *N* sulfuric acid and separation of the acid layer by means of a medicine dropper. A few drops of this acid extract are then tested with either Mayer's reagent or

silicotungstic acid to ascertain the presence of alkaloids. When samples were analyzed by both the field method and a laboratory procedure, it was found that a number of weakly positive tests recorded through use of the laboratory test were found to be negative in the field test (489). The method, of course, fails to detect quaternary alkaloids and this appears to be its major drawback.

Presumably, many of the plants collected for laboratory alkaloid testing by Webb (423, 424, 483), Amarasingham *et al.* (490), and Arthur (491) were field analyzed in a similar manner to that described above. However, their respective reports failed to point out any consistency with regard to this matter.

Alkaloid Surveys.—Although surveys for alkaloids, representing tests on more than 15,000 species of plants, have been published (5, 99, 100, 168, 176, 312, 420, 423, 424, 428–435, 478–483, 485–488, 491–546, 854) (Table VI), the data that they present are often inconsistent because of variations in testing methodology. That is, some of the procedures will detect both quaternary and tertiary alkaloids, but the former group is omitted from most survey reports. Certain procedures involve treatment of the alkaloid fraction to remove substances that often give rise to false-positive alkaloid reactions, whereas others do not include this extra step. Some methods are semiquantitative, while others lack this desirable feature. A survey of the most extensive and more frequently reported methods allows them to be classified into 6 major categories (Table VII). Perhaps the simplest method is that represented by group *A* in which either an acidic or aqueous plant extract is prepared, with or without the use of heat, followed by filtration and the addition of one or more alkaloidal reagents to separate portions of the filtrate. Most investigators assess a rating of 0, or +1 to +4 on the lack of, or degree of precipitation following use of the reagents. However, there is seldom any indication of the alkaloid equivalent of these ratings. This undoubtedly could present a problem to either a novice or one who is attempting to duplicate results in a different laboratory. On the other hand, a person experienced in alkaloid screening can usually assess this +1 to +4 rating system by a rule of thumb. The major drawback of this method is that it results in the greatest number of false-positive reactions. An inspection of the compounds presented in Fig. 2, which are representative of a great number of nonalkaloidal plant constituents capable of giving false-positive alkaloid reactions, shows that for the most part they would be soluble in either aqueous or acidic media. Also, although this method would not differentiate between quaternary and tertiary alkaloids, neither would it fail to detect one or the other.

Group *B* testing differs from group *A* only in that the filtrate is made basic and extracted with an organic solvent (usually chloroform or ether), followed by extraction of the alkaloids from the organic solution with dilute aqueous acid. The usual alkaloidal precipitants are then added to separate portions of the acid extract. This method has the advantage over the group *A* procedure of eliminating a great number of compounds from the final test extract that are capable of eliciting false-positive alkaloid reactions; however, any quaternary alkaloids present would also be eliminated. Simple modifications in this method would allow one to test for the latter group of alkaloids.

TABLE VII.—ALKALOID TEST METHODS USED IN PHYTOCHEMICAL SCREENING

Group	Method	Ref.[a]
A	Acidic or aqueous extracts.	(5, 420, 423, 424, 478–483, 490, 496, 497)
B	Acidic or aqueous extract, followed by alkali treatment, immiscible solvent extraction, and partition with dilute acid.	(507, 536)
C	Alcohol extraction followed by concentrating and addition of acid.	
	1, No additional treatment.	(100[b], 422[b], 428–435[b], 478–482, 542[b], 545[b], 546[b])
	2, Partition purification, test made only on tertiary alkaloid fraction.	(99, 100, 422, 428–435, 507, 538, 545, 546)
	3, Partition purification, tests made both for tertiary and quaternary alkaloids.	(312, 486–488, 498, 509, 511, 535, 542)
D	Extraction of alkalinized sample with organic solvent.	(502–504)
E	Prollius fluid extraction, concentration, addition of acid.	(420, 423, 424, 483, 491, 496)
F	Procedures involving chromatography.	(99, 100, 176, 537)

[a] Only laboratory methods are presented; see earlier discussions for field testing methods. [b] Preliminary test only; additional testing employed.

Since water will extract a number of nonalkaloidal constituents from plants, and because there is a possibility of free alkaloid bases existing in the plant as such and these would be water insoluble, most investigators utilize alcohol (methanol or ethanol) or alcohol–water mixtures as a primary extraction medium. Group *C* test methods involve preparation of an alcohol extract followed by removal of solvent and the addition of dilute acid to dissolve any alkaloids. Some investigators test the resultant acid extract directly (312, 478–482) and stop at this point (group *C-1*). The advantages and disadvantages for this type of testing are similar to those discussed for the group *A* methods (*vide supra*). Others will confirm initial positive reactions following a base–organic solvent–acid extraction. These are the group *C-2* methods which are designed primarily to eliminate substances capable of eliciting false-positive alkaloid reactions. The group *C-1* and *C-2* methods were designed and used most extensively by Wall and co-workers (422), but there are 2 important features that should be discussed concerning these procedures. First, the test involves precipitation of free alkaloid bases from the initial acid extract with NaOH rather than with NH_4OH. Thus, if a majority of the alkaloids in the sample were phenolic in character (highly improbable), the phenolates formed on the addition of fixed alkali would be insoluble in the immiscible organic solvent used for the extraction of the basic alkaloid-containing solution. Subsequent extraction with dilute acid would then result in a solution free from phenolic alkaloids and would therefore not be representative of the true alkaloid content of the sample. A second problem associated with this method was recognized by the workers themselves after screening the first 4000 accessions. Because they were experiencing a lesser number of positive results than would be expected from statistical averages, they increased the concentration of test solution so that 1 ml. would be equivalent to 4.0 Gm. of dry plant material (434, 435). Previous test results in the series (422, 428–433) were reported on solutions which represented only 0.2 Gm. of dry sample. This, of course, made any negative alkaloid test results reported in the first 4000 accessions (422, 428–433) open to question. Recognizing this problem, plants yielding negative results from the latter group, if available, were retested and the results included in reports on the final 2000 accessions (434, 435). The method does not include specific provisions for the detection of quaternary bases, but as indicated previously, modifications could be made so that this procedure would detect these compounds.

Various modifications of group *C-2* methodologies have been proposed in order to detect quaternary alkaloids and report them as a separate group. In group *C-3* methods the aqueous alkaline solution, after extraction with an organic solvent, is treated with a mineral acid until it is distinctly acid to litmus, followed by the subsequent addition of any of the usual alkaloidal precipitants to this acidic solution. It should be emphasized that a weak positive test at this point need not necessarily imply the presence of quaternary alkaloids. To the contrary, a weak test must be expected because of incomplete extraction of tertiary alkaloids with the organic solvent, and would be evidenced by a slight cloudiness of the solution following addition of the reagent. A positive test, on the other hand, would be noted as a definite heavy flocculation or precipitation on addition of the reagent.

Group *D* methods involve the addition of alkali to the drug, followed by extraction with an organic solvent and partition of the concentrated extract with dilute aqueous acid prior to the addition of precipitating reagents. These procedures fail to detect quaternary alkaloids, but the final test solution is relatively free from many substances associated with false-positive reactions.

Extraction of dry plant material with Prollius fluid (ether–chloroform–ethanol–NH_4OH) (25:8:2.5:1) (547), followed by evaporation of the solvent and addition of dilute acid, is the representative procedure for group *E* methods. Webb (423) has indicated that certain plants give negative tests with Prollius fluid, but +4 reactions when acidic aqueous extracts of the same plant were tested (group *A*). He suggests that this is due to a poor solubility of quaternary bases in Prollius fluid. However, several instances wherein the reversal of these positive and negative tests using Prollius fluid and dilute acid extracts were also observed (423). In our laboratories, we found this method to be unsatisfactory when compared with others in an evaluation of testing procedures using plant samples of known alkaloid content (548).

Group *F* methods involve the use of chromatography to detect alkaloids in dry plant material. These allow not only for the detection of alkaloids, but also for an estimate of the number present. In addition, the use of selective chromogenic spray reagents could serve to tentatively classify alkaloids in the samples into general groups. We have described a method

utilizing thin-layer chromatography which requires only small samples, eliminates most compounds suspected of yielding false-positive reactions, and differentiates tertiary and quaternary alkaloids (549). Several alkaloid detecting reagents used in chromatography are presented in Tables IV and V.

The comparative efficiencies of the alkaloid screening procedures of Wall *et al.* (422), Webb (423), Kiang and Douglas (503), Swanholm *et al.* (496, 497), Abisch and Reichstein (486), and Arthur (491) have been studied using 28 plant samples known to contain alkaloids and 8 samples known to be devoid of alkaloids. The latter group included several plants known to yield false-positive alkaloid reactions. Alkaloid-containing plants were selected so that they represented several different chemical classes of alkaloids (549). The surveys under comparison employed groups *A*, *C-1*, *C-2*, *D*, and *E* methodologies. It was determined that the Wall *et al.* (422) and Kiang and Douglas (503) methods were most satisfactory, and that the direct acid extraction method of Webb (422) was the most rapid and also gave acceptable results. The Prollius fluid extraction (422) and the Abisch and Reichstein (486) techniques gave the poorest results, and the latter procedure was quite time consuming (549). All plant samples known to contain alkaloids were detected using the Kiang and Douglas (503), and Webb (423) acid extraction methods. However, these 2 procedures also gave the greatest number of false-positive reactions with the plant samples known to be devoid of alkaloids (549). False-positive alkaloid reactions were completely eliminated by use of either the Wall *et al.* confirmatory test (422) or the Abisch and Reichstein method A (486). Other studies have shown that significant differences exist in these methods with regard to their ability to remove alkaloids for testing from plant material (454).

Screening for Heterosides (Glycosides).—Heterosides are organic compounds in which a hemiacetal linkage usually connects the anomeric carbon of a sugar (glycone) with an alcohol or phenolic hydroxyl of a second nonsugar molecule (aglycone). This type of linkage gives rise to the so-called *O*-heterosides (*e.g.*, salicin), the most common type of heteroside found in plants. If the anomeric carbon of the glycone is attached to an aglycone through sulfur, the *S*-heterosides are formed (*e.g.*, sinigrin). A third group are the *N*-heterosides which involve attachment of the glycone to an amino group of an aglycone (*e.g.*, vicine, crotonoside). Finally, the C-heterosides involve a carbon to carbon linkage of glycone and aglycone (*e.g.*, aloin).

As a general rule, plant heterosides are easily hydrolyzed with dilute acids or appropriate enzymes. The C-heterosides are a notable exception, as they are resistant to the usual type of acid hydrolysis, and require ferric chloride for this purpose.

A number of different sugars are known to occur in plants in combination with an equally large number of diverse aglycones. Paris (550) has recently reviewed plant heterosides with particular reference to the types and distribution in plants.

In most instances, the biological activity of heterosides can be attributed to the aglycone moiety. The glycone is mainly associated with the degree or modification of activity, primarily induced by the aglycone. However, the cardiac heterosides can be pointed out as a group that have no useful biological activity unless the heteroside is intact (310). Thus, we have the economically important saponin heterosides and the medicinally useful anthraquinone, flavonoid, cyanogenetic, *iso*thiocyanate, and cardiac groups.

From a chemical point of view, there are 3 parts of the heteroside molecule that can be used as a means of detecting this group of compounds in plant material. First, the hemiacetal linkage between aglycone and glycone is usually not associated with biological activity, nor can it be associated with any specific aglycone.[2] This part of the molecule does not appear attractive as a means of detecting plant heterosides. Because of the usual correlation of biological activity with the aglycone moiety of heterosides, and because this part of the molecule often has chemical properties amenable to ready detection, most investigators have used it as a means of screening plant material indirectly for heterosides.

If, however, heterosides must be intact to exert their potential biological activity, it would appear most fruitful to detect the hemiacetal linkage in plant extracts as an identifying feature of the presence of heterosides. Several investigators have proposed methods to accomplish this, but a lack of published applications of these to the screening of plants for heterosides, attests to their complexity or inefficiency. Bourquelot (551) proposed a method for detecting and identifying heterosides based on the determination of an "Enzymolytic Index of Reduction" obtained by measuring the optical

[2] The notable exception is concerned with deoxysugars commonly found only in combination with cardiac heterosides.

rotation of a heteroside-containing plant extract before and after hydrolysis with specific enzymes. Although the method has some value, it is time consuming and requires large amounts of plant material; therefore, it would be difficult to adapt to a large-scale screening program. Bliss and Ramstad (552), devised a simple procedure that could be adapted for routine screening. It consists of (*a*) separation of the heterosides in an extract by paper chromatography, (*b*) hydrolysis of the heterosides on the chromatogram with proper enzymes (*i.e.*, α-glucosidase–invertin; β-glucosidase–emulsin), and (*c*) location of the reducing sugars formed on the chromatogram by means of an appropriate reagent spray. This method appears to be least objectionable of many proposed. However, it will detect only those heterosides for which the selected enzymes have a hydrolytic specificity. Also, optimal reaction conditions such as time, temperature, and pH would have to be determined for a large number of substrate heterosides to propose operating conditions that would allow detection of the greatest number of compounds. Janot *et al.* (553) and Paris (554) have suggested chromatographic methods for detecting heterosides similar to the method of Bliss and Ramstad, but acid hydrolysis of the sample is included to supplement the action of enzymes. Other methods have been proposed, but either they have not been applied successfully to plant samples, or certain limiting factors make them of doubtful value for general screening (555, 556).

Knapp and Beal (557) have proposed a method involving (*a*) the selective extraction of heterosides from plant material using 80% ethanol, (*b*) oxidation of the free sugars in the extract to their corresponding carboxylic acids so that they will not be detected after hydrolysis of the heterosides, (*c*) hydrolysis of the heterosides in the extract using 0.15 *N* sulfuric acid and heat (100°) and (*d*) detection of hydrolyzed glycones by means of paper chromatography. The major objection to this procedure is that holosides, especially sucrose which is widespread in plants, are detected; thus, the method is of decreased value.

Abisch and Reichstein (486) have utilized a rather simple procedure which involves the preparation of an extract devoid of free sugars, hydrolysis of the extract with the Kiliani acid mixture, and testing of the hydrolysis products with Fehling's solution for evidence of reduction. These investigators have pointed out the nonspecificity of the test; however, in a broad screening program, false-positive reactions must be accepted, especially in the absence of a completely acceptable and specific method of detection.

It does not appear that adequate methodology has been developed to allow for an extensive screening of plants for heterosides based on the approaches described above. As indicated previously, the majority of studies involving a search for heterosides in plant material have been concerned with tests designed to detect specific aglycones. The more important of these will now be considered.

Screening for Saponins and Related Compounds

Several types of compounds must be considered whenever saponin testing is to be conducted. Of major import are the steroidal and triterpenoid saponins and their respective sapogenins, as well as saturated sterols, saturated terpenes, diterpenes, and other steroidal plant constituents (*e.g.*, cardenolides). The economic importance of steroidal sapogenins, mainly because of their facile conversion to the medicinally useful steroid hormones, has been reviewed by Correll *et al.* (11). Discussions concerning this group of compounds will be directed primarily at the detection and/or differentiation of steroidal saponins from other types of saponins and polycyclic phytoconstituents.

A number of surveys have been conducted which have been designed to detect saponins in plant material (99, 100, 168, 422, 428–435, 478–483, 490–492, 494, 499, 506, 507, 513, 529, 558–580). Saponins have several characteristic properties that can be used as a basis for simple detection tests. (*a*) They are all capable of hemolyzing red blood cells, (*b*) in aqueous media they will produce a characteristic honeycomb froth which persists for at least 30 min. after vigorous shaking of the solution; (*c*) they are toxic for fish, causing paralysis of the gills; and (*d*) they produce characteristic color reactions in the Liebermann-Burchard test. All of these properties have been utilized in one or more screening tests for the detection of saponins in plant material, and each merits discussion to show the limitations and value of these test procedures (Table VIII).

All known triterpenoid and steroidal saponins are hemolytic (584, 585), as undoubtedly are certain other plant constituents. The former group occurs both as the heteroside and as free triterpenes, whereas steroidal saponins are never found as free sapogenins in plant material (584, 585). This is an unusual phenomenon since specific steroidal saponases are known to be present in steroidal saponin-containing plants

TABLE VIII.—SURVEYS FOR SAPONINS IN PLANTS

Detection Method	Source of Plants	Species Tested, No.	Ref.
Hemolysis	India	38	(562–564, 581)
	Mexico	8	(507)
	Nigeria	22	(99, 100)
	Poland	4	(574)
Hemolysis + froth	Brazil	71	(492)
Hemolysis + steroidal sapogenin identification	Brazil	21	(559)
	Liliaceae	15	(579)
	Philippines	222	(571–573)
Froth test	India	38	(562–564, 581)
	Malaya	542	(490)
	North Borneo	205	(491)
	Papua—New Guinea	116	(558)
Froth test + Liebermann-Burchard test	Australia	1136	(558)
	New Zealand	1533	(478–483)
Fish toxicity	India	38	(562–564, 581)
Isolation of steroidal sapogenins	India	38	(562–564, 581)
	Mexico	150	(567–570)
Isolation or detection of triterpenoid sapogenins	India	6	(565)
	Poland	8	(580)
	U.S.S.R.	5 families	(577)
Undetermined methods	Bulgaria	72	(560)
	Chile	2894	(561)
	China	3	(582)
	India	4	(566)
	Japan	220	(168)
	Malgache	5	(506)
	Spain	27	(575, 583)
	Tibet	113	(513)
	U.S.S.R.	[a]	(576, 578)

[a] Original paper unavailable; data not included in abstract.

(586). Since all saponins are soluble to some extent in 80% alcohol, they are usually extracted with this solvent (584, 585), and if such a plant extract is mixed with a standardized red blood cell suspension (585) and hemolysis of the cells takes place, it can be assumed that saponins are present. On the other hand, terpenoid and steroidal saponins act similarly in this respect and cannot be differentiated on this basis alone. Wall *et al.* (585), Walens *et al.* (587), and Rothman *et al.* (588) have developed standard procedures which have been successfully used by many investigators for the detection and estimation of steroidal sapogenins, and which differentiate triterpenoid from steroidal types. The procedure involves (*a*) hemolytic detection of saponins in the plant extract (585), (*b*) isolation of crude sapogenins following hydrolysis (585), and (*c*) subjecting the crude sapogenin acetates to infrared (585) or ultraviolet (587) spectral analysis. Initially, the extraction procedure eliminates to a great degree the starch, sugars, and protein which, if present, would produce troublesome tars as a result of the acid hydrolysis (588). The presence of these materials in the extract would necessitate larger amounts of acid, with subsequent destruction of some sapogenin. It has been determined by using the recommended extraction procedure (585), that 2 *N* HCl in alcohol at reflux temperature or 0.5 *N* HCl under pressure will completely hydrolyze saponins (584, 588). Characteristic infrared and ultraviolet absorption spectra of the hydrolysates then serve to indicate whether the saponins causing hemolysis in the sample are steroidal or triterpenoid.

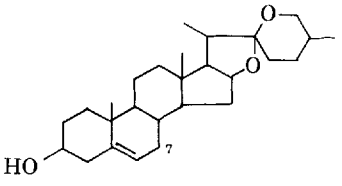
The appearance of a characteristic honeycomb froth, which persists for at last 30 min. after shaking an aqueous boiled (3–5 min.) mixture containing the plant material, is presumptive evidence for the presence of saponins. This method of detection is rapid, simple, and requires little equipment, but it will not differentiate triterpenoid from steroidal saponins. It has been used, however, by several investigators (Table VIII). If only a small froth is produced by this treatment, which is stable for only a few minutes, proteins, certain plant acids, or a low concentration of saponin may be the cause (558). Evidence exists that the froth test is not infallible since the saponin of *Castanospermum australe* is an active hemolytic agent at a dilution of 1:20,000; however, at this dilution no frothing occurs (558). The addition of aqueous sodium carbonate to a boiled aqueous plant extract which froths poorly, or not at all, may result in the production of a stable and dense froth. If this occurs, the presence of free acids (*e.g.*, stearic acid, diterpene acids, triterpene dicarboxylic acids) is indicated (558).

TABLE IX.—DIFFERENTIATION OF POLYCYCLIC SUBSTANCES IN PLANTS

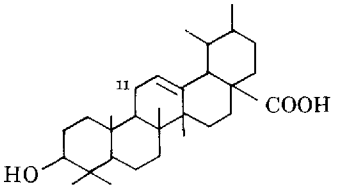
Hemolysis Test	Froth Test	Liebermann-Burchard Test	Substances Present
+	+	+ Blue or green	Saponins, probably steroidal.
+	+	+ Red, pink, purple or violet	Saponins, probably triterpenoid.
+	+	− Pale yellow	Saponins, possibly heterosides of saturated sterols or saturated triterpenes.
±	−	+ Red, pink, purple or violet	Saponins absent, free triterpenes, diterpenes, sterols or related polycyclic substances present.
−	−	− Pale yellow	Saponins absent, also unsaturated triterpenes, sterols, etc., absent, but may contain saturated sterols or saturated triterpenes.
−	+[a]	± Pale yellow, red, pink, purple or violet	Saponins absent, probably free diterpene acids.

[a] Froth only evident after addition of sodium carbonate and shaking.

Some investigators have included a test for unsaturated sterols in their phytochemical surveys. In most instances, the Liebermann-Burchard (L-B) test[3] has been used to detect this class of compounds (422, 428–435, 478–483, 491, 558, 616), and it has been used to advantage in the differentiation of triterpenoid and steroidal saponins (558). According to Simes *et al.* (558), blue or blue-green colors are formed in the L-B test with steroidal saponins, and red, pink, or purple colors result if triterpenoids are present. However, when these workers applied the L-B test either directly to powdered plant material, or to a solution of extracted material, they noted that there was variation in the colors produced, depending on the manner in which the test was conducted. For example, ursolic acid (triterpenoid) gives a blue-green color in solution, but if the test is applied directly to solid material, the colors noted are only purple or violet (558). Simes *et al.* (558) do not comment on the time required to observe these characteristic reactions, whereas Wall *et al.* (422), using chloroform extracts of plant material, point out that interfering substances such as carotene and xanthophylls produce immediate color changes in the L-B test, as also do saturated sterols. However, when interfering substances are absent, unsaturated sterols give a minimal color density at the start of the test, and slowly reach a maximum after about 15 min. This delayed color reaction has also been observed by others (589, 591). Brieskorn and Herrig (592), in an investigation of the mechanism of the L-B reaction, found the following features essential for color formation. In the steroids, two conjugated double bonds in ring B, or one double bond and an unhindered methylene group at C_7 which can undergo oxidation and dehydration, are essential. In pentacyclic triterpenes, however, it is the methylene group at C_{11} in ring C that is involved. Steroid esters were shown to give a more intense color reaction in the L-B test than the corresponding alcohols, while the opposite was true for pentacyclic triterpenes and their esters.



Steroid Sapogenin



Pentacyclic Triterpenoid Sapogenin

The use of the froth test, in conjunction with the L-B reaction, has been found useful for the detection and differentiation of triterpenoid and steroidal saponins, triterpenoid sapogenins, free sterols, and other polycyclic substances (558). An outline of methods used for these identifications using the froth test, L-B reaction, and hemolysis, is presented in Table IX.

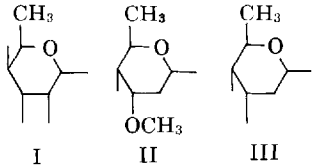
Willaman and Wadley (593) have found an interesting correlation existing between *Agave* and *Dioscorea* species with respect to the presence or absence of unsaturated sterols and steroidal saponins. They point out that strong positive sterol (L-B) tests on extracts from these 2 genera usually indicate an absence or very low steroidal saponin content. However, with *Yucca*, and undoubtedly with other groups of plants, no such association was indicated.

[3] The L-B test is usually conducted by adding a small amount of acetic anhydride–sulfuric acid mixture (19:1) to a solution of the sterol in suitable anhydrous solvent. A history of the test has been given by Dam (590).

It would appear that chromatographic differentiation of polycyclic substances in plant extracts could be used to advantage, especially since the advent of thin-layer chromatography which allows the use of corrosive reagents, since a great variety of steroid detecting reagents are available, *e.g.*, vanillin–phosphoric acid (594, 595), antimony trichloride (596–600), antimony pentachloride (596, 601), sulfuric acid (602), 50% sulfuric acid (603, 604), sulfuric acid–acetic anhydride (599, 605), chlorosulfonic acid (599), silicotungstic acid (596), phosphotungstic acid (606), zinc chloride (607), anthrone (608), furfural–sulfuric acid (609), sodium nitroprusside (610), Nessler's reagent (611), Millon's reagent (596), tetrazolium blue (612), phosphomolybdic acid (596, 613, 614), and Dragendorff's reagent (341). Axelrod and Pulliam (615) have developed several micro-methods for the detection of characteristic functional groups on the steroid nucleus which can be adapted to paper or thin-layer chromatography and which should be of great value in phytochemical screening.

Cardiac Glycosides

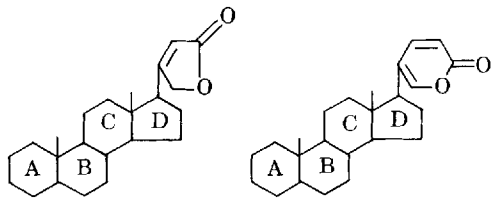
The importance of cardiac glycosides as therapeutic agents requires no elaboration. Their status in medicine, however, is not reflected in the number of published efforts to seek new and better agents of this type in the plant kingdom. As previously mentioned, a few surveys have been conducted in which plant extracts were evaluated for cardiotonic activity either through *in vitro* or *in vivo* biological tests (311–313). In the case of cardiac glycosides, the minimum structural features required for biological activity are so well defined that the substitution of a strictly chemical means of detection is an accepted procedure. All cardioactive glycosides are classified as steroids (sterols), having the cyclopentanoperhydrophenanthrene nucleus, an α-β-unsaturated lactone ring (5- or 6-membered) at C_{17}, a β-oriented hydroxyl at C_{14}, a *cis* fusion of the C and D rings at C_{13}-C_{14}, and the attachment at C_3 of one or more sugars, usually deoxyhexomethyloses (310). A 5-membered unsaturated lactone at C_{17} categorizes the glycoside



I II III

I, L-Rhamnose
II, D-Cymarose
III, D-Digitoxose

as a cardenolide, whereas a 6-membered unsaturated lactone at the same position exemplifies the bufadienolides. Of these 2 groups the cardenolides are the most frequently encountered and the most useful as therapeutic agents.



Cardenolides Bufadienolides

Detection of cardiac glycosides in plants has been effected by means of chemical tests applied either directly to a crude or semipurified extract, or following chromatographic separation of the glycosides. The latter method is to be preferred since a judicious selection of solvent systems and chromatographic conditions will free the glycosides from extraneous plant substances that either interfere with or mask the evidence of positive chemical tests.

Several methods for preparing an initial extract of the plant material have been reported, and methanol, 85% ethanol, or 80% ethanol appear to be the most useful solvents. The latter is advantageous since starch will not be extracted (617). Although a certain amount of enzymatic degradation of the native glycosides will occur, either as a result of the drying procedure or because of some processing step, this will not usually affect qualitative methods of detection since most of these are applicable to both native and secondary glycosides, as well as their aglycones. Bieleski (618) has considered the problem of halting enzyme action when extracting plant tissues, with respect to plant phosphatases, and although the situation may not be analogous, the general precautions and approach to the problem are worthwhile. The cardenolide detection method of Krider *et al.* (313) employs a paper chromatographic separation of a semipurified extract prior to the application of chemical detection methods. This purification is effected by means of lead hydroxide precipitation of nonglycosidic interfering substances. Certain extracts encountered in routine screening work, however, would require little purification, a step that is often time consuming and tedious.

Chemical tests applied to the detection of cardiotonic glycosides and/or aglycones are carried out by applying one or more reagents to a chromatogram of the extract (619–635) or to a piece of filter paper on which the extract was applied (486–488), or directly to the liquid extract (312). The available reagents are designed to detect

TABLE X.—CHROMOGENIC REAGENTS USED FOR THE DETECTION OF CARDIAC GLYCOSIDES

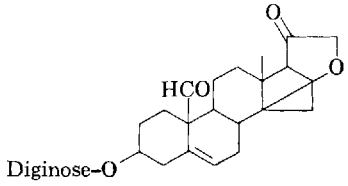
Reaction Site[a]	Common Name of Test	Major Constituent(s) of Reagent	Ref.
A	Baljet	2,4,6-Trinitrophenol–alkali	(630, 637, 833, 834)
A	Kedde	3,5-Dinitrobenzoic acid–alkali	(313, 627, 635, 638, 835)
A	Raymond	*m*-Dinitrobenzene–alkali	(630, 639, 640, 835)
A	Legal	Sodium nitroprusside–alkali	(630, 639, 641, 836)
A		1,3,5-Trinitrobenzene–alkali	(837, 838)
A		2,4-Dinitrodiphenylsulfone–alkali	(846)
A		1,2-Naphthoquinonesulfonate–alkali	(839)
A		Tetradinitrophenyl–alkali	(631)
A		*o*-Dinitrobenzaldehyde	(639)
A		1,3-Dinitrobenzaldehyde	(840)
A		Tetranitromethane	(841)
B	Kiliani	Ferric sulfate–sulfuric acid	(644)
B	Keller	Ferric chloride–acetic acid	(645, 842)
B	Keller-Kiliani	Ferric chloride–sulfuric acid–acetic acid	(632)
B	Pesez	Xanthydrol	(647, 649)
B	Tollens	Silver nitrate–ammonia	(646, 836)
B	Langejan	Orcinol–hydrochloric acid	(843)
B		Naphthoresorcinol–hydrochloric acid	(639)
B		*p*-Dimethylaminobenzaldehyde–phosphoric acid	(844)
C	Liebermann	Acetic anhydride–sulfuric acid	(589, 64)
C	Carr-Price	Antimony trichloride–acetic anhydride	(630, 649, 845)
C		Trichloracetic acid	(629, 630)
C		Trichloracetic acid–chloramine	(629, 630)
C		Sulfuric acid–ferric chloride–H_3PO_4	(847)
C		Sulfuric acid, 84%	(848)

[a] A, unsaturated lactone; B, desoxy sugar; C, steroid nucleus.

either the unsaturated lactone moiety at C_{17}, the deoxy sugar at C_3, or the steroid nucleus, the latter being the least specific for cardiotonic glycosides. These reagents are listed in Table X, together with common names frequently associated with tests involving their usage.

Most frequently utilized for the qualitative, as well as the quantitative evaluation of cardiac glycosides, have been the Baljet (2,4,6-trinitrophenol) (636, 637), Kedde (3,5-dinitrobenzoic acid) (638), Raymond (*m*-dinitrobenzene) (639, 640), or the Legal (sodium nitroprusside) reagents (641) which react with active methylene groups as found in the C_{17}-unsaturated lactone moiety (642, 643). These reagents give orange, purple, blue, and violet colors, respectively, with cardiac glycosides. Also utilized, but to a lesser extent, are reagents that react with the deoxysugar moiety of cardiac glycosides, negative tests of course being expected for aglycones. These include the Kiliani (ferric sulfate–sulfuric acid) (644), Keller (ferric chloride–acetic acid) (645), Tollens (silver nitrate–ammonia) (646), and Pesez (xanthydrol) (647) reagents. For detection of the steroid nucleus, the Liebermann (acetic anhydride–sulfuric acid) (648), and Carr-Price (antimony trichloride–acetic anhydride) (649) reagents are commonly used. Recent and thorough discussions of cardiac glycoside-detecting reagents have been published by Jensen (630), Rosenthaler (650), Cerri (651), and Frerejacque and DeGraeve (652). The latter paper is of particular value since it tabulates most of the useful reagents, gives formulas for their preparation, techniques for use, and presents remarks concerning the specificity and limitations of each.

In a phytochemical screening program for cardiac glycosides, initial positive tests on plant extracts using any one reagent should be confirmed with reagents specific for the 2 additional reactive sites (*vide supra*). For example, an initial positive Keller reaction is indicative only of the presence of a deoxy sugar. This should be followed by a second test which might be the Liebermann reaction for the steroid nucleus. Assuming that a positive reaction was also noted, a third and confirming test might be the Legal or Kedde test which denotes an unsaturated lactone at C_{17}. Positive tests with all 3 reagents offer reasonable assurance of the presence of cardioactive glycosides. If a negative test was noted with the Legal or Kedde reagent, in conjunction with positive reactions from the Liebermann and Keller tests, the presence of digitanol



Diginin—A Digitanol Glycoside

glycosides (diginin, digifolein, etc.) would be indicated since they lack the α-β-unsaturated lactone at C_{17} of the normal cardioactive glycosides and as a group are pharmacologically inactive substances (653).

Of particular importance in the interpretation of these tests is the observation of chromogenic reactions which may be transient, those which may change rapidly, or be dependent to a great degree on concentration of cardiac glycoside present. Certain of the older literature presents color reactions which are erroneous and confusing since the purity of either the glycosides being investigated, or the chemicals used in the reagents, must be questioned. Cook (589) has reviewed the color reactions of steroids in the Liebermann-Burchard reactions and presents a variety of possible colors for these compounds. In addition, he points out that in many instances, the chromogenic reactions are often delayed.

Using the tests enumerated herein may result in a few false-positive reactions for cardiac glycosides since deoxy sugars, α-β-unsaturated lactones, and steroids exist in other molecules found in plants (310). However, the use of tests for all 3 of these groups should greatly reduce the incidence of false-positive interpretations.

Table XI presents a summary of several phytochemical surveys that have been published concerning the detection of cardiac glycosides in a number of different plants. These and other data (310) point out that cardiac glycosides are present in at least 39 genera of 14 plant families.

Flavonoids and Related Compounds

The flavonoids are plant pigments based on the C_6-C_3-C_6 carbon skeleton as found in flavones, flavonols, isoflavones, flavonones, catechins, leucoanthocyanins, anthocyanins, aurones, and chalcones (Fig. 3). Although more than 33 different biological activities had been reported for some 30 of 137 known natural flavonoids up to the year 1955 (415), rutin is still the major useful member of this class of compounds as a medicinal agent. Several reviews on the biological activities and place in drug therapy of the flavonoids have been published (342, 344, 345, 415–418, 655), as have essays on their economic nonmedicinal applications (656–658). More recently, reports concerning the antiviral (659), anti-inflammatory (660), and cytotoxic (144, 661) activities of flavonoids have served to make them an exciting group of compounds for the researcher interested in biologically active phytoconstituents.

Flavonoids are widely distributed throughout the plant kingdom in the form of aglycones as well as heterosides. However, the latter are most frequently found in flowers, fruits, and leaves, whereas the former are usually most abundant in woody tissues. Some compounds are never found as heterosides, such as the nonhydroxylated and fully alkylated flavones, nobiletin, tangeretin, and meliternatin, and the polymethoxychalcone, pedicellin. These flavonoids have no hydroxyl groups with which a sugar residue can combine. It is difficult to state a general solubility rule for flavonoids because they range from water-soluble, ether-soluble (highly methylated nonheterosides), to ether- and alcohol-soluble (hydroxyflavone, flavanone, and isoflavone aglycones), to water-soluble, ether-insoluble (heterosides with up to 3 sugars) forms. However, when fresh plant material is extracted, methanol or ethanol will usually remove most flavonoids. Both flavonoid heterosides and aglycones are, as a general rule, insoluble in petroleum ether, and advantage can be taken of this fact to defat the sample prior to alcohol extraction. When dry plant material is to be extracted, several solvents should be sequentially used to insure complete removal of all flavonoids; however, in a screening program this is not usually feasible. Wall *et al.* (422, 428–435) have used 80% ethanol routinely in screening some 6000 plant accessions for flavonoids,

TABLE XI.—PHYTOCHEMICAL SURVEYS FOR CARDENOLIDES

Plant Source	Species Tested, No.	Method of Detection	Ref.
Nigeria	33	Keller-Kiliani and Kedde tests on plant extracts	(312)
Tibet	113	[a]	(513)
U.S.S.R.	[a]	[a]	(654)
Apocynaceae	22	Kedde test: paper chromatography	(620, 624)
Apocynaceae	5	Kedde, xanthydrol, $SbCl_3$ tests: extracts spotted on paper	(486, 487)
Asclepiadaceae	64	Kedde, xanthydrol, $SbCl_3$ tests: extracts spotted on paper	(488)
Moraceae	35	Kedde test: paper chromatography	(622, 625)
Periplocaceae	6	Kedde, xanthydrol, $SbCl_3$ tests: extracts spotted on paper	(488)
Misc. plants	17	Kedde test: paper chromatography	(313)
Misc. plants	10	Kedde, trichloracetic acid–chloramine and $SbCl_3$ tests: paper chromatography	(626)

[a] Original article unavailable; data not given in abstract.

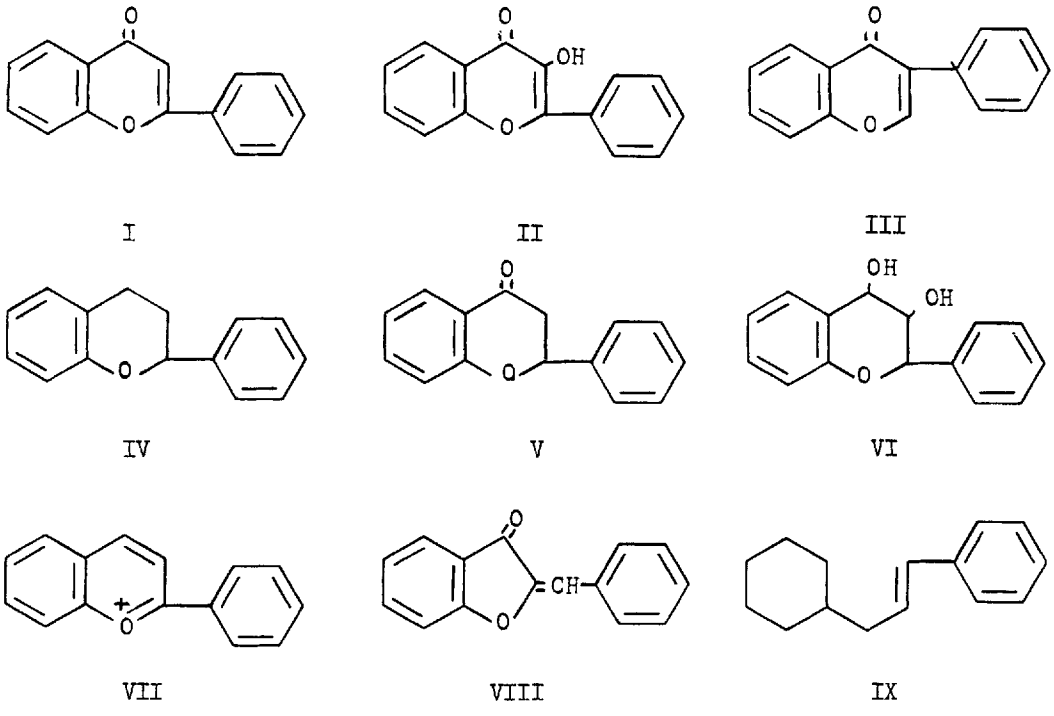


Fig. 3.—Types of flavonoids. Key: I, flavones; II, flavonols; III, isoflavones; IV, catechins; V, flavanones; VI, leuconanthocyanins; VII, anthocyanins; VIII, aurones; IX, chalcones.

the majority of the samples consisting of dry plant material.

A number of specific color reactions for various types of flavonoids have been reported that could be adapted to screening large numbers of plant samples, but specificity of a sort is usually not desirable for the initial testing. One of the most useful general tests is the so-called cyanidin reaction of Willstatter (662) which will detect compounds having the γ-benzopyrone nucleus. To an alcoholic solution of plant material is added a small piece of magnesium ribbon, followed by the dropwise addition of concentrated HCl.

Colors ranging from orange to red (flavones), red to crimson (flavanols), crimson to magenta (flavanones), and occasionally to green or blue are taken as evidence for a positive reaction for either the aglycone or heteroside. Colors usually develop within 1–2 min. following addition of the acid and are, of course, subject to variation in intensity depending on the concentration of flavonoid present in the sample (663). Problems often arise in the interpretation of positive tests when the plant extract being tested is highly pigmented. Bryant has modified the method so that heterosides and their aglycones can be differentiated (664). Xanthones have also been observed to give a positive cyanidin reaction (663).

Chalcones and aurones will not give the cyanidin reaction under the reducing conditions of this test. Direct addition of concentrated HCl to an ethanol extract of plant material containing either of these types of flavonoids, however, will result in an immediate red color (663).

Anthocyanins can be identified in plant material by extraction with cold 1% HCl followed by boiling and observation of the color at the boiling point. These plant pigments vary in color from orange-red to blue-red. Robinson and Robinson (665–668) first introduced this test which, through the use of immiscible solvent extractions, can differentiate many of the anthocyanins. A number of surveys for anthocyanins have been conducted, representing hundreds of plant species (665–680), and representative reviews on the subject of anthocyanins have been published (681–684).

Leucoanthocyanins can easily be detected by the method of Bate-Smith and Metcalfe (685) which has been utilized by Cain *et al.* (478–482) in their extensive screening of the New Zealand flora. The test involves digesting a small sample of plant material with 2 *N* HCl in 1-propanol for 15–30 min. A slow development of a strong red or violet color is indicative of a positive reaction.

Catechins give a blue or green color with ferric

chloride, but this test is of little value when crude plant extracts are being evaluated because of many other compounds that can give similar tests. Extraction of plant material with benzene, followed by extraction with ether, will yield a catechin-containing ethereal extract. Chromatography of this extract, followed by treatment of the chromatogram with 3% ethanolic *p*-toluenesulfonic acid and warming, will yield yellow spots if catechins are present (686).

A summary of flavonoid color reactions, following treatment with various reagents on paper chromatograms, is presented in Table XII.

In addition to the specific flavonoid surveys cited above, others of a more general nature have been published (99, 506, 507, 687–692). General review articles are available which should be of value to those who are interested in more specific information on testing for individual flavonoids such as the catechins (686), isoflavonoids (693–695), flavones (695–697), flavonols (697–698), dihydroflavonols (699), flavanones (700), chalcones (700), aurones (700), leucoanthocyanins (701), and the flavonoids generally (702–709).

Tannins

Two groups of phenolic constituents, hydrolyzable and condensed, comprise the tannins, substances which are important economically as agents for the tanning of leather, and for certain medicinal purposes. More recently, evidence has been presented in support of their potential value as cytotoxic and/or antineoplastic agents (146).

Hydrolyzable tannins are yellow-brown amorphous substances which dissolve in hot water to form colloidal dispersions. They are astringent and have the ability to tan hide. Chemically speaking, they are esters which can be hydrolyzed by boiling with dilute acid to yield a phenolic compound, usually a derivative of gallic acid, and a sugar. These are often referred to as pyrogallol tannins.

Condensed tannins (catechin tannins, phlobatannins) are polymers of phenolic compounds related to the flavonoids and are similar in general properties to the hydrolyzable tannins, but are not very soluble in water, and following treatment with boiling dilute acid, red-brown insoluble polymers known as phlobaphenes or tannin-reds are formed.

Tannins are detected most simply in plant extracts by the use of the so-called gelatin–salt block test (710) which has been utilized extensively in the phytochemical surveys of Wall *et al.* (422, 428–435). This test employs aqueous extracts prepared from 80% ethanol extracted plant material. A sodium chloride solution is added to one portion of the test extract, of 1% gelatin solution to a second portion, and the gelatin–salt reagent (710) to a third portion. Precipitation with the latter reagent, or with both the gelatin and gelatin–salt reagents is indicative of the presence of tannins. If precipitation is observed only with the salt solution (control), a false-positive test is indicated. Positive tests are confirmed by the addition of ferric chloride solution to the extract and should result in a blue, blue-black, green, or blue-green color and precipitate. Hoch (711) has applied some 33 different classical tannin detecting reagents to several tannin extracts; however, the nonspecificity of many of these would render them im-

TABLE XII.—COLOR REACTIONS OF FLAVONOIDS ON PAPER[a,b]

Type Flavonoid	Visible	U.V.	Ammonia Visible	Ammonia U.V.	Aluminum Chloride Visible	Aluminum Chloride U.V.	Na_2CO_3 Visible	$NaBH_4$ Visible	$ArSO_3H$[c] Visible
Flavones	Pale yellow	Dull-brown Red-brown Yellow-brown	Yellow	Bright yellow Yellow-green Dull-purple	Pale yellow	Fluorescent green Yellows Browns	Bright yellow	Colorless	Yellow
Flavonols	Pale yellow	Bright-yellow Yellow-green Browns	Yellow	Bright-yellow Yellow-green Green	Yellow	Fluorescent yellow or green	Yellow Yellow-brown Bluish	Colorless	Yellow
Isoflavones	Colorless	Faint purple Pale yellow[d]	Colorless	Faint purple Pale yellow	Colorless	Fluorescent yellow	Pale green	Colorless	
Catechins	Colorless	Colorless[d]	Colorless	Fluorescent pale blue Black	Colorless	Colorless Pale blue Yellow-white			Brown
Flavanones	Colorless	Colorless	Colorless	Colorless Pale yellow Yellow-green	Colorless	Fluorescent green-yellow Blue-white	Pale yellow-green	Magenta	Colorless
Leucoanthocyanins	Colorless	Colorless							Red Pink Purple
Anthocyanins	Pink Orange Red-purple	Dull red or purple Pink Brown[e]	Blue-gray Blue	Bluish					Unchanged
Aurones	Bright-yellow	Bright-yellow Green-yellow	Orange Orange-pink	Yellow-orange Orange Red-orange Brown	Pale-yellow	Fluorescent orange Brown Pink	Orange Brown Red	Colorless	Orange Pink
Chalcones	Yellow	Brown Black Yellow-brown	Yellow Orange Red-orange Pink	Orange Red Purple Black	Yellow Orange Yellow-orange	Fluorescent orange Brown Pink	Orange Brown Red	Colorless	Orange Pink

[a] Adapted from Seikel (849). [b] With ferric chloride and $K_3Fe(CN)_6$, all produce blue colors. [c] *p*-Toluenesulfonic acid, after heating. Dihydroflavonols change from colorless to yellow, becoming flavonols. [d] Short wavelength U.V.: isoflavones, yellow; catechins, black. [e] 3,5-Diglycosides are either fluorescent yellow or rose or bright red or purple. [f] Only derivatives of cyanidin, delphinidin, and petunidin change color.

practical for use in general phytochemical screening work.

Several phytochemical surveys for tannins in plants of Argentina (712), Bulgaria (713), China (495), Japan (168), Mexico (507), Nigeria (99, 100), Pakistan (714), Tibet (513), the U.S.S.R. (715–718), as well as from hundreds of additional miscellaneous plants (2, 5, 422, 428–435, 529), have been conducted. Happich *et al.* (2) have emphasized, from a practical viewpoint, the requirements necessary in a tannin for it to be of commercial value.

Coumarins

Coumarins are benz-α-pyrone derivatives found widely distributed in plants; however, they occur most frequently in members of the *Gramineae, Orchidaceae, Leguminosae, Umbelliferae, Rutaceae,* and *Labiatae.* They rarely, if ever, occur in the *Liliaceae, Onagraceae, Pinaceae,* or *Cactaceae.* The medicinal value of certain coumarin derivatives (bishydroxycoumarin, 8-methoxypsoralen) is well known; however, according to Soine (419), the full range of biological activities for these compounds is not appreciated by most investigators. Reviews by Bose (719) in 1958, and more recently by Soine (419) in 1964, have served to point out the biological activities of coumarins, a group which appear most interesting because of their anticoagulant, estrogenic, dermal photosensitizing, antibacterial, vasodilator, molluscacidal, anthelmintic, sedative and hypnotic, analgesic, and hypothermal effects (419, 719).

Several types of coumarin derivatives have been found in plants, the substituted coumarins, furanocoumarins, pyranocoumarins, benzocoumarins, phenylcoumarins, and others.

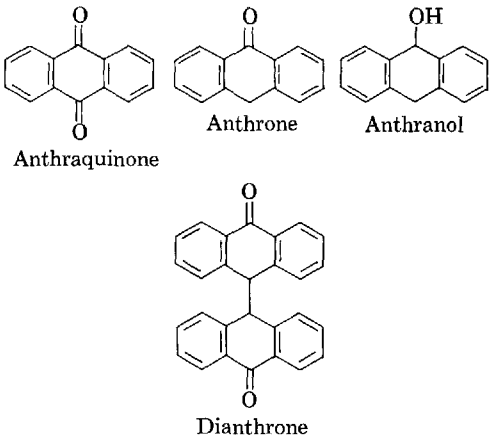
Coumarin itself can be easily detected in plant material simply by placing a small amount of moistened sample in a test tube and covering the tube with filter paper moistened with dilute sodium hydroxide solution. The covered test tube is then placed in a boiling water bath for several minutes, the paper removed and exposed to ultraviolet light. If coumarin is present, a yellow-green fluorescence appears within a few minutes (720). This procedure, however, is applicable only to coumarin and related volatile compounds. Most methods that appear useful for detecting coumarins in plant material are based on extraction of the plant sample, followed by chromatography of the extract and revelation of the coumarins with spray reagents such as diphenylboric acid, β-aminoethyl ester (721), KOH and diazotized sulfanilic acid (722), or uranyl acetate (723).

Plant material is best extracted by first quick-washing it with ice cold petroleum ether to remove lipids. Most coumarins are insoluble in this solvent, especially if it is kept cold. The defatted sample can then be extracted with warm, dilute alkali hydroxides which form the water-soluble coumarinate. Neutral organic impurities then are extracted with ether and on acidification of the aqueous coumarinate solution, the parent coumarin reforms and is removed by ether extraction. Acidic impurities are then removed by extraction with sodium bicarbonate solution. The ether solution can then be concentrated and used for chromatographic studies with specific reagents (*vide supra*), or as a spot test in which a drop of concentrate is treated, in a porcelain dish, with a drop of saturated solution of hydroxylamine hydrochloride in alcohol and 1 drop of saturated alcoholic solution of KOH. This mixture is heated over an open flame until it begins to bubble. After cooling, 0.5 *N* HCl is added to acidify the mixture, followed by a drop of 1% ferric chloride solution. A positive test consists of a violet color and is indicative of the presence of lactones (724). Other tests (725–728) have been utilized for the detection of coumarins in plant materials; however, only a slight interest has been noted in testing for coumarins, as evidenced by limited inclusion of tests for this group in phytochemical surveys (494, 513, 616).

Anthraquinones

The largest group of naturally occurring quinone substances are the anthraquinones. Although they have a widespread use as dyes, their chief medicinal value is dependent upon their cathartic action. They are of restricted distribution in the plant kingdom and are found most frequently in members of the *Rhamnaceae, Polygonaceae, Rubiaceae, Leguminosae,* and *Liliaceae.* As found in plants, they are usually carboxylated, methylated, or hydroxylated derivatives of the anthracenes, anthrone, anthranol, anthraquinone, or dianthrone. Hydroxylated anthracenes often occur as heterosides linked with various sugars through one of the hydroxyl groups (*O*-heterosides). Other types of anthracene heterosides are represented as C-heterosides in which the sugar and aglycone are linked by a carbon to carbon bond.

For the qualitative detection of anthraquinones in plant material, the Borntrager reaction, as modified by Kraus (729), appears to be simplest to perform in the application to phytochemical screening. The powdered sample (0.3 Gm.) is boiled for a few minutes with 0.5 *N* KOH (10 ml.) to which is added 1 ml. of dilute hydrogen per-



oxide solution. After cooling, the mixture is filtered and 5 ml. acidified with 10 drops of acetic acid. This acidulated mixture is then extracted by shaking with 10 ml. of benzene in a separator and the benzene layer takes on a yellow color. A 5-ml. sample of this benzene extract is shaken with 2.5 ml. of ammonium hydroxide, and a positive reaction for the presence of anthraquinones is evidenced by the formation of a red color in the alkaline layer. Normally, if C-glycosides are present in a sample being evaluated for anthraquinones, they will not be detected by the usual Borntrager reaction, as C-glycosides require special methods for cleaving the sugar from the aglycone. This can be done with ferric chloride, sodium dithionate or, as described above, with peroxide in an alkaline medium. It has been shown that this method results in a mixture of products (730); however, this is not a disadvantage for a general screening test. Other simple and rapid spot tests, which involve the direct addition of a reagent to the solid sample (powdered drug), have been described. They should be useful in phytochemical screening (731, 732), but to date have not been shown to be applicable for this type of work.

Phytochemical surveys for anthraquinones have been found only infrequently in the literature (494, 513).

Cyanogenetic Glycosides

Compounds in plants that liberate hydrocyanic acid (HCN) following hydrolysis are of major importance because of their potential danger as poisons to livestock as well as humans (733–735). Also, the use of at least one cyanogenetic glycoside, (−)-mandelonitrile-β-glucuronoside (laetrile), for the treatment of human neoplastic disease has been suggested (736), but this remains as a controversial subject. Should cyanogenetic glycosides prove useful in this respect, the plant kingdom should be expected to be a source for new and similar compounds.

It is generally recognized that a small amount of HCN occurs free in plants, but that the greatest amount is combined in glycosidic linkage (737). Although high concentrations of cyanogenetic glycosides have been reported present in the seeds of only a few species of plants, *i.e.*, those of *Rosaceae*, lesser amounts have been reported in almost every plant tissue from certain other plants. No general rule can be established with regard to the distribution of these compounds in various plant tissues. Cyanogenetic compounds are widely distributed, having been detected in more than 50 natural orders of plants, including ferns, basidiomycetes, and phycomycetes (737). Wherever cyanogenetic compounds are found in plants, specific hydrolytic enzymes are also known to exist, although a few exceptions are known (737). Also, there is some evidence that emulsin, ordinarily thought to be specific as a catalyst for the hydrolysis of all cyanogenetic glycosides, will hydrolyze certain of these compounds only with difficulty, and some are known to be unreactive in the presence of this enzyme (737).

Methods for the qualitative as well as quantitative determination of cyanogenetic glycosides in plant and animal tissues have been extensively reviewed by van der Walt, who considers such methods as the Guignard, Vortman, phenolphthalein, guaiac–copper, benzidine acetate–copper acetate, iodine–starch, Prussian blue, and the ferrous–uranic nitrate tests (735). The most widely used of these tests for the qualitative detection of cyanogenetic glycosides in plants has been the Guignard test. According to van der Walt (735), it is nonspecific since any volatile reducing agent such as hydrogen sulfide, sulfur dioxide, or aldehydes will give a positive test. Volatile reducing agents in plant extracts, however, are not frequently encountered and the Guignard test appears to be the simplest, most rapid, and accurate means by which the presence of cyanogenetic compounds can be established in plant specimens. This test, as described by Burnside (733), is conducted by placing about 2 Gm. of moist shredded plant material or crushed seeds in a small test tube, followed by the addition of 4 drops of chloroform (to enhance enzyme activity, see *reference 738*). Sodium picrate solution (5 Gm. Na_2CO_3, 0.5 Gm. picric acid, water *q.s.* 100 ml.; stable for 4 months if kept cool and well stoppered) is prepared and strips of filter paper are saturated with the solution. The strips then are blotted dry and inserted between

split cork stoppers which are then introduced into the neck of the test tube containing the reaction mixture. Care should be exercised to insure that the paper strips do not touch the inner sides of the test tube. The test tube and contents are then warmed at 30–35° for up to 3 hr. Large concentrations of HCN are detected within 15 min. as evidenced by a change in color of the yellow picrate test paper to various shades of red. Absence of a red color after 3 hr. is taken as a negative test.

Plant materials not analyzed for HCN at the time of collection can lose a large amount of their cyanogenetic glycoside content through spontaneous hydrolysis. Briese and Couch have shown that if chopped fresh plant material is stored in an aqueous mercuric chloride solution (1 Gm. $HgCl_2$ for each 100 Gm. of fresh plant material), the cyanogenetic glycosides present in the preserved specimens will remain stable for up to 6 months, presumably through an inactivation of the hydrolytic enzymes (739). van der Walt (735), however, claims that the relationship of mercuric chloride to the water volume in the preservative is more important than the mercuric chloride–plant material ratio for glycoside stabilization.

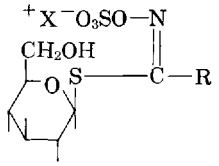
To insure that hydrolysis of cyanogenetic glycosides will indeed take place, some investigators have added emulsin (or other specific enzymes) to all plants being evaluated for the presence of these compounds. Other investigators have carried out the hydrolysis in the presence of buffer (740). Although it has been determined that cyanogenetic glycosides are hydrolyzed by dilute acids to yield HCN, this method has not been applied to the screening of plants for cyanogenetic glycosides (737). Concentrated mineral acids are to be avoided since HCN is not released under these conditions (737).

Although it appears that the Guignard test is adequate for screening plants for cyanogenetic glycosides, the precautions enumerated above should be taken into consideration if the results are to be meaningful.

Several screening programs have been conducted for the presence of cyanogenetic compounds in plants from Africa (735), Australia (423), Hong Kong (499), North Borneo (491), and the U.S.S.R. (741), in addition to others (734, 742–745).

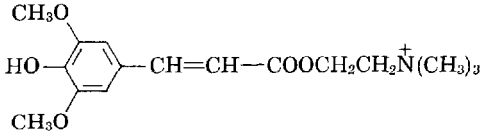
Isothiocyanate Glycosides

This group of interesting compounds will be discussed since plants containing isothiocyanates have been implicated in human as well as livestock poisoning (746), because of the antibacterial and fungistatic potential of the glycosides (747) and because of their unique, yet restricted distribution, which makes them valuable in chemotaxonomic studies (748–753). Isothiocyanate glycosides are found most abundantly in the *Cruciferae*, as well as in the related families *Capparidaceae*, *Moringaceae*, and *Resedaceae* of the Rhoeadales (746). Also, their unexpected occurrence in the taxonomically remote *Caricaceae*, *Euphorbiaceae*, *Limnanthaceae*, *Phytolaccaceae*, *Plantaginaceae*, *Salvadoraceae*, and *Tropaeolaceae* has been reported (746). Whenever mustard oil glycosides are found in a particular species, they appear to accumulate chiefly in seeds, as well as being found throughout the plant (746). The general type formula for these colorless, water-soluble compounds is illustrated.



Isothiocyanates

In all cases observed to date, glucose has been found to be the glycone moiety of isothiocyanate glycosides, thus substantiating the more common designation of this class of compounds as isothiocyanate glucosides. Because of the preponderence of potassium in plants, this element is usually found as the cation of the sulfuric acid residue. Sinalbin, the classical glucoside of white mustard, is unique in that it contains sinapine, a quaternary base, as its cationic moiety.



Sinapine

Myrosinase (myrosin), the specific enzyme catalyzing the hydrolysis of all isothiocyanate glycosides has been found in all plants containing this substrate. However, it accumulates in particular cells (idioblasts) which do not contain the glycosides (746). This enzyme has been stated to be most active at pH 6.5–7.5 and at a temperature of 30–40° (746).

About half of the known 30 or more natural mustard oils are nonvolatile isothiocyanates (cheirolin, erysolin, *p*-hydroxybenzylisothiocyanate) and as such are not steam distillable. Therefore, the practice of referring to this class of

compounds as "essential oils" should be discontinued.

Early methods for the detection of isothiocyanates consisted of hydrolysis followed by organoleptic evaluation of the liberated aglycone. As a result, only the volatile isothiocyanates were recorded. Today, however, with the availability of chromatographic techniques and rather specific detecting reagents, the analysis of plant material for isothiocyanates has become much more exacting. Although a number of microchemical tests for isothiocyanates and their glycosides have been reported (754–756), the preferred method for their detection in plant material appears to be through extraction of small samples (2–5 Gm.), followed by conversion of the natural *iso*thiocyanates into thiourea derivatives, and paper chromatographic separation of the mixture using water-saturated chloroform (757), ethyl acetate–water (758), 2-butanone–water (758), pyridine–amyl alcohol–water (759, 760), heptane–90% formic acid–*n*-butanol (759, 760), or *n*-butanol–ethanol–water (761). Visualization of the separated compounds is then accomplished by means of Grote's reagent (modified nitroprusside) (757), by silver nitrate (762, 763), or by the iodineazide reagent of Feigl (764) as modified by Kjaer (757).

A useful list of mustard oil-containing plants known up to 1938 has been published by Schmalfuss and Muller (765). Schneider (766) presents a reliable key to the older literature on isothiocyanates, and recent reviews on this topic have been published by Delaveau (748–750), Hegnauer (751), Zinner (752), and Kjaer (753, 767, 768). In addition, several surveys for isothiocyanates in plants have been conducted (767–773).

Essential Oils

Essential oils (volatile oils), in addition to their value as flavoring agents and perfumes, have been reported to have excellent antibacterial (201, 202, 204) and antifungal (202–204) properties.

A few reports have been published which include an evaluation of plant samples for the presence of essential oils (168, 491, 499, 500, 513, 774–778). For maximum efficiency, tests should be conducted on fresh material since most of the volatile constituents of plants are lost during drying. In most instances the methods that have been employed to detect essential oils have been crude since they have of necessity been conducted in the field as organoleptic examinations. More elaborate laboratory examinations have involved steam distillation followed by measurement of the water-immiscible oil and, in some cases, followed by the application of chemical tests for terpenes.

Arthur (491, 499, 500) studied more than 700 species in North Borneo and Hong Kong, and simply chopped a small amount of fresh plant with a razor, introduced this into a test tube, added hot water, and boiled the mixture. Any characteristic odor of essential oils was then recorded. Kohlmunzer (777) evaluated some 59 species of plants from genera known to have previously yielded economically important essential oils (*Salvia*, *Lavandula*, *Mentha*, *Rosmarinus*, *Thymus*, etc.). Following steam distillation and subsequent measurement of the separated oil, chemical tests for cineol were applied. On the other hand, Betts (779) has devised a method employing thin-layer chromatography for the evaluation of petroleum ether extracts, from umbelliferous fruits, in which essential oils are universally soluble. Fluorescein-treated plates of his extracts were first viewed under ultraviolet light to note the presence of dark quenching spots against a bright yellow background. Unsaturated compounds were then detected as spots by exposure of the plates to bromine vapor which converted the fluorescein to eosin, and subsequent ultraviolet examination then indicated unsaturated compounds against a dull background. Plates were then sprayed with 2,4-dinitrophenylhydrazine which revealed ketones and aldehydes as orange spots.

A simple microcohobation still for the estimation of quantities of essential oil ranging from 2–50 μl. in small (0.4 Gm.) samples of plant material has been shown to give accurate results and could be of value in screening large numbers of plant samples for essential oils on a quantitative basis (780). At least this would be some improvement over current organoleptic methodology.

SUMMARY

This review, to the best of this author's knowledge, is the first dealing with phytochemical and phytopharmacologic screening to be published. Therefore, out of necessity, a number of perhaps important topics were not discussed, and certainly some of those included were treated to a lesser degree than one might consider adequate.

It was the desire of this author to make several facts evident through a review of the areas covered in this manuscript: (*a*) that natural products have been, and still remain as, an interesting and important source of biologically active substances, the major sources of which remain untapped; (*b*) that although many methods are

available for the biological as well as chemical screening of plants, these are largely inadequate, and there is a great need for an increased interest in the development of improved methodology; (*c*) that a great deal of needless duplication of effort is evident in certain of the results presented in this review; and (*d*) correlations made and reported here point out the need for a restimulus of interest in this area of research.

The number of published reports on phytochemical and phytopharmacologic surveys is exceedingly small in view of the amount of effort that is undoubtedly being expended in these areas. Several explanations for this are suggested: (*a*) some investigators feel that this type of research should not be published because much of it represents so-called "negative" data; (*b*) others undoubtedly do not care to be associated with "screening" publications; and (*c*) a great deal of this work is being conducted in laboratories having a vested and commercial interest so that publication of their results would reveal methodology and approaches to this area of investigation that might be considered "unique" and/or classified. However, those reluctant to publish these types of data are usually exceedingly interested in hearing of, or utilizing similar results by others, in planning their own approach to phytochemical-phytopharmacologic problems, or in making certain correlations with their own data.

Natural product studies involving the search for new biologically active substances present unique challenges and problems not fully appreciated by many persons having only a limited appreciation and breadth of knowledge in biological areas. Many of the problems, either enumerated or suggested by studies reported here, can be solved if proper attention is given to the remedies. In the past, at least in this country, we have not had the organizational tools through which natural product studies could be considered in their own light. This is not true today, for with proper effort expended in its own behalf, the area of natural products can assume a more important place in the scientific community to serve humanity better.

Those of us interested in new biologically active phytoconstituents are constantly reminded, either verbally, through innuendo, or by means of restricted budgets and limited availability of research funds, of the "scientifically" unsound approaches that are used in our quest for new drugs. But, can methods other than those delineated in this review be suggested? And the work must continue.

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Excretion of Nortriptyline Hydrochloride in Man I

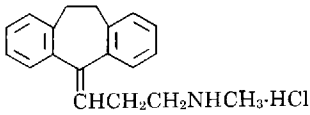
Detection and Determination of Urinary Nortriptyline

By M. E. AMUNDSON* and J. A. MANTHEY

A quantitative U.V. spectrophotometric assay procedure has been developed for the determination of urinary nortriptyline in man. A thin-layer chromatographic system is described which allows rapid detection of the presence of the drug and its metabolites in urine. The drug was found to be slowly eliminated as a mixture of free drug and metabolites.

NORTRIPTYLINE hydrochloride,[1] 5-(3-methylaminopropylidene) - 10,11 - dihydro - 5*H*-dibenzo[*a*,*d*]cycloheptene hydrochloride, has recently been introduced as an antidepressant. This communication describes an assay procedure for the quantitative determination of the drug in the urine of patients receiving therapeutic doses.

The need for urine tests for the detection and/



$CHCH_2CH_2NHCH_3 \cdot HCl$

Nortriptyline HCl

or determination of phenothiazine and related drugs in chronic mental patients has been established (1). The assay method reported here permits a reliable evaluation of drug intake by patients treated with nortriptyline hydrochloride.

EXPERIMENTAL

Drug Administration and Urine Collection.—All subjects except the normal male used for the single dose experiments were under treatment at Central State Hospital, Indianapolis, Ind., and were on a regular oral dosage schedule which ranged from 50–150 mg./day depending upon the individual patient. Twenty-four hour urine collections were made from 7:30 a.m.–7:30 a.m. For the single-dose experiments, the subject received an oral dose of 25 or 30 mg., and urine was collected at regular intervals up to as long as 72 hr. after dosage.

Assay Method.—*Method A.*—An aliquot of the urine (25 ml. for chronic studies, 100 ml. for single dose studies) was transferred to a 250-ml. separator. The urine was made distinctly alkaline with 50% sodium hydroxide and was extracted for 3 min. with 2 successive 50-ml. portions of ether. The extracted urine was saved for *Method B*. The ether was washed with 25 ml. of 0.1 *N* sodium hydroxide and 25 ml. of water, respectively. The washes were discarded. The ether was extracted for 3 min. with 2 successive 10-ml. portions of 0.1 *N* hydrochloric acid. The acid extracts were collected in a 25-ml. volumetric flask, and the solution was made to 25 ml. with additional acid. The absorbance of this solution (solution *A*) then was determined on a suitable spectrophotometer at the $\lambda_{max.}$ of approximately 240 mμ.

Method B.—The extracted urine from *Method A* was neutralized with 6 *N* hydrochloric acid and 0.2 vol. of concentrated hydrochloric acid was added. The solution was heated on a steam bath for 1 hr. and then was allowed to cool to room temperature. The solution was made alkaline with 50% sodium hydroxide using an ice bath, if necessary, to prevent excessive warming. The solution was then extracted with ether as in *Method A*. The absorbance of the acid extract (solution *B*) was determined at the $\lambda_{max.}$ of approximately 290 mμ.

Method C.—One-tenth volume of concentrated hydrochloric acid was added to an accurately measured aliquot of solution *A*. After heating for 1 hr. on a steam bath, the solution was allowed to cool and was diluted to an appropriate volume with water. The absorbance of this solution was determined at the $\lambda_{max.}$ of approximately 290 mμ.

Methods *A*, *B*, and *C* were repeated on an equal volume of control urine to establish blank urine values.

Calculations.—All results were calculated as equivalents of nortriptyline hydrochloride.

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[1] Marketed as Aventyl HCl by Eli Lilly and Co., Indianapolis, Ind.

$$\frac{\text{abs}_{240m\mu}\,(\text{soln. } A) - \text{abs}_{240m\mu}\,(\text{blank } A)}{0.482^{2}} \times \frac{10 \text{ mcg.}}{\text{ml.}} \times \frac{\text{total urine vol.}}{\text{vol. of aliquot taken}} \times \frac{1}{1000} =$$

mg. unchanged nortriptyline + "free" hydroxylated nortriptyline (X)

$$\frac{\text{abs}_{290m\mu}\,(\text{soln. } B) - \text{abs}_{290m\mu}\,(\text{blank } B)}{0.433^{3}} \times \frac{10 \text{ mcg.}}{\text{ml.}} \times \frac{\text{total urine vol.}}{\text{vol. of aliquot taken}} \times \frac{1}{1000} \times \frac{1}{0.85^{4}} =$$

mg. "bound" hydroxylated nortriptyline (Y)

$$\frac{\text{abs}_{290m\mu}\,(\text{soln. } C) - \text{abs}_{290m\mu}\,(\text{blank } C)}{0.433^{3}} \times$$

same as for calculation of Y = mg. "free" hydroxylated nortriptyline (Z)

$X - Z$ = mg. unchanged nortriptyline hydrochloride, and $X + Y$ = total mg. excreted expressed as nortriptyline hydrochloride equivalents.

Thin-Layer Chromatography.—Ether extracts of several urine samples were examined chromatographically for the presence of unchanged drug and metabolites. Standard size (20 × 20 cm.) glass plates were coated with a 250-μ layer of Silica Gel G. The plates were air-dried overnight before use. The developing solvent consisted of isopropanol–water, (88:12), one-third saturated with sodium chloride. The presence of free drug and metabolites was detected by spraying the plates with 1:1 3A ethanol–sulfuric acid followed by heating in a 90–100° oven for 75 min.

Figure 1 is a representative chromatogram of the extracts from 2 urine samples from patients who had received nortriptyline hydrochloride. The urine was extracted with ether as in *Method A* of the assay procedure. After washing with alkali and water, the ether was evaporated to dryness. The residue was redissolved in a small volume of methanol and was applied to the plates. The plates were placed in the developing solvent for approximately 2.5 hr. The plates were removed, air-dried, and sprayed with the ethanolic sulfuric acid. The plate was then placed in the oven to develop the characteristic colors given in Fig. 1.

RESULTS AND DISCUSSION

McMahon *et al.* (2) investigated the metabolism of nortriptyline-*N*-methyl-^{14}C in rats, and *N*-demethylation and hydroxylation of one of the bridgehead carbons were the major metabolic changes observed. Hucker (3) reported that the 2 major metabolic reactions of amitryptyline, the dimethylamino analog of nortriptyline, were also *N*-demethylation and hydroxylation. The metabolism of nortriptyline in man is currently under investigation and will be the subject of a future report.

The findings of McMahon *et al.* (2) were applied to the development of this assay method. When urine from a patient who had received nortriptyline was extracted with ether, a considerable amount of material which absorbed at 240 mμ was obtained. However, only a small percentage of this material was identified as unchanged nortriptyline by thin-layer chromatography (Fig. 1). Acid hydrolysis of the extracted urine yielded upon subsequent extraction a material which exhibited 290 mμ absorbance which is characteristic of the molecule in which the dimethylene bridge of nortriptyline has been replaced by a double bond as previously reported (2, 4). Acid hydrolysis of the material which had been obtained initially by ether extrac-

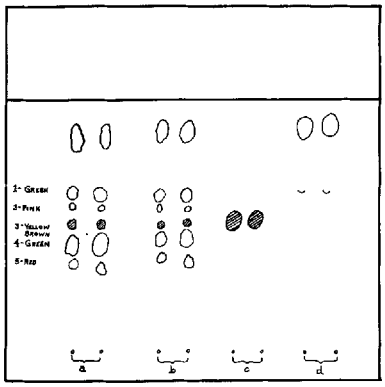

Fig. 1.—Thin-layer chromatography of urine extracts from patients receiving continued doses of nortriptyline hydrochloride. Key: a, urine extract No. 1902; b, urine extract No. 1903; c, nortriptyline; and d, control urine extract. Zone 3 identified as nortriptyline and zone 4 tentatively identified as 10-hydroxy nortriptyline. Colors shown are those observed after spraying with 1:1 ethanol–sulfuric acid and heating the plate in a 90–100° oven for 75 min.

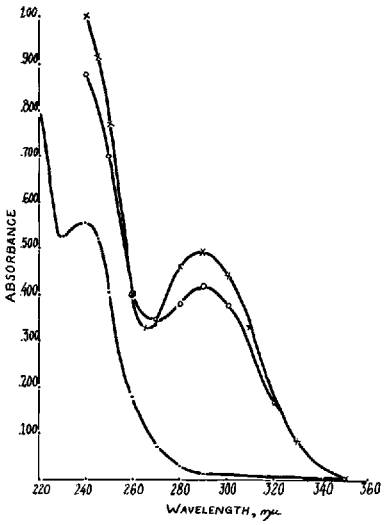

Fig. 2.—Typical U.V. absorption curves obtained on assay solutions. Key: ●, solution A = unchanged nortriptyline + "free" hydroxy derivative; ×, solution B = "bound" hydroxy derivative; and ○, solution C = "free" hydroxy derivative. Curves B and C were obtained after acid hydrolysis.

[2] 0.482 is the measured absorbance of a 10 mcg./ml. solution of nortriptyline hydrochloride in 0.1 *N* hydrochloric acid.

[3] 0.433 is the calculated absorbance of a 10 mcg./ml. solution of acid-hydrolyzed hydroxylated nortriptyline.

[4] 0.85 represents the recovery factor determined by adding hydroxylated nortriptyline to urine and hydrolyzing with acid as in *B* and *C*.

TABLE I.—URINARY EXCRETION OF NORTRIPTYLINE HYDROCHLORIDE (24-hr.)

Patient	Dose	Nortriptyline Equivalents, mg. Nortriptyline HCl, mg.	"Free" Metabolite	"Bound" Metabolite	Total	Av. % Dose
A	25 mg. bid	1.5	27.3	23.8	52.6	94.0
	25 mg. bid	2.6	18.0	20.6	41.2	
B	25 mg. qid	1.5	24.0	2.1	27.6	27.6
C	25 mg. qid	3.4	32.2	26.5	62.1	62.1
D	25 mg. qid	4.2	47.6	44.7	96.5	75.0
	25 mg. qid	2.6	28.9	22.0	53.5	
E	25 mg. qid	1.1	32.9	23.2	57.2	58.6
	25 mg. qid	3.9	43.1	21.8	68.8	
	25 mg. qid	1.9	34.5	13.5	49.9	
F	25 mg. qid	2.7	22.2	16.7	41.6	41.6
	25 mg. qid	5.3	22.0	16.3	43.6	
	25 mg. qid	4.3	30.8	10.4	45.5	
	25 mg. qid	3.0	24.6	8.3	35.9	
G	25 mg. qid	0.5	7.2	10.5	18.2	49.8
	25 mg. qid	0.8	35.7	35.3	71.8	
	25 mg. qid	1.6	37.0	35.8	74.4	
	25 mg. qid	1.0	29.5	4.5	35.0	
H	50 mg. tid	3.0	36.5	40.8	80.3	53.5
					Av.	57.8

tion of the urine exhibited the same U.V. absorption pattern. Typical curves obtained from extracts of urine before and after hydrolysis are shown in Fig. 2. It is assumed, therefore, that hydroxylation of nortriptyline does occur in man and that only part of this metabolite is subsequently conjugated.

Additional metabolites are present in the urine of man as evidenced by the chromatogram shown in Fig. 1. Only small amounts of unchanged drug were excreted (zone 3). Four metabolites were apparent in the extracts. Zone 4 has been tentatively identified as 10-hydroxynortriptyline and is present in the greatest concentration. The remaining 3 metabolites are currently under investigation. The relatively large zones near the solvent front apparently have no relationship to the metabolism of nortriptyline and represent extraneous material carried through the extraction procedure.

Triplicate assays of a urine sample by the above procedure showed a total excretion of 61.2 ± 1.8 (S.D.) mg. of nortriptyline equivalents.

Pure 10-hydroxy nortriptyline was not available to use as a standard in the assay procedure. The 0.433 absorbance value used in the calculations was calculated from the data of Villani *et al.* (4) on amitryptyline and its derivatives and represents the theoretical absorbance of a 10 mcg./ml. solution of the hydrochloride salt of the unsaturated molecule which results from the acid hydrolysis of the 10-hydroxy metabolite. Addition of a known concentration of impure 10-hydroxy material, obtained by extraction of a patient's urine to a control urine and subsequent acid hydrolysis and extraction, resulted in a recovery of 85% based on the calculated absorbance of 0.433.

Several of the patients received other drugs in addition to nortriptyline hydrochloride. Phenaglycodol[5] (1200 mg. daily), chlorothiazide[6] (500 mg. daily), perphenazine[7] (24 mg. daily), trifluoperazine[8] (45 mg. daily), and fluphenazine[9] (15 mg. daily) did not interfere with the assay procedure. Chlorpromazine[10] (300 mg. daily) did interfere in the extraction procedure, as was evidenced by the U.V. absorption curve obtained on the extract.

It was not possible to obtain control urine specimens from the patients: therefore, the results obtained from these patients are uncorrected. A 25-ml. sample of control urine from a normal female gave insignificant absorption readings when carried through methods *A* and *C*, and the blank resulting from hydrolysis of the extracted urine sample (*Method B*) was equivalent to 1.1 mcg. of nortriptyline equivalents per ml. of urine. Control urine collected from the subject, who was given a single dose of the drug, gave values of 1.5, 2.1, and 2.1 mcg. of nortriptyline equivalents per ml. of urine for methods *A*, *B*, and *C*, respectively, and the results were corrected. In this instance 100 ml. of urine was used as the aliquot for assay. Since 25-ml. aliquots were used for all assays on the chronic administration study, blank values would be relatively insignificant.

Table I is a summary of the data obtained on 24-hr. urine collections from patients on a chronic dosage regimen of nortriptyline hydrochloride. All patients had been receiving the drug for several months. In the limited number of patients studied there appears to be a correlation between the dose administered and the amount of drug recovered in the urine. Individual fluctuations in the rate of excretion of the drug are quite apparent, however. It is evident that only small amounts of unchanged nortriptyline are excreted and that a somewhat larger amount of the hydroxy metabolite is excreted in the "free" than in the "bound" or conjugated form. In the 8 patients studied, an average of about 58% of the administered dose was excreted in the urine in a 24-hr. period.

Single doses of 25 and 30 mg. were given to a normal, healthy male to determine the excretion pattern after a single dose. These data are given in Table II. In this subject the maximum excretion

[5] Marketed as Ultran by Eli Lilly and Co.
[6] Marketed as Diuril by Merck Sharp and Dohme.
[7] Marketed as Trilafon by Schering Corp.
[8] Marketed as Stelazine by Smith Kline & French. Marketed as Prolixin by E. R. Squibb & Sons.
[10] Marketed as Thorazine by Smith Kline & French.

TABLE II.—NORTRIPTYLINE HYDROCHLORIDE EXCRETION AFTER SINGLE DOSES

Dose, mg.	Collection Interval, hr.	Nortriptyline HCl	"Free" Metabolite	"Bound" Metabolite	Total	% of Dose
25	0–24	Negligible	4.6	4.9	9.5	38.0
30	0–24	1.0	3.2	3.4	7.6	25.3
	24–48	0.8	2.1	2.0	4.9	16.3
	48–72	0.8	0.7	1.7	3.2	10.7
	Totals	2.6	6.0	7.1	15.7	52.3

TABLE III.—NORTRIPTYLINE HYDROCHLORIDE EXCRETION AFTER 3 SUCCESSIVE DAILY 10-mg. DOSES

Day	Dose, mg.	Nortriptyline HCl	Nortriptyline Equivalents, mg.[a] "Free" Metabolite	"Bound" Metabolite	Total
1	10	0.96	0.98	1.91	3.85
2	10	0.95	0.84	2.35	4.14
3	10	0.90	2.36	2.52	5.78
4	None	0.93	2.03	3.22	6.18

[a] Urine was collected during the 24-hr. interval between doses and during the 24-hr. interval following the final dose.

rate was found to occur in the 4–8-hr. interval after dosage. The drug is slowly excreted in the urine and substantial levels were found 2–3 days after dosage. Within the 0–24-hr. interval a lower percentage of the administered dose is recovered in the urine than from those patients on chronic administration of the drug.

The data in Table III show the gradual increase in the amount of drug excreted daily as the dose is maintained over a period of 3 days. These data were obtained on the same subject as was used in the single dose study.

From the data presented, it is evident that the assay method described is capable of permitting a reliable evaluation of nortriptyline hydrochloride ingestion. If one is interested only in whether a patient has taken his medication and is not interested in the total amount of nortryptyline present in the urine, it would be necessary to carry out only *Method A* of the assay procedure. The presence of the drug can also be detected by the TLC system described.

SUMMARY

1. A quantitative U.V. spectrophotometric assay method has been developed for the determination of nortriptyline hydrochloride and its principal metabolites in the urine of man.

2. The drug is slowly excreted in the urine primarily as conjugated and unconjugated 10-hydroxy nortriptyline. Little unchanged nortriptyline is excreted.

3. An average of 58% of the daily dose was recovered in 24-hr. urine samples from patients on a continuing dosage regimen of the drug. An average of 34% of the dose was recovered from the 24-hr. urine of a subject given single doses.

4. A thin-layer chromatographic system is described which permits rapid identification of the presence of the drug and/or metabolites in the urine.

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Adrenolytic Activity of Atropine, (+)-Hyoscyamine, Atroscine, Homatropine, and Related Compounds

By F. P. LUDUENA and MARY-JANE BRANIN

The adrenolytic activity of atropine, (+) and (−) hyoscyamine, (−)-hyoscine, atroscine, and related compounds was determined by their ability to antagonize the lethal effect of epinephrine in rats. (+)-Hyoscyamine was found to be more active than atropine, and atropine was slightly more active than atroscine (racemic hyoscine). The *levo*-isomers, (−)-hyoscyamine and (−)-hyoscine, were inactive. These results indicate that (+)-hyoscyamine and (+)-hyoscine are responsible for the effect of atropine and atroscine, respectively. Homatropine and benztropine, but not tropine itself, were active. Benztropine was approximately one-fiftieth as active as phentolamine. A pair of esters of tropine had weak adrenolytic activity, but their pseudotropine isomers were inactive. Atropine aminoxide (genatropin) and atropine methyl nitrate had no adrenolytic activity.

IT HAS long been known that atropine, in high doses, inhibited or reduced the effect of epinephrine and sympathetic stimulation on various arterial beds (1). Those results included the effect of epinephrine on arterial strips from horses, oxen, and rabbits and perfused arterial

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beds of frogs, dogs, and rabbits. Bussell (1) showed that atropine reduced the effect of epinephrine on the blood pressure of the spinal cat, the dog perfused leg, the cat nictitating membrane, the perfused rabbit ear, and the rabbit uterus. He found that (−)-hyoscyamine was inactive or had very little activity. More recent studies have confirmed these results. Graham (2) demonstrated that atropine and (−)-homatropine blocked the constricting effect of epinephrine on the duck duodenum, while (−)-hyoscyamine and (−)-hyoscine were inactive. He also reported that the relaxation of the hen's caecum induced by epinephrine was not changed by atropine. Ruegg (3) found that atropine reduced the effect of epinephrine and norepinephrine on the rabbit iris dilator *in vitro*.

The general conclusion to be drawn from the literature data is that atropine blocks or reduces the effect of epinephrine and other catecholamines on sympathetic α-receptors and has no effect on β-receptors. In other words, atropine has adrenolytic (α-sympatholytic) activity.

The experiments described below were carried out to compare the adrenolytic activity of atropine with that of related compounds and, since (−)-hyoscyamine had been reported to be inactive, to determine whether the activity of atropine (racemic hyoscyamine) was that of the *dextro*-isomer in the mixture.

EXPERIMENTAL

Method.—The method was based on the ability of adrenolytics to antagonize the lethal effect of epinephrine in rats. The rationale for the selection of the rat as experimental animal, the intravenous route of administration, and the injection of epinephrine with the antagonist in the same solution has been discussed earlier (4). Two slightly different techniques were used. *Method A*, described earlier (4), follows.

Method A.—Male white rats, weighing between 80–110 Gm., were injected intravenously with a 200-mcg./Kg. (2.7–5 × LD_{50}) dose of epinephrine together with one of a series of graded doses of the compound to be tested. Using 0.3-log intervals, the dose of the compound under study was varied, while the concentration of epinephrine was kept constant. To obtain the ED_{50}, the percentage mortality was plotted against dose on log probit paper.

Method B.—*Method B* was employed when the adrenolytic activity of the compound was too weak to be observed by *Method A*. Similarly, a combined injection of epinephrine and the compound to be tested was administered intravenously to male white rats. In this case, however, the dose of the compound was kept constant, while the concentration of epinephrine was varied, at 0.2-log intervals. Again, plotting per cent mortality *versus* dose, the LD_{50} of epinephrine, as affected by the weak adrenolytic, was determined. In comparing this value with values obtained by determining the toxicity of epinephrine alone, slight adrenolytic activity could be detected. In Table II the results obtained with the 2 techniques are shown in different columns. In the case of *Method B*, the control LD_{50} for epinephrine, obtained with animals from the same batch, is included also. Doses of phentolamine, smaller than the ED_{50} obtained by *Method A*, were tested by *Method B*. The results in Table II show that even the smaller of the 2 doses used reduced the intravenous toxicity of epinephrine.

Compounds Used.—Atropine sulfate [(±)-hyoscyamine (Merck)], (+)-hyoscyamine 1-2-oxobornanesulfonate, (−)-hyoscyamine sulfate, homatropine hydrobromide (Merck), benztropine methanesulfonate[1] (3-diphenyl methoxytropane methane sulfonate), apoatropine hydrochloride, and tropine hydrochloride.

Compound I (tropyl diphenylacetate hydrochloride), compound II (pseudo-tropyl diphenylacetate hydrochloride), compound III (tropyl-2-cyclopentyl - 4 - methylpentanoate hydrochloride), compound IV (pseudo - tropyl -2 - cyclopentyl - 4-methylpentanoate hydrochloride), atropine aminoxide chlorhydrate (American Roland Co.), atropine methyl nitrate,[2] and (−)-hyoscine-hydrobromide (scopolamine hydrobromide) (Merck.).

(±)-Hyoscine (atroscine) prepared by racemization of (−)-hyoscine by Dr. Benjamin F. Tullar, diphenhydramine[3] (Parke, Davis and Co.), compound V (3-phenyltropane hydrochloride), phentolamine HCl,[4] papaverine hydrochloride (Merck), and pronethalol hydrochloride[5] (nethalide HCl).

RESULTS

Examination of the results summarized in Table I shows that some of the compounds tested had adrenolytic activity.

Of the 2 optical isomers of atropine only (+)-hyoscyamine antagonized the lethal effect of epinephrine; (−)-hyoscyamine in a dose of 40 mg./Kg. failed to protect the rats injected with a 200-mcg./Kg. dose of epinephrine.

The activity of (+)-hyoscyamine was low; phentolamine tested in the same manner was approximately 400 times more active.

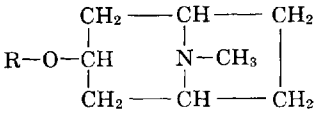
The results showed clearly that there is no correlation between cholinolytic and adrenolytic activities. The cholinolytic action of (−)-hyoscyamine was found by Long *et al.* to be 110–250 times higher than that of the (+) isomer. This (−)/(+)-activity ratio is considerably higher than those reported by others (6–8). [Lower ratios may indicate (−)-isomer contamination of the (+)-hyoscyamine sample.]

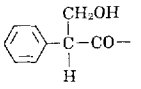
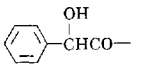
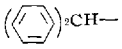
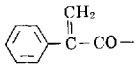
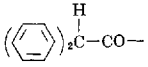
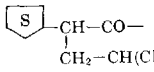
Atropine aminoxide (genatropine) and atropine methyl nitrate had no adrenolytic activity.

Scopolamine [(−)-hyoscine] was inactive at the highest dose tested (40 mg./Kg.). Racemic hyoscine (atroscine) was slightly less active than atropine; this indicates that (+)-hyoscine is the

[1] Marketed as Cogentin by Merck Sharp & Dohme.
[2] Marketed as Eumydrin by S. B. Penick Co.
[3] Marketed as Benadryl by Parke, Davis and Co.
[4] Marketed as Regitine HCl by Ciba Pharmaceutical Co.
[5] Marketed as Aldetlin HCl by Imperial Chemical Industries Ltd.

TABLE I.—ADRENOLYTIC ACTIVITY OF ATROPINE AND RELATED COMPOUNDS



Compd.	Salt	Structure R	Tropine (T) Pseudo-Tropine (P)	Adrenolytic Activity A ED_{50}, mg./Kg.[a]	Adrenolytic Activity B Increase in Epinephrine LD_{50} Epinephrine Alone, mcg./Kg. (Base)	Epinephrine + x mg. Drug/Kg. (mcg./Kg. Base)	Drug/Kg. (Base)
Atropine (± hyoscyamine)	Sulfate, H_2O				42.7 ± 5	232 ± 30	33.4
(+)-Hyoscyamine	1-2-Oxobornane-sulfonate		T	8.5 ± 1.5			
(−)-Hyoscyamine	Sulfate				Inactive		34.2
Homatropine	Hydrobromide		T		37.5 ± 4.2	148 ± 11	30.9
Benztropine	Methanesulfonate		T	1.14 ± 0.21			
Apoatropine	Hydrochloride		T		Inactive		10
Tropine	Hydrochloride				Inactive		100
I	Hydrochloride		T		42.7 ± 4.5	180 ± 13.5	10
II	Hydrochloride		P		Inactive		10
III	Hydrochloride		T		37.5 ± 4.2	180 ± 24	10
IV	Hydrochloride		P		Inactive		10
Atropine aminoxide	Hydrochloride				Inactive		40

[a] In terms of the bases.

TABLE II.—ADRENOLYTIC ACTIVITY OF ATROPINE AND RELATED COMPOUNDS

Compd.	Salt	Structure	Adrenolytic Activity: A — ED_{50}, mg./Kg. (In Terms of the Bases)	Adrenolytic Activity: B — Epinephrine Alone, mcg./Kg. (Base)	Adrenolytic Activity: B — Increase in Epinephrine LD_{50} — Epinephrine + x mg. Drug/Kg. (mcg./Kg. Base)	Adrenolytic Activity: B — Increase in Epinephrine LD_{50} — x
Atropine methyl	Nitrate				Inactive	10
(−)-Hyoscine (scopolamine)	Hydrobromide · $3H_2O$				Inactive	40
(±)-Hyoscine (atroscine)	Hydrochloride			42.7 ± 4.5	134 ± 17.5	40
Diphenhydramine	Hydrochloride				Inactive	10
V	Hydrochloride				Inactive	10
Phentolamine	Hydrochloride		0.022 ± 0.0038			
Phentolamine	Hydrochloride			37.5 ± 5	87 ± 16	0.0057
Phentolamine	Hydrochloride			37.5 ± 5	55 ± 4.6	0.0027
Papaverine	Hydrochloride				Inactive	4
Pronethalol	Hydrochloride				Inactive	4

active isomer and that substitution of scopine for the tropine ring in atropine does not abolish adrenolytic activity.

Pronethalol and papaverine, at the doses tested, did not protect rats from the lethal effect of epinephrine.

The most active of the compounds tested was benztropine, its activity being approximately one-fiftieth of that of phentolamine. Homatropine and 2 other tropanol esters (I and III) were slightly less active than atropine. The pseudotropanol analogs (II and IV) of the last 2 compounds had no activity.

Substitution of a dimethylamino ethanol group in compound I for the tropanol moiety abolished adrenolytic activity. (I *versus* diphenhydramine.)

Most of the compounds tested had cholinolytic activity. Since paralysis of the vagi and cholinergic vasodilators by cholinolytics may increase the magnitude and the duration of epinephrine hypertensive effect, the following experiment was performed. Graded doses of epinephrine were injected together with a dose of 1 mg./Kg. of (−)-hyoscyamine. As expected, the toxicity of epinephrine was slightly increased. [LD_{50} of epinephrine alone was 37.5 ± 4 mcg./Kg.; when 1 mg./Kg. of (−)-hyoscyamine was added to each of the graded doses of epinephrine the LD_{50} was 27 ± 2.7 mcg./Kg.] These results suggest that the adrenolytic effect of the active compounds tested had to be exerted against an epinephrine toxicity somewhat greater than that obtained on control rats.

DISCUSSION

The experiments reported above have confirmed, by a different method, reports in the literature which showed that atropine (1) and homatropine (2) had adrenolytic activity and that (−)-hyoscyamine was inactive (1, 2). Although (+)-hyoscyamine had not been tested before, the fact that (−)-hyoscyamine was inactive strongly suggested that the known weak adrenolytic activity of atropine was due to (+)-hyoscyamine. Our experiments have demonstrated that (+)-hyoscyamine is, indeed, an adrenolytic of a potency greater than that of atropine.

Since (−)-hyoscine (scopolamine) is inactive the activity of racemic hyoscine (atroscine) must be due, as in the case of atropine, to the action of the *dextro*-isomer.

In the series of compounds studied, with the exception of atroscine, the adrenolytic activity is associated with the presence of the tropanol moiety in the molecule, although tropine itself is inactive. Substitution of scopine for the tropine ring in atropine did not abolish activity but only reduced it. The presence of a tertiary amine group appears to be important—both atropine aminoxide and atropine methyl nitrate were inactive.

The activity of (+)-hyoscyamine was approximately $^1/_{100}$ of that of phentolamine. Assuming that the activity of atropine is 50% of that of (+)-hyoscyamine, the phentolamine/atropine activity ratio of 800 differs from that reported by Fleckenstein (9), who found a molar activity ratio of 375 in experiments on the perfused rabbit ear. He also found that diphenhydramine, inactive in our test, was as active as atropine. Since in Fleckenstein's experiments the adrenolytic was perfused, the con-

centration of diphenhydramine at the biophase, when distribution equilibrium had been reached, may have been much higher than that of atropine. In our experiments higher doses of diphenhydramine could not be administered because of toxicity.

The steric configuration of the tropyl moiety in atropine and atroscine plays an important role, since in both cases only the *dextro*-isomers are active. Cushny (10) reported that atropine was 200 times more active as a cholinolytic than its desoxy analog. This ratio is within the range of the *levo/dextro* hyoscyamine activity ratios obtained by Long *et al.* (5), suggesting, in accordance with Easson and Stedman's (11) theory, that the side chain OH group contributes to the attachment to the parasympathetic receptor. With the information available we can only theorize on the influence of the OH group in the tropyl moiety on adrenolytic activity. Only one of the enantiomorphs may have a configuration that provides an extra bond (hydrogen) for attachment to α-sympathetic receptors. Or possibly, adrenolytic activity may occur in atropine and racemic hyoscine only when the OH group is "pointing away" from the receptor, as appears to be the case with the adrenolytic activity of (+)-isoproterenol (12). In this case the OH group is not essential since the β-desoxy analog is equally active. The desoxy analogs of atropine and hyoscine could not be tested because they were unavailable. However, a closely related compound of atropine, apoatropine, was found inactive at 10 mg./Kg. (The toxicity of the compound prevented the use of higher doses.)

In examining the method we employed, the question may be raised of whether the antagonism of the lethal effect of epinephrine is a measure of adrenolytic activity (blockade of α-sympathetic receptors), or could protection against epinephrine toxicity be obtained with other types of drugs? To investigate this problem, 2 drugs which were known to antagonize some of the effects of epinephrine without acting on α-receptors—a smooth muscle relaxant, papaverine, and a β-receptor blocker, pronethanol—were tested.

As discussed in a previous paper (4), the data in the literature indicate that the fatal pulmonary edema which follows the intravenous injection of high doses of epinephrine is due to a pronounced rise of arterial and pulmonary venous pressure. Theoretically, antagonists of the effect of epinephrine on the blood vessels or the heart could act as epinephrine antidotes. However, pronethalol, a β-receptor blocker, in doses of 1, 2, or 4 mg./Kg., had no antidotal effect when injected together with epinephrine (100 mcg./Kg.). Nonadrenolytic vasodilators, such as (−)-isoproterenol (12) and papaverine, do not reduce the toxicity of epinephrine in the rat.

In addition to adrenolysis, the only pharmacological property which is known to protect rats (4) against the lethal effect of epinephrine is that of methacholine, *i.e.*, muscarinic activity. Obviously, the positive readings reported above cannot be due to muscarinic activity, since the active compounds have an antagonistic, not an agonistic action on the muscarinic receptors.

The compounds we tested had weak protective action compared with phentolamine, and as in all cases of weak inhibitory activity, the identification of the type of activity involved is not clear cut. The ED_{50} of phentolamine and those of other adrenolytics (4) are considerably lower than the doses required to reverse the pressor effect of epinephrine. Therefore, if the term "adrenolytic" is applied only to those that reverse the pressor effect of epinephrine, the weakest members of the α-receptor blockers may not be included, although they may show antiepinephrine effect on isolated vessel preparations (rabbit uterus, vas deferens, etc.). Atropine and homatropine were known to reduce the effect of epinephrine on various test objects; our tests confirmed the α-receptor blocking effect of homatropine and identified the optical isomer responsible for the effect of atropine. The chemical similarity of hyoscine and the fact that the racemic mixture, but not the levorotatory isomer, was active strongly suggest that the same mechanism of action is involved. Less is known about the pharmacological actions of compounds I and III which were found weakly active in our tests. However, the chemical relationship with active compounds in our series and the absence of an alternative mechanism of action suggest that the protection against epinephrine toxicity is due to α-receptor blockade.

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Factors Affecting the Absorption of Riboflavin in Man

By GERHARD LEVY and WILLIAM J. JUSKO

The urinary recovery of riboflavin as a function of dose after oral administration to fasted normal humans shows that the process responsible for the absorption of this vitamin is saturable. The saturation effect is not evident when riboflavin doses as high as 30 mg. are administered after a meal. The enhanced absorption of riboflavin in the presence of food appears to be due to a decrease in intestinal transit rate which causes the vitamin to be retained at absorption sites in the small intestine for a longer period of time. The site specificity and saturability of riboflavin absorption suggest that the vitamin is absorbed by specialized transport rather than by passive diffusion. This conclusion is supported by kinetic and physical-chemical data, as well as by the results of studies of the effect of route of administration on the recovery of riboflavin in the urine.

SEVERAL GROUPS of investigators have found a linear relationship between the dose of riboflavin administered orally in solution or in other rapidly available forms and the urinary recovery of this vitamin (1–4). Melnick *et al.* (1) recovered an average of about 46% of the dose after administration of up to 10 mg. of riboflavin in solution. Brewer *et al.* (2) recovered about 50% in the dose range of 2 to 7 mg. but demonstrated that doses under 2 mg. are retained more completely. Morrison and Campbell (3) reported an average urinary recovery of 61% in the dose range of 1 to 20 mg., and Morrison *et al.* (4) recovered 56% of the dose after oral administration of 5 and 10 mg. of riboflavin. It has been suggested (5), on the basis of these observations, that riboflavin is absorbed by passive diffusion. In the course of a study of the effect of viscosity on gastrointestinal absorption in man (6), in which riboflavin was used as a model drug, results were obtained which appeared to be completely incompatible with the published observations of others. A thorough review of the data revealed only one potentially significant difference between the experimental designs employed by other investigators and that used in this laboratory: the other investigators administered riboflavin after a meal, while in this laboratory the vitamin was given on an empty stomach. Consequently, a formal study of the effect of time of administration (*i.e.*, before or after breakfast) on the urinary recovery of riboflavin was instituted. The study was designed to yield kinetic data as well, and was expanded subsequently to include an evaluation of the effect of route of administration on riboflavin excretion.

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EXPERIMENTAL

Absorption Study.—Four healthy male volunteers, ranging in age from 22–37 years, served as test subjects. Each subject received 5, 10, and 30 mg. of riboflavin U. S. P. in 100 ml. of 0.02 *N* acetic acid, followed by a small amount of water either on an empty stomach (after an overnight fast) or immediately after a standard breakfast consisting of 60 Gm. of cornflakes with sugar and 500 ml. of milk. One subject received also doses of 3 and 20 mg. of riboflavin on an empty stomach. Total urine collections were carried out at 0, 0.5, 1.0, 1.5, 2, 3, 4, and 6 hr. after riboflavin administration, and at convenient timed intervals thereafter up to 24 hr. Immediately after collection the urines were placed in brown opaque plastic bottles, glacial acetic acid (about 3 ml./100 ml. of urine) was added, and the bottles were placed in a refrigerator. The subjects were instructed to drink about 100 ml. of water every hour to maintain adequate urine output. When riboflavin was given on an empty stomach, breakfast was withheld for 2 hr. after the start of the experiment. The subjects were asked to avoid eating certain foods known to contain appreciable amounts of riboflavin and to refrain from taking vitamin preparations or drugs for at least 48 hr. prior to and during the experiment.

A similar protocol was followed when riboflavin was administered by other than the oral route. The vitamin was administered intravenously by rapid injection of a commercial preparation stated to contain 5 mg. of riboflavin (actual assay: 4.5 mg.) solubilized with nicotinamide (item No. 398, Eli Lilly & Co.). Rectal administration of riboflavin was carried out by means of a polyethylene syringe fitted with a 25-cm. catheter tube which was inserted fully in the rectum. The rectal solution consisted of 5 mg. riboflavin dissolved in 50 ml. of 0.5% methylcellulose (Methocel 4000 60 HG, Dow Chemical Co.) in water. This solution was retained in the colon.

At least two 24-hr. blank urine collections, with and without breakfast, were carried out in each subject. Control urine collections were carried out also after oral administration of 200 mg. of nicotinamide.

Analytical Methods.—Riboflavin in the urine was determined fluorometrically by the method of Burch *et al.* (7) as well as by a method based on the U. S. P. XVI assay procedure (8), using the Turner fluorometer, model 111, with primary filter 47-B and

secondary filter 2A-12. The Burch assay involves extraction of riboflavin into benzyl alcohol, while the modified U. S. P. XVI procedure does not require extraction. In the latter procedure, 5 ml. of suitably diluted urine is mixed with 1 ml. of pH 4.8 acetate buffer (1 *N*). One milliliter of 4% potassium permanganate and, subsequently, 1 ml. of 3% hydrogen peroxide are added. The fluorescence intensity of this solution is determined before and after reduction of riboflavin with sodium hydrosulfite. All data were corrected for blank values which averaged 0.6 mg./24 hr. by the Burch procedure and 1.0 mg./24 hr. by the modified U. S. P. procedure. Nicotinamide was found not to interfere in the assay of riboflavin, both *in vitro* (aqueous solutions) and *in vivo* (urines after nicotinamide administration).

Determination of Partition Coefficients.—Chloroform and 0.1 *N* hydrochloric acid or pH 7.0 phosphate buffer (0.1 *M*) were used as the organic and aqueous phases, respectively. The organic–aqueous phase volume ratio was 50:1, and the initial concentration of riboflavin in the aqueous phase was 5 mg./100 ml. The phases were shaken at 37° for 14 hr. The aqueous phase then was removed, centrifuged, adjusted to pH 4.8 with 0.1 *N* acetate buffer, and assayed fluorometrically by the modified U. S. P. method. Control experiments without riboflavin were carried out in parallel and yielded negligible fluorescence readings.

RESULTS

Both assay procedures employed in this investigation yielded essentially identical results. For example, the average urinary recovery after oral administration of 5 mg. of riboflavin after breakfast was 61.3% by the Burch assay and 60.5% by the modified U. S. P. procedure. The significance of these findings will be discussed in a subsequent report on the metabolic fate of riboflavin and riboflavin-5′-phosphate, respectively (9). All data presented in this report are based on the results obtained with the assay procedure of Burch *et al.* (7).

The urinary recoveries of riboflavin as a function of dose when given on an empty stomach and after a standard meal, respectively, are listed in Tables I and II. As shown in Fig. 1, there was a linear relationship between dose and the amount excreted when the vitamin was taken immediately after a standard breakfast. The average recovery of riboflavin was 62%, which is essentially identical to the 61% recovery obtained by Morrison and Campbell (3). When riboflavin was taken on an empty stomach, the per cent recovered in the urine decreased with increasing dose. In agreement with the observations of others [for example, Everson *et al.* (10)], there were consistently high and low excretors of riboflavin, respectively, regardless of dose and experimental conditions. The relationship between dose and urinary recovery of riboflavin given on an empty stomach is depicted in a Lineweaver-Burk-type plot (11) for the "high excretor," subject *M*, and the "low excretor," subject *J* (Fig. 2). The linear relationship obtained in each case indicates a limited capacity for riboflavin absorption under the experimental conditions.

Figure 3 depicts the excretion rate of riboflavin as a function of time after oral administration of 10 mg. either on an empty stomach or after breakfast. The plot on Cartesian coordinates shows the dif-

TABLE I.—EFFECT OF DOSE ON URINARY RECOVERY[a] OF RIBOFLAVIN GIVEN ON AN EMPTY STOMACH

Subject	Dose, mg. 3	5	10	20	30
J	48.0	35.8	24.5	19.5	10.0
L		33.4	22.2		13.4
A		52.7	22.8		18.2
M		67.9	49.8		21.1

[a] Per cent of dose.

TABLE II.—EFFECT OF DOSE ON URINARY RECOVERY[a] OF RIBOFLAVIN GIVEN AFTER A STANDARD BREAKFAST

Subject	Dose, mg. 5	10	30
J	49.8	61.0	52.4
L	59.2	54.6	50.0
A	60.1	68.9	59.9
M	76.1	68.4	80.4

[a] Per cent of dose.

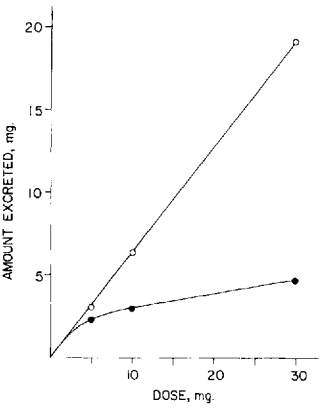

Fig. 1.—Urinary recovery of orally administered riboflavin as a function of dose when given on an empty stomach (●) and after a standard breakfast (○). Mean of 4 subjects.

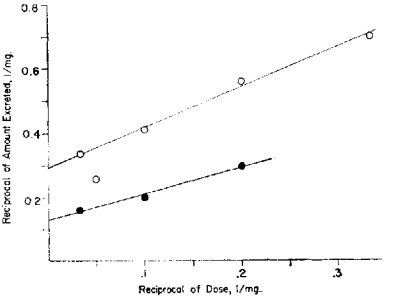

Fig. 2.—Lineweaver-Burk-type plot of the reciprocal of the amount of riboflavin recovered in the urine *vs.* the reciprocal of the oral dose given on an empty stomach. Key: ○, subject *J*; ●, subject *M*.

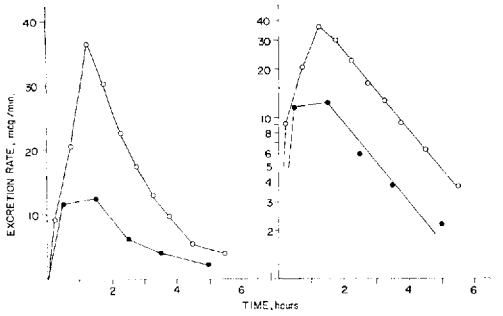

Fig. 3.—Excretion rate of riboflavin as a function of time after oral administration of 10 mg. of riboflavin in aqueous solution to 4 subjects on an empty stomach (●) and after a standard breakfast (○). Data are plotted on Cartesian coordinates (left) and in semilogarithmic form (right).

ference in the areas under the respective curves (which reflects the different amounts absorbed and excreted under the different experimental conditions) and indicates the very rapid absorption of the vitamin. The shapes of the 2 curves suggest that absorption under the 2 experimental conditions occurred at about the same rate initially, but continued for a longer period of time when the vitamin was given after breakfast. The semilogarithmic plot in Fig. 3 shows an exponential decline of excretion rate as a function of time, with an average half-life of 1.1 hr. (range of individual half-lives: 0.9 to 1.4 hr.). There was a consistent decrease in slope after 6 hr., when only a very small fraction of the total excreted amount remained to be excreted.

The effect of route of administration on riboflavin recovery is shown in Table III for subject *J*. The dose had to be restricted to 5 mg. to permit intravenous and rectal administration of the vitamin in solution. The viscosity of the rectal solution was increased by addition of methylcellulose to permit retention of the solution without leakage. The possibility of complex formation between riboflavin and the polymer was ruled out by equilibrium dialysis (6).

The kinetics of elimination of riboflavin after intravenous administration are depicted in Fig. 4. The experimental data could be resolved into a rapid and a slow component with half-lives of about 0.2 and 8 hr., respectively.

DISCUSSION

Site Specificity of Riboflavin Absorption.—Evidence which suggests that riboflavin is absorbed mainly in the proximal region of the intestinal tract has been reviewed by Campbell and Morrison (5).

TABLE III.—EFFECT OF ROUTE OF ADMINISTRATION ON URINARY RECOVERY OF A 5-mg. DOSE OF RIBOFLAVIN IN A FASTING SUBJECT

Rt. of Admin.	Recovery, %
Oral, empty stomach	35.8
Oral, after breakfast	49.8
Intravenous[a]	72.0
Rectal[b]	6.2

[a] Actual dose, 4.5 mg. [b] Retention enema.

For example, they point out that riboflavin absorption is decreased if the vitamin is administered in certain coated tablets or sustained-release preparations which do not release riboflavin relatively promptly. Moreover, administration of riboflavin in a number of sustained-release preparations having markedly different release characteristics has yielded excretion rate *versus* time curves which tend to drop off at about the same (early) times as curves obtained after giving the vitamin in rapidly available form.

Colonic absorption of riboflavin is practically insignificant (Table III). The present findings are in agreement with those of Everson *et al.* (10) and Campbell and Morrison (5). Najjar *et al.* (12) noted some absorption of riboflavin after administering 20 mg. as a rectal enema, but presented no quantitative data. It has been shown that rectally administered enemas and even suppositories spread in a retrograde manner at least to the mid-descending and frequently to the ascending colon (13, 14). In the present study, particular emphasis was placed upon introducing the riboflavin solution as high up the colon as possible by use of a 25-cm. catheter. The very low absorption of riboflavin after rectal administration is considered, therefore, to reflect an intrinsically poor absorbability of this vitamin in the colon, rather than being due to limited contact with colonic membranes.

It has been suggested (4) that the apparent site specificity of riboflavin absorption could be due to degradation of the vitamin in the lower bowel. This suggestion is based on a report by Selye (15) who stated, on the basis of very limited and indirect evidence, that riboflavin is rapidly destroyed in the large intestine of rats. The theory that the site-specificity of riboflavin absorption is due to degradation of the vitamin in the large intestine is rather untenable on kinetic grounds. If the degradation process is first order, a constant fraction of each dose, regardless of size, would be destroyed. If the process is apparent zero order (perhaps due to a limited metabolic capacity of a microbial population involved in this process), the fraction of a dose which will be destroyed would decrease with increasing dose. The experimental data (Table I) show exactly the opposite relationship. To explain this phenomenon on the basis of degradation in the lower gastrointestinal tract requires that one invoke such

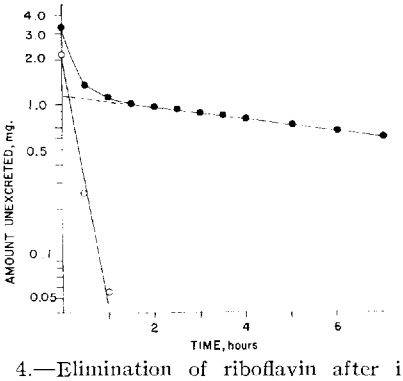

Fig. 4.—Elimination of riboflavin after intravenous administration (subject *J*). Key: ○, difference between experimental data; ●, extrapolated line.

possibilities as substrate inhibition of an enzymic system, or a dose-dependent effect of riboflavin on gastrointestinal motility. Neither of these possibilities is realistic in terms of present knowledge or in relation to the enhanced absorption observed when riboflavin is taken after a meal. Another remote possibility is that the decreased absorption of riboflavin with increasing dose is due to the dimerization of the vitamin at higher concentrations and that this is practically abolished in the presence of other interacting substances ingested as food. However, there is evidence that biologic membranes have a dissociating effect on homo-complexes and that dimerization will not affect absorption rate (28).

Saturability of Riboflavin Absorption.—The observation that the urinary recovery of riboflavin is decreased with increasing dose when the vitamin is taken on an empty stomach (Table I) suggests strongly that the process responsible for riboflavin absorption is saturable. There is no evidence that renal excretion of riboflavin is saturable in the concentration range encountered in the present experiments (*cf.* subsequent paragraph concerning elimination kinetics), and this possibility may, therefore, be ruled out as accounting for the experimental observations. Since given individuals appear to have relatively constant intestinal transit rates (16), the amount of an incompletely absorbed substance (given in solution) which is absorbed can be used as a relative measure of absorption rate in the individual. On this basis, Lineweaver-Burk plots have been prepared by plotting the reciprocal of the amount of riboflavin absorbed on an empty stomach against the reciprocal of the dose. Figure 2 shows such plots for a high and a low excretor of riboflavin. Each of these plots is linear and indicates an absorption maximum, as is characteristic of active transport processes (17). The magnitude of the extrapolated maximum values cannot be compared directly between different individuals, because they are a function also of intestinal transit rate and of the fraction of absorbed riboflavin which is excreted in the urine. These characteristics show appreciable intersubject variations. For example, the data listed in Table II show that subject *M* intrinsically excretes a larger fraction of riboflavin than does subject *J*.

The Effect of Food.—Food and viscous liquids slow gastric emptying and intestinal transit (18, 19). It is reasonable to assume that the presence of food causes riboflavin to be in contact with optimum absorption sites in the proximal region of the intestinal tract for a longer period of time, and thus, brings about the complete absorption of the vitamin over a wider dose range. The data in Table II indicate that riboflavin given after breakfast is absorbed either completely or that the same fraction of the dose is absorbed[1] in the dose range of 5 to 30 mg. While it would be desirable to demonstrate that saturation of riboflavin absorption is possible even when the vitamin is given after meals, results of such experiments would be equivocal since the limited solubility of riboflavin would require that the large doses necessary for such a study be given in suspension. If the amount of undissolved riboflavin is greater than that which can be dissolved relatively rapidly in gastric and upper intestinal fluids, the absorption kinetics would be apparent zero order for physicochemical rather than for physiologic reasons. However, if experiments now in progress in this laboratory show that riboflavin-5′-phosphate (FMN) has similar absorption characteristics as does riboflavin, large doses of the more water-soluble FMN will be given in solution after a meal to see if saturation effects can be demonstrated.[2]

It is of interest that apparently only Morrison and Campbell (3) have administered riboflavin also on an empty stomach. They used a 5-mg. dose only and reported "similar" urinary recovery to that obtained after breakfast, but did not present quantitative data. Their findings are explained readily by reference to Fig. 1, which shows that recovery of riboflavin given before or after breakfast is indeed "similar" at doses of 5 mg.; the differences become appreciable only when larger doses are given.

The Evidence for Specialized Intestinal Transport of Riboflavin in Man.—Two of the major characteristics of specialized intestinal transport processes are site specificity and saturability (20). These characteristics are evident in the absorption of riboflavin. The vitamin has a molecular weight of almost 400 which precludes rapid absorption by the pore route. Its chloroform–water partition coefficient, when the pH of the aqueous phase is either 1 or 7, is very small (< 0.001). Substances with such properties are not absorbed or only very poorly absorbed by passive diffusion (20). Yet riboflavin is absorbed very rapidly, as is evident from Fig. 3 and from the report by Wiegand *et al.* (21), who have estimated that the rate constant for riboflavin absorption (in doses of 5 mg. or less) is in excess of 30 reciprocal hr.! Although Wiegand *et al.* point out that a rate constant of this magnitude is not meaningful (it indicates that absorption is 95% complete in 6 min. or less), the value does reflect the very rapid absorption of the vitamin. It is inconsistent with present knowledge that such rapid absorption of a large, lipoid-insoluble substance can occur other than by some form of specialized transport.

Animal Experiments.—Spencer and Zamcheck (22) have studied riboflavin absorption with everted intestinal sacs from rats and hamsters and by the *in vivo* ligated loop technique in rats. They did not find any accumulation of riboflavin against a concentration gradient and noted that the intestinal sac appeared relatively impermeable to the vitamin. However, they used an almost saturated solution of riboflavin, and the high concentration alone can account for their inability to find serosal to mucosal concentration ratios greater than unity. The observed low permeability of the intestinal sac to riboflavin shows, regardless of other considerations, that the absorption of this substance by passive diffusion is at best very poor. Spencer and Zamcheck conclude that "the scant evidence available suggests that riboflavin may cross the intestine by diffusion rather than by specific transport," but add that "it is possible that in the presence of lower concentra-

[1] This would be the case if riboflavin is partially destroyed in the intestinal tract.

[2] After completion of this manuscript, the recent paper by Stripp [*Acta Pharmacol. Toxicol.*, **22**, 353(1965)] was received. He found that 50- to 500-mg. doses of FMN, given in solution after a meal, yielded the same riboflavin in blood levels and the same amounts excreted despite the 10-fold difference in dose. This demonstrates unequivocally the existence of an absorption maximum even when the vitamin is administered after a meal.

tions of the vitamin and with the admixture of food materials there may be other mechanisms of riboflavin absorption by the intestine" (22).

The significance of the study by Turner and Hughes (23), who used rat intestine preparations, is also limited by the high concentration of riboflavin used. They concluded that in rats all B group vitamins are absorbed by passive diffusion, but it is of interest that Spencer and Brody (24) have shown subsequently that at least one of these, biotin, is absorbed by specialized transport in the white mouse, hamster, and squirrel, though not in the rat, rabbit, and guinea pig. Thus the possibility of species differences alone limits the applicability of the results of animal studies to the elucidation of the mechanism of riboflavin absorption in man. A study by Middleton and Grice (25) is of interest because these workers administered riboflavin by stomach tube to intact rats. Their results show a relative site specificity for riboflavin absorption as well as definite apparent zero-order absorption kinetics which is compatible with the existence of a saturable transport process.

Kinetics of Riboflavin Elimination in Man.—Excretion of riboflavin after intravenous administration was very rapid initially and decreased after the first hour (Fig. 4). The experimental curve could be resolved into a rapid and a slow exponential component. Similar results are obtained upon graphical analysis of the data of Axelrod *et al.* (26), who administered intravenously 0.2 or 0.4 mg. of riboflavin per Kg. body weight to 4 subjects. They found that 30 to 40% of the dose was excreted within 1 hr. after injection. Evaluation of rate constants for their subject III yield essentially the same values as were obtained in the experiment depicted in Fig. 4. The data of Axelrod *et al.* (26) show riboflavin excretion rates of more than 6 mg./hr. and give no indication of possible saturation of excretory function.[3] The existence of an initial rapid elimination phase followed by a much slower phase is also evident from the study of Najjar and Holt (27), who administered 1 mg. riboflavin intravenously to 10 subjects after an overnight fast. They recovered 32 to 72% of the dose in the urine. Najjar *et al.* (12) established in a subsequent study that large doses of intravenously administered riboflavin do not cause an increase in fecal riboflavin output. This suggests that the incomplete recovery of absorbed riboflavin is due to biotransformation and/or metabolic retention of riboflavin, rather than to fecal excretion. All the available data suggest that riboflavin, when given intravenously, is relatively slowly distributed and therefore very rapidly eliminated initially. A variable fraction is either metabolized or retained in some form (probably as flavoprotein), and a small fraction is eliminated rather slowly.

The kinetics of riboflavin elimination after oral administration yield a somewhat different picture (Fig. 3). Excretion rate decreases exponentially with time, with an apparent half-life of about 1.1 hr. However, a slower elimination component was noted after 6 hr. in the present study and is evident also in the study of Morrison *et al.* (4). This slow component is probably identical to that found after intravenous administration and may reflect the elimination of riboflavin from a "deep" compartment. The more rapid early phase of riboflavin elimination after oral administration may reflect a combination of a distally decreasing intestinal absorption gradient and slow diffusion of the vitamin from blood into tissues, or it may represent an effect of route of administration on the distribution of riboflavin in the body.

[3] The same is evident from Fig. 3 of the present study.

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Synthesis and Preliminary Pharmacological Evaluation of Alkylpiperazine Esters

By ELDA CRESCENZI, ERNESTA MARAZZI-UBERTI, and GERMANO COPPI

Twenty-nine alkylpiperazine esters have been synthesized and tested for the action on the CNS and for anti-inflammatory, analgesic, antipyretic, antispasmodic, and antimicrobic activities. Many of the compounds tested, in particular XV, display analgesic, anti-inflammatory, and antipyretic properties.

MANY ALKYLPIPERAZINE esters, mainly acrylates, have been synthesized and investigated chiefly for their anti-inflammatory, antispasmodic, and antimicrobic activities. This research was suggested by the known anti-inflammatory activity of β-4-biphenylylacrylic acid (1, 2), and of other acrylic acids (3), by the autonomotropic properties exerted by amino-alcohol esters (4), and by the antimicrobic properties observed for various acrylic acids (5–9) and quaternary ammonium salts of their basic esters (10). The activity on the CNS was also tested together with the analgesic and antipyretic activities.

The compounds dealt with in the present paper are mainly symmetrical dialkylpiperazine bis-acrylates and asymmetrical alkylpiperazine mono-acrylates. Only X, XI, and XXIX are esters of saturated aliphatic acids. They have been synthesized for the purpose of determining the influence produced by the disappearance of the double olefine bond on the pharmacological properties studied.

The synthesis methods used are the general ones for esters; *i.e.*, the reaction of acid chlorides with alcohols, or the reaction of acids or their sodium salts with chloro-derivatives. In the latter case, the use of isopropyl alcohol, in which the sodium salts of the acids are soluble to a certain extent, is particularly advantageous. I and II were isolated from the reaction mixture directly as the hydrochlorides.

The new esters are colorless crystalline solids, except for XVIII, which is a very viscous oil. Their properties are given in Tables I and II. The hydrochlorides of the esters are sparingly soluble in water, except for those in which an atom of nonpiperazine nitrogen is also present.

EXPERIMENTAL

Chemistry.—Melting points were taken on a Townson-Mercer melting point apparatus and are corrected.

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Intermediates.—The references for the synthesis of the required acrylic acids have already been reported by the authors in another paper (3). 3-(3-Pyridyl)-propionic acid was prepared according to Merz and Stolte (11), and 2-(4-biphenylyl)-butyric acid according to Cavallini and Massarani (12).

The intermediate piperazines, *i.e.*, 1,4-bis-(1-methyl-2-chloroethyl)-piperazine (b. p. 150–152°/15 mm.), 1-(4-chlorophenyl)-4-(2-chloroethyl)-piperazine dihydrochloride (m. p. 201–203° dec.), 1-benzyl-4-(2-chloroethyl)-piperazine dihydrochloride (m. p. 304–308° dec.), 1-(4-methoxyphenyl)-4-(2-chloroethyl)-piperazine dihydrochloride (m. p. 220–222° dec.), and 1-(4-tolyl)-4-(2-chloroethyl)-piperazine dihydrochloride (m. p. 221–223° dec.) were prepared according to the general methods for the preparation of chloroamines described by Wilson and Tishler (13). 1-(4-Fluorophenyl)-4-(2-chloroethyl)-piperazine dihydrochloride was prepared according to Janssen (14) and 1-(2-chloroethyl)-piperazine dihydrochloride according to Hromatka and Engel (15).

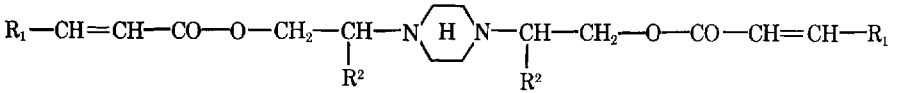
Esters

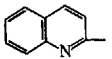
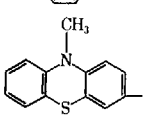
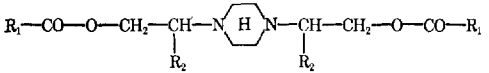
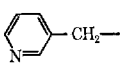
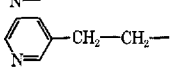
1,4-bis[2-(Crotonyloxy)ethyl]-piperazine Dihydrochloride (I).—*Method A.*—A mixture of 48.9 Gm. of α-crotonic acid, 64 Gm. of 1,4-bis (2-chloroethyl)-piperazine (13), 45 Gm. of anhydrous potassium carbonate, and 1300 ml. of anhydrous benzene are refluxed for 16 hr., with stirring. After cooling to room temperature, potassium chloride and the excess potassium carbonate are removed by filtration and the benzene solution thoroughly washed, first with dilute sodium carbonate solution, and then with water. Finally, after drying over sodium sulfate, hydrogen chloride is bubbled through. The filtered product, washed with anhydrous ether and dried under vacuum at 50°, weighs 71.7 Gm. After crystallization from ethanol, it melts at 221.5–223.5° dec.

1,4-bis{2-[3-(1-Naphthyl)acrylyloxy]ethyl}-piperazine Dihydrochloride (II).—*Method B.*—A 8.71-Gm. quantity of 1,4-bis(2-hydroxyethyl)-piperazine (4) is added to a solution of 21.66 Gm. of 3-(1-naphthyl)-acrylyl chloride (10) in 100 ml. of anhydrous benzene. The reaction mixture is refluxed for 2 hr., and, after standing overnight at room temperature, the solid precipitate is filtered off and dried at 50° under vacuum. The product (17.9 Gm.) is crystallized from ethanol and gives colorless crystals, m.p. 269–271° dec.

1,4-bis{2-[3-(3-Pyridyl)acrylyloxy]ethyl}-piperazine (IV).—*Method C.*—A 77-Gm. quantity of sodium 3-(3-pyridyl)-acrylate (3) and 43 Gm. of 1,4-

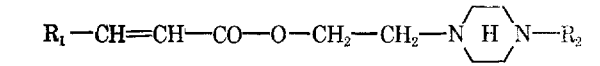
TABLE I.—SYMMETRICAL DIALKYLPIPERAZINE BIS-ESTERS

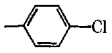
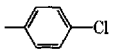
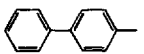
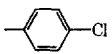
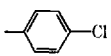
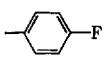
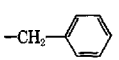
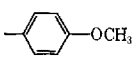
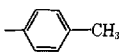
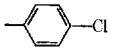
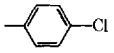
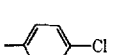
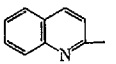
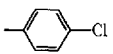


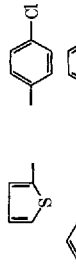
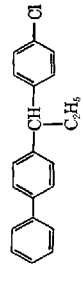
Compd.	R_1	R_2	Method	Reflux, hr.	Yield,[a] %	M.p., °C.	Recrystn. Solvent	Formula	Anal., % Calcd.	Anal., % Found
I	CH_3	H	*A* *B*	16	66 72	221.5–223.5[b]	Ethanol	$C_{16}H_{26}N_2O_4 \cdot 2HCl$	C, 50.13 H, 7.36 N, 7.30	C, 50.41 H, 7.27 N, 7.21
II		H	*B*	2	59	269–271[b]	Ethanol	$C_{34}H_{34}N_2O_4 \cdot 2HCl$	C, 67.21 H, 5.97 N, 4.61	C, 67.28 H, 5.81 N, 4.54
III		H	*C*	48	81	130.5–131.5	Benzene–cyclohexane	$C_{24}H_{28}N_4O_4$	C, 66.03 H, 6.47 N, 12.84	C, 65.91 H, 6.49 N, 12.79
IV		H	*C*	15	72	123–124	Isopropanol	$C_{24}H_{28}N_4O_4$	C, 66.03 H, 6.47 N, 12.84	C, 65.98 H, 6.55 N, 12.66
V		H	*C*	60	75	135–136.5	Benzene–cyclohexane	$C_{24}H_{28}N_4O_4$	C, 66.03 H, 6.47 N, 12.84	C, 66.11 H, 6.46 N, 12.91
VI		H	*C*	48	69	165–166.5	Benzene–cyclohexane	$C_{32}H_{32}N_4O_4$	C, 71.62 H, 6.01 N, 10.44	C, 71.80 H, 6.06 N, 10.28
VII		H	*C*	48	49	116–117	Benzene–cyclohexane	$C_{32}H_{32}N_4O_4$	C, 71.62 H, 6.01 N, 10.44	C, 71.66 H, 6.07 N, 10.40
VIII		H	*C*	60	60	178–180	Ethanol–benzene	$C_{40}H_{40}N_4S_2O_4$	C, 68.15 H, 5.72 N, 7.95	C, 68.30 H, 5.80 N, 7.88
IX		CH_3	*C*	60	39	149–150	Benzene–cyclohexane	$C_{28}H_{32}N_4O_4$	C, 67.22 H, 6.94 N, 12.06	C, 67.16 H, 6.88 N, 12.01
										
X		H	*C*	48	45	69–71	Benzene–cyclohexane	$C_{22}H_{28}N_4O_4$	C, 64.06 H, 6.84 N, 13.58	C, 63.91 H, 6.82 N, 13.37
XI		H	*C*	48	89	[c]	...	$C_{24}H_{32}N_4O_4$	C, 65.43 H, 7.32 N, 12.72	C, 65.29 H, 7.33 N, 12.64

[a] Crude product. [b] The compound melts with decomposition. [c] Liquid product, which decomposes on heating.

TABLE II.—ASYMMETRICAL ALKYLPIPERAZINE MONO-ESTERS



Compd.	R_1	R_2	Method	Reflux, hr.	Yield,[a] %	M.p., or B.p., °C., mm.	Recrystn. Solvent	Formula	Anal., % Calcd.	Found
XII			*D*	48	72	125–126.5	Isopropanol	$C_{21}H_{23}ClN_2O_2$	C, 68.00 H, 6.25 Cl, 9.56 N, 7.55	C, 68.16 H, 6.21 Cl, 9.67 N, 7.44
XIII			*D*	48	80	150–155	Isopropano	$C_{21}H_{23}ClN_2O_3$	C, 65.20 H, 5.99 Cl, 9.16 N, 7.24	C, 65.33 H, 5.83 Cl, 9.23 N, 7.24
XIV			*D*	48	65	174.5–176	Ethylacetate	$C_{27}H_{27}ClN_2O_2$	C, 72.55 H, 6.08 Cl, 7.93 N, 6.26	C, 72.51 H, 6.11 Cl, 7.98 N, 6.19
XV			*D*	48	81	110–110.5	Isopropanol	$C_{20}H_{22}ClN_3O_2$	C, 64.59 H, 5.96 Cl, 9.53 N, 11.30	C, 64.54 H, 5.96 Cl, 9.55 N, 11.31
XVI			*D*	48	79	107–108	Ethanol	$C_{20}H_{22}FN_3O_2$	C, 67.58 H, 6.24 N, 11.82	C, 67.79 H, 6.20 N, 11.80
XVII		H	*D*	48	49	240–245[b]	...	$C_{14}H_{19}N_3O_2.3HCl$	C, 45.35 H, 5.98 Cl, 28.69 N, 11.33	C, 45.33 H, 6.01 Cl, 28.71 N, 11.16
XVIII			*D*	48	51	165–170 (0.05)	...	$C_{21}H_{25}N_3O_2$	C, 71.77 H, 7.17 N, 11.96	C, 71.71 H, 7.10 N, 12.03
XIX			*D*	48	66	88–89	Hexane–benzene	$C_{21}H_{25}N_3O_3$	C, 68.64 H, 6.86 N, 11.44	C, 68.60 H, 6.94 N, 11.29
XX			*D*	42	82	132–133	Isopropanol	$C_{21}H_{25}N_3O_2$	C, 71.77 H, 7.17 N, 11.96	C, 71.75 H, 7.18 N, 12.11
XXI			*D*	48	83	116–117	Benzene–cyclohexane	$C_{20}H_{22}ClN_3O_2$	C, 64.59 H, 5.96 Cl, 9.53 N, 11.30	C, 64.66 H, 5.94 Cl, 9.48 N, 11.28
XXII			*D*	48	69	116–116.5	Isopropanol	$C_{20}H_{22}ClN_3O_2$	C, 64.59 H, 5.96 Cl, 9.53 N, 11.30	C, 64.66 H, 5.88 Cl, 9.51 N, 11.16
XXIII			*D*	48	85	106.5–107.5	Isopropanol	$C_{25}H_{25}ClN_2O_2$	C, 71.33 H, 5.98 Cl, 8.42 N, 6.65	C, 71.59 H, 6.09 Cl, 8.44 N, 6.60
XXIV			*D*	48	89	140–141.5	Ethylacetate	$C_{24}H_{24}ClN_3O_2$	C, 68.31 H, 5.73 Cl, 8.40 N, 9.96	C, 69.01 H, 5.66 Cl, 8.32 N, 10.02

XXV		D	60	48	100.5–101.5	Isopropanol	$C_{24}H_{29}ClN_2O_2$	C, 68.31 H, 5.73 Cl, 8.40 N, 9.96	C, 68.16 H, 5.74 Cl, 8.43 N, 9.91
XXVI		D	48	88	126–127	Isopropanol	$C_{19}H_{23}ClN_2O_3$	C, 63.24 H, 5.86 Cl, 9.82 N, 7.76	C, 64.02 H, 5.71 Cl, 9.88 N, 7.66
XXVII		D	48	89	134.5–136	Ethylacetate	$C_{19}H_{23}ClN_2SO_2$	C, 60.54 H, 5.61 Cl, 9.40 N, 7.43	C, 60.81 H, 5.72 Cl, 9.39 N, 7.42
XXVIII		D	48	87	165–167	Ethylacetate	$C_{23}H_{24}ClN_3O_2$	C, 67.38 H, 5.90 Cl, 8.65 N, 10.25	C, 67.33 H, 5.90 Cl, 8.78 N, 10.11
XXIX		D	48	82	87–88	Ethanol	$C_{28}H_{31}ClN_2O_2$	C, 72.63 H, 6.75 Cl, 7.66 N, 6.05	C, 72.80 H, 6.71 Cl, 7.49 N, 6.06

bis(2-chloroethyl)piperazine are refluxed for 15 hr. in 700 ml. of isopropanol. At the end the reaction mixture is cooled to room temperature, and the suspended solid filtered off. The mother liquor from the reaction is concentrated to a small volume, and the solid separating is filtered and added to the previously isolated precipitate. The combined solids are taken up in chloroform, rejecting the insoluble portion, and the chloroform solution, after filtration with charcoal, is evaporated under reduced pressure, to give 63.7 Gm. of product. After crystallization from isopropanol, IV melts at 123–124°.

1 - (4 - Chlorophenyl) - 4 - [2 - (cinnamoyloxy) - ethyl]-piperazine (XII).—*Method D.*—A solution of sodium ethylate, prepared from 1.87 Gm. of sodium and 45 ml. of ethanol, is added dropwise to a suspension of 14 Gm. of 1-(4-chlorophenyl)-4-(2-chloroethyl)-piperazine dihydrochloride in 70 ml. of ethanol, at 0°. The mixture is stirred for 30 min. at room temperature and then concentrated under reduced pressure, until a thick mush is obtained. A 140-ml. quantity of isopropanol and 7.16 Gm. of sodium *trans*-cinnamate are added to this, then refluxed for 48 hr. with continuous stirring. At the end the mixture is cooled to room temperature, and the suspended solid filtered. The mother liquor from the reaction is concentrated to a small volume, and the separated solid filtered and combined with the previously isolated precipitate. The combined solids are taken up in chloroform, rejecting the insoluble portion, and the chloroform solution, washed with water and filtered with charcoal, is evaporated under reduced pressure to give 11.2 Gm. of product which, after crystallization from isopropanol, melts 125–126.5°.

PHARMACOLOGICAL RESULTS

The new compounds were submitted to preliminary pharmacological screening comprising the action on the CNS (16), the anti-inflammatory (17), analgesic (18), antipyretic (19), antispasmodic (20), antibacterial and antifungal actions (10), and the acute toxicity. The highest dosage level that did not provoke an obvious toxic symptomatology in experimental animals was used for each test. In all cases the compounds were administered by intraperitoneal injection. Phenylbutazone and morphine were used as standards for comparison of the anti-inflammatory, analgesic, and antipyretic actions.

The results of the activity tests considered most interesting are reported in Tables III and IV; they show that many of the compounds have a mild general depressive action on the CNS. Several compounds (II, III, IV, V, VI, VII, VIII, IX, XVII, XVIII, XIX, XX, XXI) display significant inhibition of formalin-induced edema. As for the analgesic action, XII, XV, XVI, XXI, XXII, and XXIX greatly increase the pain threshold of mice, in particular, XV, VI, VII, IX, XVIII, and XXI show a significant antipyretic effect. All the compounds have been found to be inactive regarding antispasmodic, antibacterial, and antifungal actions. It is interesting to note that, within the sphere of the authors' investigations, the over-all pharmacological outline of the 3 esters of saturated aliphatic acids (X, XI, XXIX) does not substantially differ from that of the acrylic esters.

In the light of the above results, the authors con-

TABLE III.—PHARMACOLOGICAL RESULTS OF SYMMETRICAL DIALKYLPIPERAZINE BIS-ESTERS

Compd.	LD_{50} (Approx.) Mouse, mmole/Kg., i.p.	mmole/ Kg., i.p.	Action on the CNS, Mouse	Analgesic Activity, Mouse: mmole/ Kg., i.p.	Analgesic Activity, Mouse: Increase of Reaction Time, %	Anti-Inflammatory Activity, Rat: mmole/ Kg., i.p.	Anti-Inflammatory Activity, Rat: Inhibition of Edema, %	Antipyretic Activity, Rat: mmole/ Kg., i.p.	Antipyretic Activity, Rat: Max. Temp. Decrease, °C.
I	0.313–0.365	0.130	Moderate muscle hypotonia, moderate ipsilateral flexor reflex decrease	0.130	29	0.130	Inact.	...	...
II	1.646–2.140	0.658	Nothing noticeable	0.658	47	0.658	53	0.658	1.7
III	0.527–0.664	0.458	Moderate CNS depression, moderate motor incoordination, muscle hypotonia	0.458	54	0.458	37	...	...
IV	2.062–2.978	0.458	Moderate pinna reflex decrease	0.458	74	0.458	40	0.458	1.9
V	1.993–2.864	0.458	Nothing noticeable	0.458	39	0.458	32	...	...
VI	1.267–1.770	0.745	Nothing noticeable	0.745	21	0.745	60	0.373	2.2
VII	0.671–0.801	0.373	Moderate CNS depression	0.373	72	0.373	50	0.373	3.2
VIII	0.312–0.440	0.284	Nothing noticeable	0.284	20	0.284	49	...	...
IX	2.045–2.691	0.861	Nothing noticeable	0.861	49	0.861	53	0.430	2.3
X	>7.758	0.970	Nothing noticeable	0.970	18	0.970	21	...	...
XI	>7.264	0.908	Nothing noticeable	0.908	39	0.908	26	...	...
Morphine[a]	...	...	...	0.0133	67	...	...	...	...
Phenylbutazone	...	...	...	...	...	0.32	18	0.32	1.6

[a] Hydrochloride.

TABLE IV.—PHARMACOLOGICAL RESULTS OF ASYMMETRICAL ALKYLPIPERAZINE MONO-ESTERS

Compd.	LD_{50} (Approx.) Mouse, mmole/Kg., i.p.	mmole/ Kg., i.p.	Action on the CNS, Mouse	Analgesic Activity, Mouse: mmole/ Kg., i.p.	Analgesic Activity, Mouse: Increase of Reaction Time, %	Anti-Inflammatory Activity, Rat: mmole/ Kg., i.p.	Anti-Inflammatory Activity, Rat: Inhibition of Edema, %	Antipyretic Activity, Rat: mmole/ Kg., i.p.	Antipyretic Activity, Rat: Max. Temp. Decrease, °C.
XII	7.550–9.707	2.157	Moderate CNS depression, muscle hypotonia	2.157	96	2.157	16	...	...
XIII	3.619–4.653	0.517	Nothing noticeable	0.517	38	0.517	Inact.	...	...
XIV	3.244–3.982	0.447	Nothing noticeable	0.447	42	0.447	Inact.	...	...
XV	0.753–0.914	0.134	Moderate CNS depression, muscle hypotonia	0.134	124	0.134	Inact.	...	...
XVI	1.069–1.322	0.281	Moderate CNS depression, muscle hypotonia, moderate ipsilateral flexor reflex decrease	0.281	97	0.281	12	...	...
XVII	0.536–0.689	0.383	Moderate CNS depression	0.383	42	0.383	35	...	...
XVIII	0.825–0.967	0.569	Moderate muscle hypotonia	0.569	74	0.569	45	0.569	3.3
XIX	0.952–1.170	0.136	Moderate CNS depression, moderate ipsilateral flexor, corneal, and pinna reflexes decrease	0.136	61	0.136	33	...	...
XX	0.936–1.195	0.071	Moderate ipsilateral flexor, corneal, and pinna reflexes decrease	0.071	69	0.071	34	...	...
XXI	1.829–2.124	0.134	Moderate CNS depression	0.134	106	0.134	41	0.134	2.4
XXII	1.398–1.748	0.067	Moderate CNS depression, moderate muscle hypotonia	0.067	94	0.067	Inact.	...	...
XXIII	6.889–7.958	0.950	Nothing noticeable	0.950	23	0.950	Inact.	...	...
XXIV	>7.584	3.792	Moderate CNS depression, muscle hypotonia	0.474	38	0.474	Inact.	...	...
XXV	0.355–0.592	0.237	Moderate CNS depression	0.237	72	0.237	Inact.	...	...
XXVI	6.097–7.760	4.434	Moderate CNS depression	0.554	28	0.554	Inact.	...	...
XXVII	5.572–7.297	4.246	Nothing noticeable	0.531	16	0.531	Inact.	...	...
XXVIII	5.245–6.709	0.976	Moderate CNS depression	0.976	37	0.976	Inact.	...	...
XXIX	>6.911	0.864	Nothing noticeable	0.864	123	0.864	Inact.	...	...
Morphine[a]	...	...	...	0.0133	67	...	...	...	...
Phenylbutazone	...	...	...	...	...	0.32	18	0.32	1.6

[a] Hydrochloride.

sider that many of the compounds tested, especially XV, deserve a more detailed pharmacological study for their analgesic, anti-inflammatory, and antipyretic properties.

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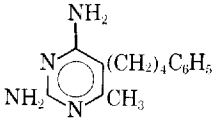
Analogs of Tetrahydrofolic Acid XXXII

Hydrophobic Bonding to Dihydrofolic Reductase IV. Inhibition by *p*-Substituted Benzoic and Benzoyl-L-glutamic Acids

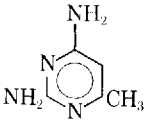
By B. R. BAKER*, THOMAS J. SCHWAN, JAROSLAV NOVOTNY, and BENG-THONG HO

A series of *p*-substituted benzoic acids and benzoyl-L-glutamic acids were synthesized and evaluated as inhibitors of dihydrofolic reductase in order to gain information on the position of the hydrophobic bonding region of the enzyme with respect to the position of the substrate, dihydrofolate, when the latter is complexed to the enzyme. Hydrophobic bonding by the *p*-substituted benzoyl-L-glutamic acids was reached 4–8 atoms from the *p*-position, thus indicating that the hydrophobic bonding region was not between the pyrimidyl and *p*-aminobenzoyl moieties of the substrate, dihydrofolate; in contrast, hydrophobic bonding with *p*-substituted benzoic acids was reached 1–4 atoms from the *p*-position, thus indicating that the *p*-substituted benzoic acids were complexed in a different region of the enzyme than the *p*-substituted benzoyl-L-glutamic acids.

THE DISCOVERY of strong hydrophobic bonding to dihydrofolic reductance with alkyl pyrimidines and 1,2-dihydro-*s*-triazines (1) has led to a major program in this laboratory on the nature, stereochemistry, and position of this hydrophobic bonding. That the aryl group of 1-aryl-1,2-dihydro-*s*-triazines and 5-arylpyrimidines of the pyrimethamine[1] type is also most probably complexed to dihydrofolic reductase by hydrophobic bonding has received strong experimental support (2). Furthermore, the 5-alkyl group of 5-alkyl-2,4-diamino-6-pyrimidines had maximum hydrophobic bonding with the 3-methylbutyl group (3); less binding was observed with 2-methylbutyl, butyl, and 1-methylbutyl, in that order. Furthermore, cyclohexyl was as good as *n*-butyl, but cyclopentyl was considerably poorer; thus, there are definite conformational requirements for alkyl groups to give maximum hydrophobic bonding (3). The magnitude of hydrophobic bonding to dihydrofolic reductase can be enormous; the phenylbutyl group of I alone had a free energy of binding of 6.0 Kcal./mole, equivalent to 80% of the total binding of the substrate, dihydrofolate. This binding by the phenylbutyl group could be calculated from the increment of 40,000 observed between I and II in their relative ability to inhibit dihydrofolic reductase.



I



II

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Previous paper: Baker, B. R., and Lourens, G. J., *J. Heterocyclic Chem.*, **2**, 344(1965).

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[1] Marketed as Daraprim by Burroughs Wellcome Co.

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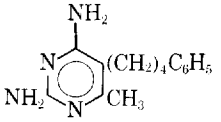
Analogs of Tetrahydrofolic Acid XXXII

Hydrophobic Bonding to Dihydrofolic Reductase IV. Inhibition by *p*-Substituted Benzoic and Benzoyl-L-glutamic Acids

By B. R. BAKER*, THOMAS J. SCHWAN, JAROSLAV NOVOTNY, and BENG-THONG HO

A series of *p*-substituted benzoic acids and benzoyl-L-glutamic acids were synthesized and evaluated as inhibitors of dihydrofolic reductase in order to gain information on the position of the hydrophobic bonding region of the enzyme with respect to the position of the substrate, dihydrofolate, when the latter is complexed to the enzyme. Hydrophobic bonding by the *p*-substituted benzoyl-L-glutamic acids was reached 4–8 atoms from the *p*-position, thus indicating that the hydrophobic bonding region was not between the pyrimidyl and *p*-aminobenzoyl moieties of the substrate, dihydrofolate; in contrast, hydrophobic bonding with *p*-substituted benzoic acids was reached 1–4 atoms from the *p*-position, thus indicating that the *p*-substituted benzoic acids were complexed in a different region of the enzyme than the *p*-substituted benzoyl-L-glutamic acids.

THE DISCOVERY of strong hydrophobic bonding to dihydrofolic reductance with alkyl pyrimidines and 1,2-dihydro-*s*-triazines (1) has led to a major program in this laboratory on the nature, stereochemistry, and position of this hydrophobic bonding. That the aryl group of 1-aryl-1,2-dihydro-*s*-triazines and 5-arylpyrimidines of the pyrimethamine[1] type is also most probably complexed to dihydrofolic reductase by hydrophobic bonding has received strong experimental support (2). Furthermore, the 5-alkyl group of 5-alkyl-2,4-diamino-6-pyrimidines had maximum hydrophobic bonding with the 3-methylbutyl group (3); less binding was observed with 2-methylbutyl, butyl, and 1-methylbutyl, in that order. Furthermore, cyclohexyl was as good as *n*-butyl, but cyclopentyl was considerably poorer; thus, there are definite conformational requirements for alkyl groups to give maximum hydrophobic bonding (3). The magnitude of hydrophobic bonding to dihydrofolic reductase can be enormous; the phenylbutyl group of I alone had a free energy of binding of 6.0 Kcal./mole, equivalent to 80% of the total binding of the substrate, dihydrofolate. This binding by the phenylbutyl group could be calculated from the increment of 40,000 observed between I and II in their relative ability to inhibit dihydrofolic reductase.



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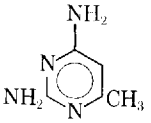

II

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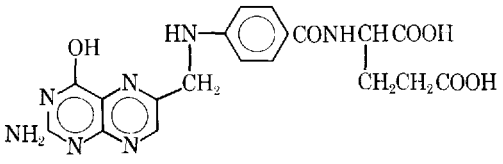
[1] Marketed as Daraprim by Burroughs Wellcome Co.



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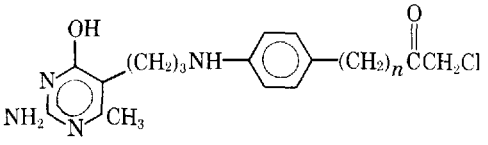

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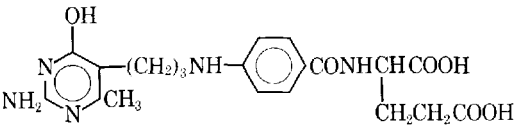
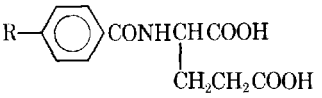


V



VI, R = NH_2
VII, R = H

The discovery of this hydrophobic bonding creates a serious problem from the standpoint of design of an active-site-directed irreversible inhibitor (4) of dihydrofolic reductase—namely, where is the hydrophobic region with respect to the binding region for the *p*-aminobenzoyl moiety of folic acid (III) on dihydrofolic reductase? Is this hydrophobic region between the *p*-aminobenzoyl and the pyrimidine moieties or is it elsewhere, such as near the region where the 4-oxo group of folic acid is in the enzyme–inhibitor complex? If the hydrophobic region is not between these 2 moieties, then the anilino group of IV would probably not be complexed with the *p*-aminobenzoyl locus. If the anilino group of IV is complexed with a different locus, then an inhibitor of type IV (5) would not have its alkylating function sufficiently neighboring to the glutamate binding points of folic acid (III) to alkylate irreversibly such a binding point. Furthermore, if the anilino group of IV is complexed to a hydrophobic region, then by definition there is apt not to be a nucleophilic group in this region of the enzyme. A variety of approaches to answer this important question were initiated, since it could be expected to be difficult to obtain an unequivocal answer; one approach is the subject of this paper.

DISCUSSION

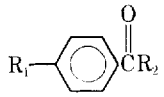
p-Aminobenzoyl-L-glutamic acid (VI) was measured as an inhibitor of dihydrofolic reductase. With the consideration that VI did not have the pyrimidyl moiety of the prototype inhibitor, V (6), the 12 m*M* concentration of VI needed for 50% inhibition (Table I) compared favorably with V, where 0.10 m*M* was needed for 50% inhibition (5). The 120-fold difference in binding between V and VI is a difference of 2.9 Kcal./mole. Since it can be calculated that 2-amino-6-methyl-4-pyrimidinol has a free energy of binding to dihydrofolic reductase of 3.0 Kcal./mole (1), the agreement is fairly reasonable for the amount of inhibition that can be expected when the pyrimidyl moiety is removed from V to give VI; these results support the suggestion that *p*-aminobenzoyl-L-glutamate (VI) binds to the same region of dihydrofolic reductase that complexes this moiety of V.

With the now reasonable assumption that *p*-aminobenzoyl-L-glutamic acid (VI) binds at the same locus as the *p*-aminobenzoyl-L-glutamate moiety of folic acid (III) then hydrophobic bonding by alkyl, aryl, or aralkyl groups substituted at the *p*-position of VII should be observed if the hydrophobic bonding region were between the pyrimidyl and *p*-aminobenzoyl-L-glutamate moieties of folic acid (III). Note that the *p*-amino group of VI contributed little to inhibition since benzoyl-L-glutamic acid (VII) was about as good an inhibitor (Table I). Little, if any, hydrophobic bonding occurred when a 3-bromopropyl (X) or a phenyl group (XIV) was introduced into the *p*-position. However, when the *p*-substitutent was lengthened to *n*-octyl (XII), about a ninetyfold increase in binding occurred (Table I). These data clearly show that the hydrophobic region begins at least 3 atoms away from the *p*-position of benzoyl-L-glutamic acid (VII). Since the distance between the pyrimidyl and benzoyl-L-glutamate moieties of folic acid (III) consists of a 4-atom chain, it is clear that the hydrophobic region is elsewhere than between these 2 moieties. It should again be emphasized that this interpretation contains the assumption that VII and the *p*-aminobenzoyl-L-glutamate moiety of folic acid (III) are complexed to the same region on the enzyme. Although this interpretation is quite reasonable, it is not unequivocal.

A previous study on the relative contribution of the functional groups (XXIII–XXVI) of the carboxy-L-glutamate moiety of the prototype inhibitor (V) to folic reductase (7), is now open to question on the validity of the interpretations, since it was made prior to the discovery of the strong hydrophobic bonding to dihydrofolic reductase (1). A particularly plaguing inconsistency was the fact that pteroic acid was eightyfold less effective than folic acid (III) as an inhibitor of dihydrofolic reductase (8), whereas XXVI was as good or better an inhibitor than V (5, 7). Although V may be complexed in the same manner as folic acid (III) to dihydrofolic reductase, the possibility existed that the less polar relatives (XXIII–XXVI) were complexed with the hydrophobic region rather than the *p*-amino benzoyl-L-glutamate region. Furthermore, it was previously noted that XXIV was almost as effective as V when assayed with the dihydrofolic reductase system (5); in the folic reductase system, XXIV was one-sixth as effective as V and XXV was about one-half as effective as V (7).

In Table I it can be noted that *p*-aminobenzoyl-L-glutamic acid (VI) was a greater than seventeenfold better inhibitor than *p*-aminohippuric acid (XXI)

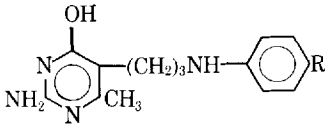
TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



Compd.	R_1	R_2[a]	mM Concn.	% Inhibition	Estimated mM Concn. for 50% Inhibition[b]
VI	NH_2	GL	12	50	12
VII	H	GL	16	50	16
VIII	H	OH	75	0	>300[c]
IX	$Br(CH_2)_3$—	OH	17	50	17
X	$Br(CH_2)_8$—	GL	4.5	50	4.5
XI	n-C_8H_{17}—	OH	1.0[d,e]	15	5.6
XII	n-C_8H_{17}—	GL	0.17[d]	50	0.17
XIII[f]	C_6H_5—	OH	6.7	50	6.7
XIV	C_6H_5—	GL	6.5[d]	50	6.5
XV	$C_6H_5CH_2$—	OH	10	50	10
XVI	$Br(CH_2)_4$—	OH	6.2	50	6.2
XVII	$Cl(CH_2)_5$—	OH	6.0	50	6.0
XVIII[f]	C_6H_5CO—	OH	13[d]	50	13
XIX	$CONHC_6H_4$—$(CH_2)_2CH(CH_3)_2$-p—	OH	0.20[d,e]	0	>0.80[c]
XX	$CONHC_6H_4CH_2$-p—$(CH_2)_2CH(CH_3)_2$-p—	OH	0.60[d,e]	0	>2.4[c]
XXI[f]	NH_2	—$NHCH_2COOH$	50	0	>200[c]
XXII[g]	NH_2	—$NH(CH_2)_3COOH$	75	0	>300[c]
XXVI	m-$NH_2C_6H_4CH_2NH$-p—	OH	0.76	50	0.76
XXVII	m-$NH_2C_6H_4CH_2N$-p—$COCH_3$	OH	9	0	>36[c]
XXXVIII	m-$NO_2C_6H_4CH_2NH$-p—	OH	1.8	50	1.8

Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction that was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM Versene as previously described (20). Solutions of inhibitors were prepared in the 0.05 M Tris buffer by adjustment of the pH to 7.4 with 0.1 N KOH, unless otherwise indicated. [a] GL = L-glutamate. [b] The concentration for 50% inhibition was determined by plotting V_0/VI against I for several concentrations of I that would give 30–70% inhibition, where V_0 = velocity without inhibitor, VI = velocity with inhibitor, and I = concentration of inhibitor; the concentration for 50% inhibition was obtained where $V_0/VI = 2$ (21, 22). When 50% inhibition could not be reached due to lack of solubility, the line was extended to the 50% inhibition point; the less the maximum inhibition, the greater is the error in the estimated concentration for 50% inhibition. [c] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is greater than 4 times the concentration measured. [d] Solution of inhibitor prepared in 1:1 N,N-dimethylformamide–Tris buffer by adjusting the pH to 7.4 with Tris base in 50% aqueous N,N-dimethylformamide; the assay was run in 10% N,N-dimethylformamide. [e] Maximum solubility in cell in 10% N,N-dimethylformamide. [f] Commercial sample. [g] See *Reference 7* for preparation.

and greater than a 33-fold better inhibitor than γ-(*p*-aminobenzoyl)butyric acid (XXII). If it is again assumed that *p*-amino benzoyl-L-glutamate *per se* as well as this moiety of V are complexed to the binding locus for the *p*-aminobenzoyl-L-glutamate moiety of folic acid (III), it follows that XXI–XXVI are complexed elsewhere, perhaps in the hydrophobic region.



V, R = —CONHCH(CH$_2$CH$_2$COOH)COOH

XXIII, R = H

XXIV, R = CONHCH$_2$COOH

XXV, R = CONHCH(CH$_2$CH$_2$COOH)CH$_2$

XXVI, R = COOH

In order to obtain experimental evidence for or against this hypothesis, some *p*-substituted benzoic acids were investigated for their ability to inhibit dihydrofolic reductase.

Note that benzoic acid (VIII) showed no inhibition at a concentration of 75 mM, but that *p*-substitution with a bromopropyl gave a compound (IX) that showed 50% inhibition at 17 mM. Lengthening the chain to bromobutyl (XVI) or chloropentyl (XVII) gave still better inhibitors, with 50% inhibition at about 6 mM. Furthermore, *p*-phenyl-(XIII), *p*-benzyl-(XV), and *p*-benzoylbenzoic (XVIII) acids showed 50% inhibition in the 6–13 mM range. Thus, these hydrophobic groups could give as much as a greater than fiftyfold increase in binding compared to benzoic acid (VIII). Whether there were limitations on the length of this *p*-group for hydrophobic bonding could not be shown by XI, XIX, or XX due to insolubility; however, it was apparent that longer groups in XI and XX could not have given much further increment in hydrophobic bonding. Thus the hydrophobic region begins soon after the first atom in the *p*-position of benzoic acid, when these benzoic acids are complexed to dihydrofolic reductase; in contrast, the *p*-position of benzoyl-L-glutamate must have greater than a 4-

atom chain before hydrophobic bonding is detected.

That hydrophobic bonding can occur also with *N*-substituents on *p*-aminobenzoic acid is shown with the *m*-aminobenzyl and the *m*-nitrobenzyl derivatives, XXVI and XXXVIII (Table I); XXVI is actually the best benzoate-type inhibitor in Table I. Surprisingly, *N*-acetylation on the *p*-amino group led to a greater than 47-fold loss in binding to dihydrofolic reductase. A possible interpretation of this result will be discussed (9).

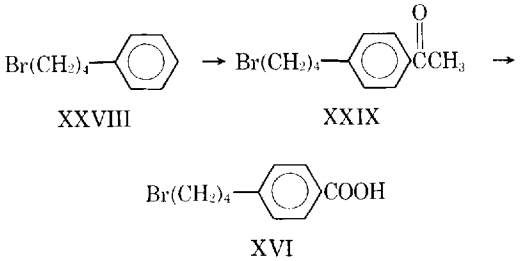
Regardless of the mode of binding of benzoyl-L-glutamic acid (VII) and the substituted benzoic acids, it is clear that the benzoyl group of the 2 classes of inhibitors are complexed to different regions of the enzyme. Whether the benzoyl-L-glutamates are complexed to the normal region for this moiety in folic acid, and the benzoic acids are complexed in hydrophobic region cannot yet be proven unequivocally. However, as a working hypothesis, such modes of binding have led to useful results which could not have been accrued otherwise. These results on the nature and position of hydrophobic bonding are the subjects of additional papers to be submitted in the near future.

EXPERIMENTAL

Methods.—*p*-(4-Bromobutyl)benzoic acid (XVI) was synthesized from 4-(bromobutyl)benzene (XXVIII) by Friedel-Crafts acetylation to XXIX followed by hypobromite oxidation; similarly, XVII was synthesized. (Scheme I.) The synthesis of IX (10) and XI (11) by this route have been previously recorded.

The *p*-substituted benzoyl-L-glutamic acids (X, XII, and XIV) were synthesized by condensation of the appropriate acid chloride in an organic solution with an aqueous sodium carbonate solution of L-glutamic acid; the known benzoyl-L-glutamic acid (VII) (12) was also synthesized in this manner. All but XIV were isolated as the bis-cyclohexylammonium salts, which were readily crystallized and purified, in contrast to the free acids which were difficult to crystallize or purify, or both.

4′-Nitrodiphenic acid (XXXII) was synthesized by modification of a procedure in the patent literature (13, 14) by Friedel-Crafts acetylation of XXX in nitrobenzene to XXXI followed by sodium hypobromite oxidation to XXXII without isolation of XXXI. Catalytic reduction to XXXIII proceeded smoothly in a 50% ethanolic solution of the sodium salt in the presence of Raney nickel; this method was considered to be more convenient than the ammonium sulfide reduction described previously (15). Acylation with 4-methylvaleroyl chloride to XIX in acetone in the presence of potassium carbonate proceeded satisfactorily. Similarly, acylation of



XXVIII XXIX XVI

Scheme I

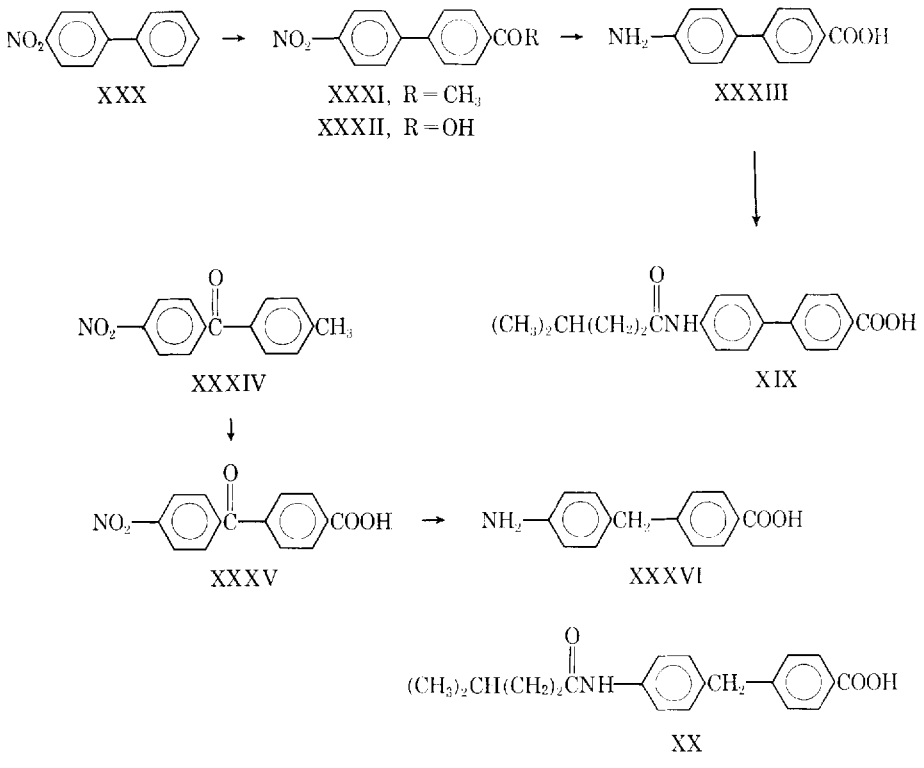
XXXVI to XX was performed. The required intermediate amino acid (XXXVI), although known in the literature (15), was prepared by an alternate route. Oxidation of 4-methyl-4′-nitrobenzophenone (XXXIV) (16) with chromium trioxide in acetic acid gave the acid (XXXV) in 55% yield of analytically pure material. Oxidation of XXXIV to XXXV with potassium permanganate in aqueous alkali or in acetone proceeded poorly due to overoxidation to water-soluble products. Huang-Minlon reduction of the carbonyl group of XXXV with hydrazine in diethyleneglycol containing potassium hydroxide also gave concomitant reduction of the nitro group; the desired amino acid (XXXVI) was obtained in 82% yield. (Scheme II.)

Condensation of *m*-nitrobenzaldehyde with *p*-aminobenzoic acid in ethanol gave the anil (XXXVII) in 96% yield. Reduction of a suspension of the anil in methanol with sodium borohydride gave the benzylamine (XXXVIII) in 98% yield. Further hydrogenation in 2-methoxyethanol with a platinum oxide catalyst afforded the desired amino acid (XXVI). Acetylation of XXXVIII with boiling acetic anhydride gave an anhydride of the desired *N*-acetyl derivative which was readily converted to XXXIX with 1 *N* sodium hydroxide in quantitative over-all yield. Catalytic reduction in ethanol afforded the requisite amino acid (XXVII) in good yield. (Scheme III.)

Synthesis.—Melting points were taken on a Fisher-Johns apparatus or a Mel-Temp block, and those below 230° are corrected. Infrared spectra were determined in KBr disk with a Perkin-Elmer 137B spectrophotometer unless otherwise indicated; ultraviolet spectra were determined with a Perkin-Elmer 202 spectrophotometer.

p-(4-Bromobutyl)benzoic Acid (XVI).—To a stirred suspension of 5.6 Gm. (42 mmoles) of anhydrous aluminum chloride in 21 ml. of carbon disulfide, cooled in an ice bath and protected from moisture, was added 3.0 ml. (42 mmoles) of acetyl chloride over a period of 10 min. Then a mixture of 10.2 Gm. (48 mmoles) of 4-bromobutylbenzene (17) and 6.5 ml. (90 mmoles) of acetyl chloride was added as rapidly as reflux would allow. After the addition was complete, the mixture was stirred for 2.5 hr., then poured into a mixture of 50 Gm. of ice and 10 ml. of 12 *N* aqueous hydrochloric acid. To the mixture was added 40 ml. of benzene; an insoluble yellow solid was removed by filtration. The separated aqueous layer was extracted with three 10-ml. portions of benzene. The combined benzene extracts were washed successively with 10% hydrochloric acid, 2 *N* aqueous potassium hydroxide, and water. Dried with magnesium sulfate, the benzene solution was spin-evaporated *in vacuo* leaving 12.6 Gm. of the crude acetophenone, XXIX (18).

To an ice cold solution of 1.55 ml. (0.02 mmole) of bromine in 28 ml. of water containing 3.3 Gm. of sodium hydroxide was added 15 ml. of dioxane. Then a solution of 2.5 Gm. (10 mmoles) of crude XXIX in 5 ml. of dioxane was added over a period of 45 min. After being stirred for an additional 2 hr. in the ice bath, the solution was acidified with 10 ml. of 12 *N* aqueous hydrochloric acid. A brown oil separated that soon solidified. The product was collected on a filter and washed with water until the washings were colorless; yield, 1.21 Gm. (46% based on XXVIII), m.p. 135–137°.



Scheme II

Recrystallization from benzene–petroleum ether (b.p. 30–60°) gave white needles, m.p. 136–137°. $\nu_{max.}$ 2680–2550 (acidic OH); 1680 (C=O); 859 cm.$^{-1}$ (*p*-C_6H_4).

Anal.—Calcd. for $C_{11}H_{13}BrO_2$: C, 51.4; H, 5.08. Found: C, 51.2; H, 5.06.

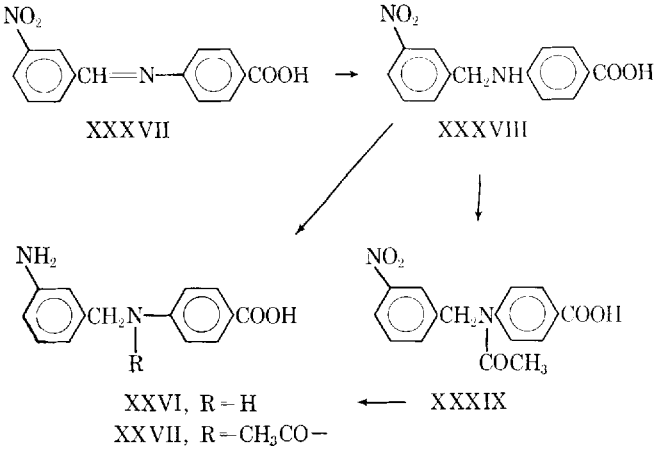
Similarly, IX (10) and XI (11) were prepared.

p-(5-Chloropentyl)benzoic Acid (XVII).—This compound was prepared in 46% over-all yield from 5-chloropentylbenzene as described for XVI. Recrystallization from benzene–petroleum ether (b.p. 30–60°) gave white crystals, m.p. 109–111°. $\nu_{max.}$ 1690 (C=O); 860, 840 cm.$^{-1}$ (*p*-C_6H_4).

Anal.—Calcd. for $C_{12}H_{15}ClO_2$: C, 63.6; H, 6.65. Found: C, 63.3; H, 6.49.

That this was a *p*-substituted benzoic acid, as indicated by the infrared spectrum, was further verified by oxidation to terephthalic acid.

p-(n-Octyl)benzoyl-L-glutamic Acid (XII) (Cyclohexylammonium Salt).—A solution of 351 mg. (1.5 mmoles) of XI, 10 ml. of chloroform, 0.9 Gm. of thionyl chloride, and a trace of pyridine was refluxed for 30 min. when gas evolution was complete. Solvent was removed by evaporation *in vacuo*. A solution of the residual acid chloride in 3 ml. of acetone was added in 1 portion to a magnetically



Scheme III

stirred solution of 147 mg. (1 mmole) of L-glutamic acid, in 0.67 ml. of 3 *N* sodium hydroxide (2 mmoles) and 5 ml. of water containing 160 mg. (1.5 mmoles) of sodium carbonate. After being stirred overnight at ambient temperature, the reaction mixture was spin-evaporated until the acetone was removed, then the solution was diluted with 50 ml. of water and adjusted to about pH 5 with dilute hydrochloric acid. After standing for a few hours, the mixture was filtered, and the recovered XI was washed with water. The filtrate was adjusted to about pH 1 with 3 *N* hydrochloric acid. The semisolid (XII) which separated (224 mg., 0.62 mmole) was dissolved in 5 ml. of methanol, and 155 mg. of cyclohexylamine (1.55 mmoles, 25% excess) was added. The solution was spin-evaporated *in vacuo* yield, 348 mg. (62%) of crude product. The residue was recrystallized twice from absolute ethanol–ether; yield, 71 mg. (13%) of analytically pure material, m.p. 170–175°. $\lambda_{max.}$ (pH 1, 13) 245 mμ; $\nu_{max.}$ (Nujol) 2200 (NH^+), 1670–1640, 1560–1500 cm.$^{-1}$ (COO^-, amide I and II, C=C).

Anal.—Calcd. for $C_{32}H_{55}N_3O_5$: C, 68.4; H, 9.87; N, 7.48. Found: C, 68.5; H, 9.96; N, 7.59.

No attempt was made to isolate additional material from the mother liquors.

p-(3-Bromopropyl)benzoyl-L-glutamic Acid (X) (Cyclohexylammonium Salt).—This compound was prepared as described for XII except that five 40-ml. extractions with ethyl acetate were employed since the product was fairly water soluble. Recrystallization from methanol–acetone afforded a 30% yield of the bis-cyclohexylammonium salt of X, m.p. 145–150°. A second recrystallization from the same solvent pair gave white crystals, m.p. 149–152°. $\nu_{max.}$ (Nujol) 2220 (NH^+); 1610–1640, 1590–1510 cm.$^{-1}$ (COO^-, amide I and II, C=C).

Anal.—Calcd. for $C_{27}H_{44}BrN_3O_5$: C, 56.8; H, 7.77; N, 7.36. Found: C, 56.6; H, 7.55; N, 7.12.

p-Phenylbenzoyl-L-glutamic Acid (XIV).—A mixture of 1.93 Gm. (10 mmoles) of 4-biphenylcarboxylic acid, 20 ml. of chloroform, 4 ml. (50 mmoles) of thionyl chloride, and a trace of pyridine was refluxed for 16 hr., then spin-evaporated *in vacuo*. The residual crystalline acid chloride was dissolved in 15 ml. of carbon tetrachloride, then added over a period of 1 hr. to a stirred solution of 1.47 Gm. (10 mmoles) of L-glutamic acid, and 3.41 Gm. (32.5 mmoles) of sodium carbonate in 15 ml. of water that was layered with 8 ml. of carbon tetrachloride. After being stirred for an additional 24 hr., the layers were separated; the aqueous layer was adjusted to pH 5.5 with 3 *N* hydrochloric acid, then washed with chloroform to remove 4-biphenylcarboxylic acid. The aqueous solution then was adjusted to pH 1.5. The white, difficultly filterable precipitate was collected by centrifugation, then washed with water. Recrystallization from aqueous acetone gave 1.08 Gm. (33%) of white crystals, m.p. 178–179°. The compound moved as a single spot on thin-layer chromatography on Silica Gel G with propanol–water (37:13) when viewed under ultraviolet light. The compound has $\nu_{max.}$ 3320 (amide NH), 3000, 2650, 2550 (broad acidic OH); 1700 (carboxyl C=O), 1630, 1600, 1580, 1520, 1500 (amide I and II, C=C); 850 (*p*-C_6H_4); 750, 685 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1) 273 mμ (ε 25,700); (pH 13) 272 mμ (ε 28,500).

Anal.—Calcd. for $C_{18}H_{17}NO_5$: C, 66.0; H, 5.23; N, 4.28. Found: C, 65.8; H, 4.99; N, 4.22.

Benzoyl-L-glutamic Acid (VII) (Cyclohexylammonium Salt).—A solution of L-glutamic acid (10 mmoles) in aqueous sodium carbonate was acylated with 1.42 ml. (15 mmoles) of benzoyl chloride in 8 ml. of carbon tetrachloride as described for the preparation of XIV. After removal of the benzoic acid at pH 1, the filtrate was spin-evaporated to dryness *in vacuo*. The residue was extracted with acetone. The filtered solution was spin-evaporated *in vacuo* leaving 2.20 Gm. (88%) of VII as an oil which solidified after several days, m.p. 125–130°. [Lit. m.p. 139–140° (12).]

To a solution of 638 mg. (2.5 mmoles) of VII in 5 ml. of ethanol was added 550 mg. (5.5 mmoles) of cyclohexylamine. Some of the cyclohexylammonium salt (449 mg., m.p. 186–190°) separated immediately, and an additional 600 mg. (total 94%), m.p. 180–190°, was isolated from the filtrate. Recrystallization from absolute ethanol–ether gave 849 mg. (75%) of white crystals, m.p. 191–193°. $\nu_{max.}$ 3250 (NH), 3000–2600, 2200 (NH^+); 1660, 1640, 1580–1520 (COO^-, amide I and II, C=C); 712, 692 cm.$^{-1}$ (C_6H_5); $\lambda_{max.}$ (pH 1, 7) 229 (ε 14,500), 245 mμ (sh) (ε 8000).

Anal.—Calcd. for $C_{24}H_{39}N_3O_5$: C, 64.4; H, 8.74; N, 9.35. Found: C, 64.4; H, 8.70; N, 9.40.

4′-Nitro-4-biphenylcarboxylic Acid (XXXII).—To a stirred solution of 30 Gm. (0.15 mole) of 4-nitrobiphenyl in 100 ml. of nitrobenzene protected from moisture was added 39 Gm. (0.29 mole) of anhydrous aluminum chloride. The mixture was heated to 50–53°, then a solution of 19.5 Gm. of acetyl chloride in 17 ml. nitrobenzene was added over a period of 8 hr. After being heated an additional 4 hr. at 62–64°, the mixture was stirred overnight at ambient temperature, then poured into 400 ml. of iced water. The separated nitrobenzene layer was washed with water (3 × 50 ml.), then poured in a thin stream into an ice-cooled stirred solution of 72 Gm. of bromine in 100 ml. of water containing 50 Gm. of sodium hydroxide and 300 Gm. of ice. The stirred mixture was gradually warmed to 70° over a period of about 30 min., then maintained at 60–70° for 1.5 hr.; during this time it was necessary to remove the heating bath occasionally to keep the temperature from rising above 70°. The mixture then was stirred under a reflux condensor in a bath at 100–105° for 2 hr. The orange sodium salt was collected by filtration after chilling at 15° for 3 hr. The sodium salt was extracted with three 800-ml. portions of boiling water, filtering the solution each time through glass wool to remove a brown insoluble by-product. The combined filtrates were reheated to dissolve the sodium salt, then acidified to pH 2–3 with 12 *N* hydrochloric acid; yield, 10.2 Gm. (28%), m.p. 338–342° dec. Recrystallization from 2-methoxyethanol gave 6.65 Gm. (18%), m.p. 345–348° dec. [Lit. m.p. 340°, 345° (13, 14).]

4′-Amino-4-biphenylcarboxylic Acid (XXXIII).—A solution of 243 mg. (1 mmole) of XXXII in 100 ml. of 50% ethanol and 2 ml. of 2 *N* sodium hydroxide was shaken with hydrogen at 2–3 Atm. in the presence of about 1 Gm. of Raney nickel; reduction was complete in 30 min. The filtered solution was adjusted to pH 6 with glacial acetic acid, then spin-evaporated *in vacuo* to about 50 ml. The product

was collected on a filter and washed with water. Yield, 170 mg. (80%), m.p. 245–248°. [Lit. m.p. 243–246° (15).]

4-(4′-Nitrobenzoyl)benzoic Acid (XXXV).—4-Methyl-4′-nitrobenzophenone (XXXIV) was prepared from *p*-nitrobenzoyl chloride, toluene, and aluminum chloride; the yield, after recrystallization from ethanol was 95%, m.p. 120–121° (16).

To a stirred solution of 9.05 Gm. (37.5 mmoles) of XXXIV in 100 ml. of glacial acetic acid was added 9.5 Gm. (95 mmoles) of chromium trioxide in portions over a period of 1 hr. The mixture was refluxed for 16 hr., then poured into 1 L. of ice water. The product was collected on a pad of Celite, then washed with water until the washings were colorless. The filter cake was stirred at about 90° with 300 ml. of 3 *N* aqueous sodium hydroxide, then filtered hot. The filtrate was rewarmed to dissolve the sodium salt, then the hot solution was acidified to about pH 1 with hydrochloric acid. The product was collected on a filter and washed with water; yield, 6.71 Gm. (66%). Recrystallization from methanol gave 5.86 Gm. (55%) of light yellow crystals, m.p. 257–258°. $\nu_{max.}$ 3000, 2650, 2550 (broad acidic OH); 1670 (carboxyl C=O); 1650 (ketone C=O); 1600 (C=C); 1520 cm.$^{-1}$ (NO_2); $\lambda_{max.}$ (pH 1) 227 (ϵ 13,300), 273 mμ (ϵ 26,100); (pH 13) 276 mμ (ϵ 24,200)

Anal.—Calcd. for $C_{14}H_{19}NO_5$: C, 62.0; H, 3.34; N, 5.16. Found: C, 61.8; H, 3.35; N, 4.96.

4-(4′-Aminobenzyl)benzoic Acid (XXXVI).—To a solution of 6.87 Gm. of potassium hydroxide in 120 ml. of diethyleneglycol was added 9.49 Gm. (35 mmoles) of XXXV and 7 ml. of hydrazine hydrate. After being refluxed for 2 hr., the solution was slowly distilled until 10 ml. of liquid was collected over 2 hr. After being refluxed 1 hr. more, the solution was diluted with several volumes of water, then acidified to pH 4.8 with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water; yield, 6.51 Gm. (82%), m.p. 220–223°, that was suitable for further transformations. Recrystallization of a sample from methanol with the aid of Norit gave slightly pink crystals, m.p. 227–228°. [Lit. m.p. 228° (15).]

p-Benzylbenzoic Acid (XV).—Reduction of *p*-benzoylbenzoic acid, as described for the preparation of XXXVI, gave (after recrystallization from aqueous methanol) a 60% yield of product, m.p. 159–160°; an additional 24%, m.p. 152–153°, was isolated from the filtrate. [Lit. m.p. 156–157° (19).]

4-[4′-(4-Methylvaleramido)benzyl]benzoic Acid (XX).—To a stirred mixture of 908 mg. (4 mmoles) of XXXVI and 4.16 Gm. (30 mmoles) of potassium carbonate and 20 ml. of acetone was added 0.94 ml. (7.2 mmoles) of 4-methylvaleroyl chloride. After being stirred for 3 hr. at 40–50° under a reflux condensor, the mixture was poured into 20 ml. of 1 *N* aqueous hydrochloric acid, then cooled. The product was collected on a filter and washed with water; yield, 982 mg. (75%), m.p. 229–231°. Recrystallization from ethanol gave 769 mg. (59%) of white prisms, m.p. 237–238°. $\nu_{max.}$ 3300 (NH); 2800, 2650, 2540 (broad acidic OH); 1680 (carboxyl C=O); 1650 (amide I); 1600, 1580, 1570 (C=C); 1520 (amide II); 830 cm.$^{-1}$ (*p*-C_6H_4); $\lambda_{max.}$ (pH 1) 258 mμ (ϵ 10,800); (pH 13) 247 mμ (ϵ 25,400).

Anal.—Calcd. for $C_{20}H_{23}NO_3$: C, 73.8; H, 7.12; N, 4.30. Found: C, 74.0; H, 7.26; N, 4.35.

4′-(4-Methylvaleramido)-4-biphenylcarboxylic Acid (XIX).—This compound was prepared in 60% yield from XXXIII as described for the preparation of XX. Recrystallization from ethanol with the aid of Norit gave white plates, m.p. 318–320°; the principal peaks in the infrared spectrum were similar to those of XX. $\lambda_{max.}$ (pH 1) 300 mμ (ϵ 19,000); (pH 13) 288 mμ (ϵ 38,500).

Anal.—Calcd. for $C_{19}H_{21}NO_3$: C, 73.3; H, 6.80; N, 4.50. Found: C, 73.0; H, 6.92; N, 4.27.

N-(m-Nitrobenzylidene)-p-aminobenzoic Acid (XXXVII).—To a stirred hot solution of 3.47 Gm. (20 mmoles) of *p*-aminobenzoic acid in 10 ml. of 95% ethanol was added a hot solution of 3.02 Gm. (20 mmoles) of *m*-nitrobenzaldehyde in 10 ml. of ethanol. The product rapidly separated. The mixture was heated to the b.p., then cooled to room temperature. The product was collected on a filter and washed with ethanol; yield, 5.19 Gm. (96%), m.p. 252–253°. Recrystallization from aqueous methanol gave yellow crystals, m.p. 251–257°. $\nu_{max.}$ 1680 (carboxyl C=O); 1640, 1600, 1575 (C=C, C=N); 1520, 1350 cm.$^{-1}$ (NO_2).

Anal.—Calcd. for $C_{14}H_{10}N_2O_4$: C, 62.2; H, 3.73; N, 10.4. Found: C, 62.1; H, 3.81; N, 10.4.

N-(m-Nitrobenzyl)-p-aminobenzoic Acid (XXXVIII).—To a stirred suspension of 4.05 Gm. (15 mmoles) of XXXVII in 80 ml. of methanol was added in portions over a period of about 30 min., 2.3 Gm. (60 mmoles) of sodium borohydride. The amber solution was refluxed for 15 min., then spin-evaporated *in vacuo*. The residual sodium salt was dissolved in 50 ml. of water, then the solution was acidified to pH 6. The product was collected on a filter and washed with water; yield, 4.01 Gm. (98%), m.p. 237–238°. Recrystallization from aqueous 2-methoxyethanol gave yellow crystals, m.p. 247–248°. $\nu_{max.}$ 3400 (NH); 1670 (carboxyl C=O); 1600, 1575 (C=C, NH); 1520, 1350 (NO_2); 838 (*p*-C_6H_4); 769 cm.$^{-1}$ (*m*-C_6H_4).

Anal.—Calcd. for $C_{14}H_{12}N_2O_4$: C, 61.7; H, 4.44; N, 10.3. Found: C, 61.7; H, 4.60; N, 10.4.

N-Acetyl-N-(m-nitrobenzyl)-p-aminobenzoic Acid (XXXIX).—A mixture of 2.50 Gm. (9.18 mmoles) of XXXVIII and 15 ml. of acetic anhydride was refluxed for 30 min., then poured into 30 Gm. of iced water. The oily product was extracted with chloroform (3 × 10 ml.). The combined extracts, dried with magnesium sulfate, were spin-evaporated *in vacuo*, leaving an oil which solidified on trituration with petroleum ether to a solid (3.16 Gm.), m.p. 91–94°; the infrared spectrum showed anhydride bands at 1800 and 1725 cm.$^{-1}$. The anhydride was warmed with 30 ml. of 1 *N* aqueous sodium hydroxide until solution was essentially complete. The solution was clarified by filtration, then acidified to pH 2 with 3 *N* hydrochloric acid. The product was collected on a filter and washed with water; yield, 2.84 Gm. (99%), m.p. 209–211°. Recrystallization from aqueous ethanol afforded 2.69 Gm. (93%) of nearly white needles, m.p. 212–213°. $\nu_{max.}$ 2600–2500 (broad acidic OH); 1680 (carboxyl C=O); 1625 (amide C=O); 1625, 1580, 1525 (C=C); 1525, 1340 (NO_2); 870 (*p*-C_6H_4); 785 cm.$^{-1}$ (*m*-C_6H_4).

Anal.—Calcd. for $C_{16}H_{14}N_2O_5$: C, 61.1; H, 4.49; N, 8.92. Found: C, 61.1; H, 4.29; N, 8.81.

N-(m-Aminobenzyl)-p-aminobenzoic Acid (XXVI).—A solution of 2.72 Gm. (10 mmoles) of

XXXVIII in 200 ml. of 2-methoxyethanol was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide catalyst; reduction was complete in 90 min. The filtered solution was spin-evaporated *in vacuo* and the residue was recrystallized from aqueous 2-methoxyethanol; yield, 2.04 Gm. (84%) of buff-colored crystals, m.p. 193–194°. Recrystallization from aqueous 2-methoxyethanol gave nearly white crystals with unchanged m.p. $\nu_{max.}$ 3550 (NH); 1680 (carboxyl C=O); 1620, 1550, 1505 (C=C, NH); 825 (*p*-C_6H_4); 765 (*m*-C_6H_4); no NO_2 near 1520 or 1340 cm.$^{-1}$.

Anal.—Calcd. for $C_{14}H_{14}N_2O_2$: C, 69.4; H, 5.82; N, 11.5. Found: C, 69.5; H, 6.00; N, 11.3.

N - Acetyl - N - (m - aminobenzyl) - p - aminobenzoic Acid (XXVII).—A solution of 942 mg. (3 mmoles) of XXXIX in 100 ml. of ethanol was shaken with hydrogen at 2–3 Atm. in the presence of 60 mg. of platinum oxide catalyst; reduction was complete in about 15 min. The filtered solution was spin-evaporated *in vacuo* leaving 849 mg. (99%) of a glassy residue which showed 2 spots on TLC in methanol. After separation by preparative TLC, the material still could not be crystallized.

To a solution of 400 mg. of the crude product in 5 ml. of absolute ethanol was added 172 mg. (25% excess) of cyclohexylamine. Addition of 20 ml. of ether caused the separation of a gum (228 mg.). The supernatant liquid was decanted and deposited 127 mg. of crystals on standing which had m.p. 170–174°. Two recrystallizations from absolute alcohol–ether gave 90 mg. of pure cyclohexylammonium salt, as white crystals, m.p. 168–170°. $\nu_{max.}$ 3450, 3400 (NH); 2200, 2150 (NH^+); 1660 (amide C=O), 1600 (COO^-); 1625, 1540, 1500 cm.$^{-1}$ (NH, C=C).

Anal.—Calcd. for $C_{16}H_{16}N_2O_3 \cdot C_6H_{11}NH_2$: C, 68.9; H, 7.62; N, 11.0. Found: C, 68.7; H, 7.80; N, 10.8.

The free acid could be obtained as a glass free of other organic matter in 73% over-all yield by preparative thin-layer chromatography on Silica Gel HF_{254} with methanol as solvent. Since the compound would not dissolve in acetone or chloroform, methanol was used for elution; the resultant product had a C/N ratio of 6.89 (calcd. 6.86), but could not be freed of about 10% of extracted silica.

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Analogs of Tetrahydrofolic Acid XXXIII

Hydrophobic Bonding to Dihydrofolic Reductase V. Inhibition by Some Pyrimidines Bridged to Benzoic Acid

By B. R. BAKER*, BENG-THONG HO, JAMES K. COWARD, and DANIEL V. SANTI

Folic acid (I), pteroic acid (IV), and a series of 2-amino-6-methyl-4-pyrimidinols bridged from its 5-position to the *p*-position of benzoic acid with aminopropyl (VII), butyl (X), carbamoylpropyl (XIII), sulfonamidopropyl (XV), and *N*-acetylaminopropyl (XVI) were compared as inhibitors of dihydrofolic reductase. Evidence was presented that VII and X probably had their side chains off of the 5-position of the pyrimidine complexed to the hydrophobic region of the enzyme, whereas XIII, XV, and XVI probably had their side chains complexed to the locus on the enzyme that normally binds the *p*-aminobenzoyl moiety of folic acid (I).

In the preceding paper of this series (1), experimental evidence was presented that the hydrophobic bonding region of dihydrofolic reductase (2) is probably not between the binding regions for the pyrimidyl and *p*-aminobenzoyl-L-glutamate moieties of folic acid (I) or dihydrofolic acid. If the hydrophobic bonding area is in some other region, then of the hundreds of compounds, such as type II, evaluated as dihydrofolic reductase inhibitors and presented in previous

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XXXVIII in 200 ml. of 2-methoxyethanol was shaken with hydrogen at 2–3 Atm. in the presence of 100 mg. of platinum oxide catalyst; reduction was complete in 90 min. The filtered solution was spin-evaporated *in vacuo* and the residue was recrystallized from aqueous 2-methoxyethanol; yield, 2.04 Gm. (84%) of buff-colored crystals, m.p. 193–194°. Recrystallization from aqueous 2-methoxyethanol gave nearly white crystals with unchanged m.p. $\nu_{max.}$ 3550 (NH); 1680 (carboxyl C=O); 1620, 1550, 1505 (C=C, NH); 825 (*p*-C_6H_4); 765 (*m*-C_6H_4); no NO_2 near 1520 or 1340 cm.$^{-1}$.

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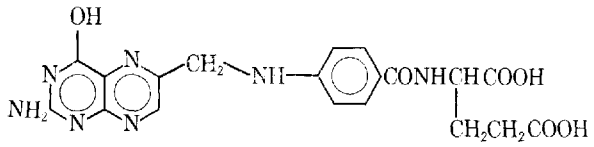
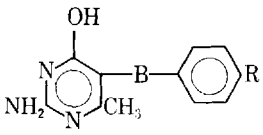


I



II, R = H
III, R = COOH

papers of this series, which have side chains on the heterocycles that complex with this hydrophobic region and which have side chains that complex with the *p*-aminobenzoyl locus? As a side chain is made more and more polar or has added groups that can complex at one or more of the points of the glutamate locus, at what stage does the side chain shift from the hydrophobic bonding region to the more polar *p*-aminobenzoyl-L-glutamate binding region? Such information is considered crucial for rational design of active-site-directed irreversible inhibitors (3), since the covalent bond-forming group on the inhibitor must be juxtapositioned to a nucleophilic group on the enzyme in order to operate; such a nucleophilic group is polar by definition and is less apt to be present in a hydrophobic region of the enzyme. One possible approach to the problem is to compare effects of binding to dihydrofolic reductase when a given pyrimidine bridged to a benzene ring such as II is substituted on the phenyl ring by a *p*-carboxyl group as in III. The results of such a study are the subject of this paper.

DISCUSSION

In a previous study (4) it was noted that the pyrimidyl prototype inhibitor (VI) of dihydrofolic reductase with the intact *p*-aminobenzoyl-L-glutamate moiety (5) was consistently slightly less effective than the corresponding inhibitor (VII) derived from *p*-aminobenzoic acid (Table I). In contrast, folic acid (I) was complexed about eightyfold more effectively than pteroic acid (V) as inhibitors of dihydrofolic reductase (6). These conflicting data on removal of the L-glutamate moiety from the 2 types of inhibitors were in part resolved in the preceding paper of this series (1); that is, it appeared that the propylaminobenzoate side chain of VII was complexed in the hydrophobic region of dihydrofolic reductase rather than at the *p*-aminobenzoyl locus normally complexing this moiety of folic acid (I). The prototype inhibitor (VI) appeared to be complexed in the same manner as folic acid (I). It should also be noted that removal of the carboxyl group of VII gave an inhibitor (VIII) that was only tenfold less effective than VII.

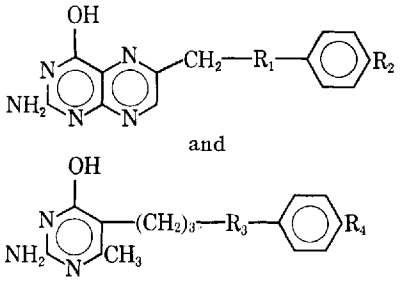
Since both VII and VIII are believed to have their pyrimidyl side chains complexed in the hydrophobic region, 3 other types of side chains previously studied were converted to their *p*-carboxy derivatives to determine if a similar increase in magnitude of binding observed with VII and VIII would be obtained. If a similar increment were obtained, then this increment could be used as evidence for the hypothesis that the side chain in question was complexed to the hydrophobic region of the enzyme. The following 3 side chains were selected: (*a*) the phenylbutyl side chain (IX), since this side chain gave 27-fold better inhibition than the anilinopropyl side chain (VIII) (7); (*b*) the benzamidopropyl side chain (XI), since this side chain gave an inhibitor about as effective as VIII (8); and (*c*) the *p*-tolylsulfonamidopropyl side chain (XIV), since this side chain gave an inhibitor less effective than VIII (8).

Introduction of the *p*-carboxyl (X) into the phenylbutyl side chain (IX) gave a twelvefold enhancement in binding (Table I), thus indicating that the phenylbutyl side chains (IX and X) and the anilinopropyl side chain (VII and VIII) were complexed in the same region of the enzyme, presumably the hydrophobic region. In contrast, introduction of the carboxyl group on the benzamidopropyl side chain (XII) to give XIII or on the benzenesulfonamidopropyl side chain (XIV) to give XV showed no enhancement in binding; these data would indicate that these two side chains of XII and XIV are not complexed in the hydrophobic region, and could be presumed to be complexed to the *p*-aminobenzoyl locus where folic acid (I) is normally complexed.[1] However, such an interpretation would require considerable substantiation since other explanations are possible, for example, (*a*) the chain length between the pyrimidyl and benzene moieties of XII and XIV are 1 atom longer than in the case of the anilinopropyl (VIII) or phenylbutyl series (X); (*b*) the ground-state staggered conformations about the $C_6H_5CH_2CH_2$ group of X or the $C_6H_5NHCH_2$— group of VIII are different than the ground-state planar conformations of the $C_6H_5CONCH_2$ group of XII or conformation of the $C_6H_5SO_2NCH_2$ group of XIV which is not quite so near planarity as the amide.

Bertino *et al.* (6) recently noted that 10-formyl folic acid (IV) was a twentyfold better inhibitor of dihydrofolic reductase than folic acid (I) (Table I). It was interesting to observe that acetylation of VII on the "10-position" corresponding to folic acid gave an inhibitor (XVI) that was less than twofold better than VII and as an inhibitor. A similar small increment has been observed previously on acetylation of 2-amino-5-(3-anilinopropyl)-6-methyl-4-pyrimidinethiol (9). This lack of appreciable increase in binding after acylation could be interpreted to indicate that folic acid (I) and presumably pteroic acid (V) are complexing differently to dihydrofolic reductase than are the 5-anilinopropyl-pyrimidine types (VII and VIII).

[1] Since the *N*-butyl benzamidopropyl and *N*-butyl *p*-tolylsulfonamidopropyl side chains previously studied (8) are less polar, these may be complexed by hydrophobic bonding to the hydrophobic region.

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY

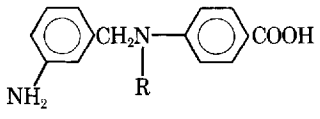


Compd.	R_1	R_2	R_3	R_4	μM Concn. for 50% Inhibition[a]	$K_i \times 10^6$[b]
I	—NH—	—CONHCHCOOH $(CH_2)_2COOH$	...	...	3[c]	0.11
IV	O=CH —N—	—CONHCHCOOH $(CH_2)_2COOH$	...	...		0.0061
V	—NH—	—COOH	...	...		8
VI	...	...	—NH—	—CONHCHCOOH $(CH_2)_2COOH$	100[d,e]	
VII	...	...	—NH—	—COOH	77[d,e]	
VIII	...	...	—NH—	H	800[f,g]	
IX	...	...	$—CH_2—$	H	30[f,h]	
X	...	...	$—CH_2—$	—COOH	2.5[e]	
XI	...	...	$—CH_2—$	$—COOC_2H_5$	13[g]	
XII	...	...	—HNCO—	H	480[g,i]	
XIII	...	...	—NHCO—	—COOH	480[e]	
XIV	...	...	$—NHSO_2—$	$CH_3—$	2400[g,i]	
XV	...	...	$—NHSO_2—$	—COOH	2800[e]	
XVI	...	...	O=CCH₃ —N—	COOH	53[e]	

[a] Dihydrofolic reductase was a 45–90% saturated ammonium sulfate fraction from pigeon liver which was prepared and assayed with 6 μM dihydrofolate and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM Versene as previously described (12). The technical assistance of Mrs. Shirley Humphrey, Miss Karen Smith, and Mrs. Gail Salomon is acknowledged. [b] Data of Bertino *et al.* (6). They used the dihydrofolic reductase from Ehrlich ascites carcinoma cells with dihydrofolate as substrate at pH 7.5; dihydrofolate had apparent K_m 1.3×10^{-6}. [c] Data from *Reference 15*. [d] Data from *Reference 4*. [e] For assay the inhibitor was dissolved in 0.05 M Tris buffer containing 10 mM mercaptoethanol and 1 mM Versene, then the pH was readjusted to 7.4 with 0.1 N KOH. [f] Data from *Reference 7*. [g] The inhibitor was dissolved in N,N-dimethylformamide and the assay was run in the presence of 10% N,N-dimethylformamide. [h] The same results were obtained with or without 10% N,N-dimethylformamide in the assay. [i] Data from *Reference 8*.

That 10-formyl folic acid (IV) is a twentyfold better inhibitor of dihydrofolic reductase than folic acid (I) (6) has considerable bearing on the mode of binding of folic acid to dihydrofolic reductase. In the $—C_6H_4—\underset{|}{N}—CH_2—$ moiety of folic acid (I) the bond angles about the nitrogen are pyramidal, whereas the same bonds are nearly planar in the 10-formyl folic acid (IV) due to the amide resonance. Therefore, it is probable that either folic acid or the enzyme or both undergo an energetically unfavorable conformational change to accommodate the nonplanarity of the N_{10} bonds in folic acid (1); this energy formation of the complex between the enzyme and folic acid (I), being reflected in less net binding energy. Then the planar N_{10} of 10-formyl folic acid is presumably in a more favorable ground-state conformation for complexing with the enzyme, being reflected in better net energy of binding than in the case of folic acid (1).

It was previously noted that XVII was a relatively good inhibitor of dihydrofolic reductase showing 50% inhibition at 0.76 mM (1), about the same as the 5-anilinopropyl pyrimidine (VIII) (1). The aminobenzyl group and part of the benzoate group of XVII were believed to be complexed to the hydrophobic region, but the carboxyl group was probably not in contact with the hydrophobic region of the enzyme surface (1). It was noted that N-acetylation of XVII on the p-amino group gave a compound (XVIII) that failed to inhibit dihydrofolic reductase at 12 times the concentration required for XVII to show 50% inhibition. The planarity about the "N_{10}" (*para*)nitrogen of XVIII is apparently unfavorable for complexing to the hydrophobic region



XVII, R = H
XVIII, R = CH_3CO

of enzyme particularly when a terminal carboxylate is present.

Since acylation of folic acid (I) gives an inhibitor (IV) that is 20 times more effective than I, but acylation of XVII gives an inhibitor (XVIII) that is less than one-fortieth as effective as XVII, it is quite reasonable that XVI has its side chain complexed at the *p*-aminobenzoyl locus; this is in contrast to VII, which probably has its side chain complexed to the hydrophobic region. Again this deduction has the tenuous assumption that VII and XVII have the same conformation in the complex.

A problem that still remains is how a carboxyl group can increase the phenyl binding to the hydrophobic region since the carboxyl is ionized at the pH of the assay to the highly polar carboxylate anion; it is therefore obvious that the carboxylate cannot be in direct contact with the hydrophobic region. That the carboxyl of a compound such as X does not exert an inductive effect on binding of the phenyl is clear from the relative binding of X and its ester, XI. The ester (XI) is only one-fifth as effective as the carboxylate (X), but the ester is still 2–3 times as effective as the parent phenyl compound, IX. It was previously noted that an *n*-octyl group gave no more hydrophobic bonding than an *n*-butyl group, but placement of a phenyl on the terminus of the *n*-butyl group did give an increment in binding (2); these results were interpreted to indicate that a flat interaction between the phenyl and the enzyme was required which an aliphatic group could not duplicate. If the hydrophobic region ends at the end of the phenylbutyl group of IX, then an additional carboxylate may form a hydrogen bond with the enzyme in this region; the ester group of XI could then form a somewhat weaker hydrogen bond to account for the relative order of activity of X > XI > IX. Such a hypothesis might be verified by replacing the carboxyl group of X by a bromomethyl to determine if an active-site-directed irreversible inhibitor (3) would be obtained that would presumably operate by alkylation of this carboxylate binding point to the enzyme.

To put these working hypotheses presented here on a firmer basis would require considerably more investigation of related compounds. However, these working hypotheses in this and in the accompanying papers appeared attractive enough to warrant pursuit of the design of active-site-directed irreversible inhibitors (3) that can bind to both the hydrophobic and the *p*-aminobenzoyl loci of the enzyme. Such studies are being pursued as direct evidence for these 2 binding loci. Initial results with several potential active-site-directed irreversible inhibitors built on the hypothesis have been successful; when the results are on completely firm ground, they will be presented in future papers.

Although it cannot be unequivocally stated at this time that a molecule such as 2-amino-5-(*p*-carboxyphenylbutyl)-6-methyl-4-pyrimidinol (X) has its side chain complexed to the hydrophobic region of the enzyme, the unequivocal result remains that X is the most effective 2-amino-4-pyrimidinol type of dihydrofolic reductase inhibitor yet observed.

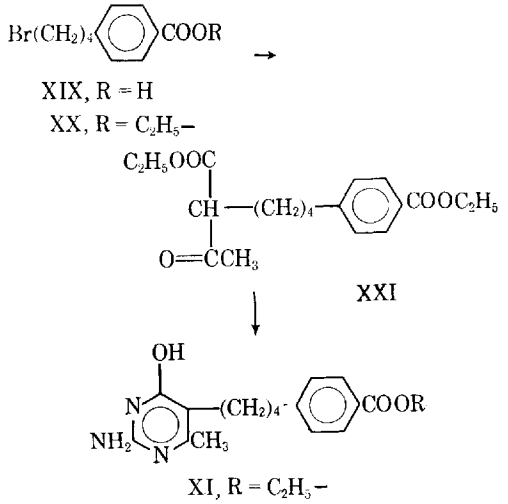
EXPERIMENTAL

Synthesis.—Ethyl *p*-(4-bromobutyl)benzoate (XIX) was prepared by esterification of the corresponding acid (XX) (1) in 92% yield. Alkylation of ethyl acetoacetate with XX was performed with sodium hydride in *tert*-butyl alcohol to avoid alcoholysis of the alkylated keto ester (XXI) (9). The crude keto ester (XXI) was condensed with guanidine carbonate in *tert*-butyl alcohol (9) to give a 21% over-all yield of the crystalline pyrimidine ester (XI). Saponification of XI with aqueous 1 *N* sodium hydroxide proceeded in 97% yield to the desired *p*-carboxyphenylbutyl pyrimidine (X). (Scheme I.)

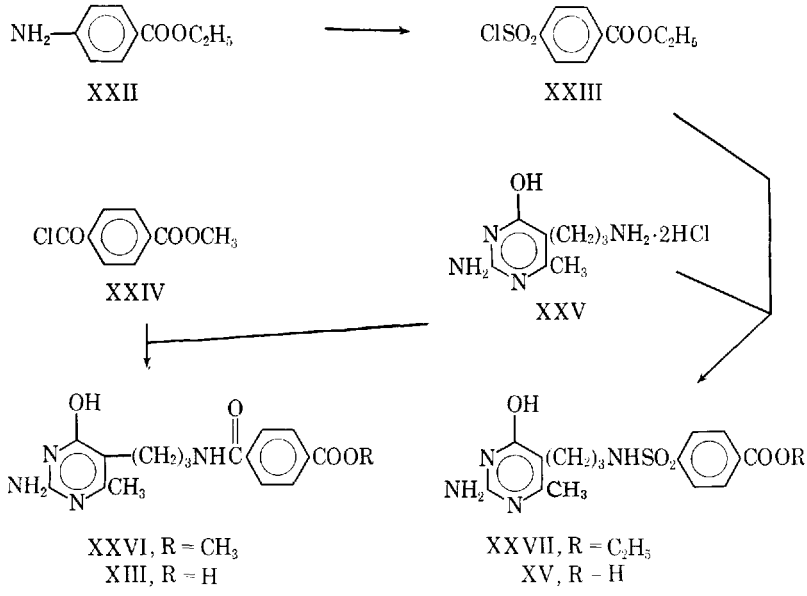
p-Carbomethoxybenzoyl chloride (XXIV) was prepared from dimethyl terephthalate by modification of the procedure of Williams *et al.* (10). Acylation of the pyrimidyl propylamine (XXV) (8) as its free base with XXIV in aqueous acetone proceeded smoothly to the pyrimidyl ester (XXVI) in 79% yield. Saponification of XXVI with 1 *N* aqueous sodium hydroxide at room temperature afforded XIII in 80% yield. (Scheme II.)

p-Carbethoxybenzenesulfonyl chloride (XXIII) was prepared in 81% yield by the Sandmeyer reaction (11); that is, the treatment of the diazonium salt derived from ethyl *p*-aminobenzoate (XXII) with sulfur dioxide catalyzed by cuprous chloride. Reaction of the sulfonyl chloride (XXIII) with the free base of XXV in aqueous acetone also proceeded smoothly in 81% yield. Saponification with 1 *N* aqueous sodium hydroxide gave the requisite carboxy pyrimidine (XV) in 80% yield. (Scheme II.)

Acetylation of the anilino NH-group of VII (14) with acetic anhydride in pyridine was sufficiently slowed by the electron-withdrawing *p*-carboxyl group so that acetylation of the 2-amino group (12) became competitive and selective acetylation of the anilino group was not feasible. In previous successful selective acylations of this type, an electron-withdrawing *p*-group was not present (7). Therefore, both amino groups were allowed to acetylate (13) giving XXVIII in 86% yield. Since the acetyl on the 2-amino group is considerably more labile than the acetyl group on the anilino moiety,



Scheme I



Scheme II

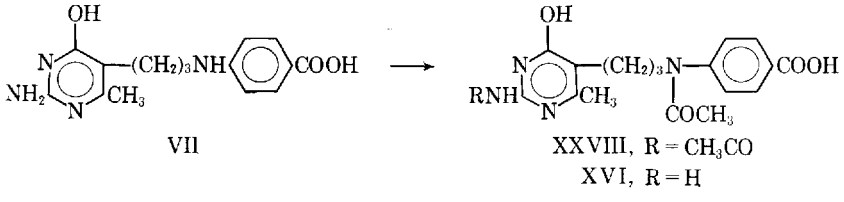
selective removal was achieved with *n*-butylamine in boiling methanol (13). The reaction was readily followed by the difference in ultraviolet spectra of XXVIII and XVI in 0.1 *N* acid, particularly at 247 mμ. After a 3-hr. treatment, XVI could be isolated analytically pure in 74% yield from XXVIII. (Scheme III.)

Methods.—Melting points were taken on a Mel-Temp block in capillary tubes and those below 230° are corrected. Infrared spectra were determined in KBr pellet, unless otherwise indicated, with a Perkin-Elmer 137B spectrophotometer. Ultraviolet spectra were determined in water, unless otherwise indicated, with a Perkin-Elmer 202 spectrophotometer.

2 - Amino - 5 - (p - carbethoxyphenylbutyl) - 6-methyl-4-pyrimidinol (XI).—A solution of 1.00 Gm. (3.89 mmoles) of XIX (1) in 10 ml. of ethanol and 1 ml. of ethanesulfonic acid was refluxed for 24 hr., then spin-evaporated *in vacuo*. The residue was dissolved in 20 ml. of chloroform, and the solution was washed with 3 *N* aqueous ammonia (2 × 10 ml.), then water (2 × 10 ml.). Dried with magnesium sulfate, the chloroform solution was spin-evaporated *in vacuo* leaving 1.02 Gm. (92%) of XX as an oil with $\nu_{max.}$(film) 1720 (ester C=O), 1270, 1100 cm.$^{-1}$ (ester C—O—C).

To a solution of 800 mg. (6.1 mmoles) of ethyl acetoacetate in 15 ml. of *tert*-butyl alcohol was added portionwise 273 mg. (6.1 mmoles) of a 53.5% dispersion of sodium hydride in mineral oil. When solution was complete, 1.51 Gm. (5.3 mmoles) of XX was added, then the solution was refluxed gently with magnetic stirring for 20 hr. The mixture, acidified with glacial acetic acid, was spin-evaporated *in vacuo*. The residue was partitioned between 20 ml. of chloroform and 10 ml. of water. The separated aqueous layer was extracted with 5 ml. of chloroform. Dried with magnesium sulfate, the combined extracts were spin-evaporated *in vacuo*, finally at less than 1 mm. (bath 90°) to remove the excess ethyl acetoacetate; yield, 872 mg. of crude XXI with $\lambda_{max.}$ (1 *N* methanolic sodium methoxide) 244, 284 mμ.

A mixture of 870 mg. (2.6 mmoles) of crude XXI, 234 mg. (1.3 mmoles) of guanidine carbonate, and 10 ml. of *tert*-butyl alcohol (9) was refluxed gently with magnetic stirring for 60 hr. The solution was clarified by filtration, neutralized with glacial acetic acid, then spin-evaporated *in vacuo*. The residue was partitioned between 25 ml. of chloroform and 10 ml. of water. The product which separated was collected on a filter and washed with water; yield, 341 mg., m.p. 163–165°. An additional 23 mg. (total 21% based on XX), m.p. 156–159°, was obtained by concentration of the chloroform layer.



Scheme III

Recrystallization of 290 mg. from aqueous ethanol gave 227 mg. (16%), m.p. 148–150°. A second recrystallization afforded 201 mg. (14%) of white crystals, m.p. 150–152°. $\nu_{max.}$ 3400, 3200 (NH); 1715 (ester C=O); 1650, 1630, 1600, 1530, 1510 (C=O, C=N, C=C, NH); 1280, 1110 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (ethanol) 290 mμ.

Anal.—Calcd. for $C_{15}H_{23}N_3O_3$: C, 65.6; H, 7.04; N, 12.8. Found: C, 65.4; H, 6.92; N, 12.9.

2 - Amino - 5 - (p - carboxyphenylbutyl) - 6-methyl-4-pyrimidinol (X).—A solution of 164 mg. (0.5 mmole) of XI in 3 ml. of 1 *N* aqueous sodium hydroxide was heated at 100° for 30 min. The cooled solution was clarified by filtration, then adjusted to pH 4–5 with acetic acid. The product was collected on a filter and washed with water; yield, 146 mg. (97%), m.p. 295–297°. Recrystallization from aqueous 2-methoxyethanol afforded 108 mg. (72%) of white crystals, m.p. 311–312°, unchanged on further recrystallization. $\nu_{max.}$ 3350, 3150 (NH); 1690, 1670, 1600, 1545–1500 (C=O, NH, C=N, C=C); no ester band near 1280 cm.$^{-1}$; $\lambda_{max.}$ (pH 1) 243, 274 mμ (inflection); (pH 13) 235, 280 mμ.

Anal.—Calcd. for $C_{16}H_{19}N_3O_3$: C, 63.8; H, 6.35; N, 13.9. Found: C, 63.6; H, 6.55; N, 13.7.

p-Carbomethoxybenzoyl Chloride (XXIV).—Dimethyl terephthalate was partially hydrolyzed as described by Williams *et al.* (10); they record a crude yield of 66%, but no melting point is given until after purification. The crude product contained considerable terephthalic acid. Solution in acetone at ambient temperature was more satisfactory than water (10) for removing the insoluble terephthalic acid.

A suspension of 270 mg. (1.5 mmoles) of *p*-carbomethoxybenzoic acid in 7 ml. of chloroform, 0.29 ml. (6 mmoles) of thionyl chloride, and a drop of pyridine was refluxed for 3 hr., solution being complete at that time. Spin-evaporation *in vacuo* gave the acid chloride as a crystalline solid with $\nu_{max.}$ (Nujol) 1780 cm.$^{-1}$ (acid chloride C=O) that was used immediately in the next step.

2 - Amino - 5 - (p - carbomethoxybenzamidopropyl)-6-methyl-4-pyrimidinol (XXVI).—To a magnetically stirred solution of 255 mg. (1 mmole) of XXV (8) and 0.67 ml. of 3 *N* aqueous sodium hydroxide and 0.5 ml. of water containing 159 mg. (1.5 mmoles) of sodium carbonate was added 1 ml. of acetone; then a solution of the crude XXIV from 270 mg. of acid in 2.5 ml. of acetone was added. After being stirred for 4 hr., during which time the product separated, the mixture was diluted with several volumes of water. The product was collected on a filter and washed with water; yield, 272 mg. (79%). Recrystallization from 2-methoxyethanol gave 210 mg. (61%) of white crystals, m.p. 240–242° dec. $\nu_{max.}$ 3300, 3100 (NH); 1720 (ester C=O); 1690, 1630 (sh), 1610, 1525 (C=O, NH, C=N, C=C), 1280 1100 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (pH 13) 276 mμ (sh).

Anal.—Calcd. for $C_{17}H_{20}N_4O_4 \cdot H_2O$: C, 56.3; H, 6.12; N, 15.5. Found: C, 56.0; H, 6.10; N, 15.9.

2 - Amino - 5 - (p - carboxybenzamidopropyl) - 6-methyl-4-pyrimidinol (XIII).—A suspension of 638 mg. (1.85 mmoles) of crude XXVI in 10 ml. of 1 *N* aqueous sodium hydroxide was magnetically stirred for 20 hr. at ambient temperature, during which time solution took place. Acidification to about pH 5 with 4 *N* aqueous hydrochloric acid precipitated the product which was collected on a filter and washed with water. The crude product was dissolved in 1 *N* aqueous sodium bicarbonate; the solution was clarified by filtration, then acidified to about pH 5. The product was collected by centrifugation and washed well with water; yield, 486 mg. (80%), of white solid, m.p. 290–295° dec. $\nu_{max.}$ 3450–3300 (NH); 1700 (carboxyl C=O); 1660, 1620, 1550 (C=O, C=N, C=C, N—H); no 1280 or 1100 cm.$^{-1}$ ester C—O—C bands; $\lambda_{max.}$ (pH 13) 276 mμ. The compound moved on paper as a single spot (R_f 0.7) in 5% aqueous K_2HPO_4.

Anal.—Calcd. for $C_{16}H_{18}N_4O_4 \cdot H_2O$: C, 55.2; H, 5.78; N, 16.1. Found: C, 54.8; H, 5.79; N, 16.3.

2 - Amino - 5 - (p - carbethoxybenzenesulfonamidopropyl) - 6 - methyl - 4 - pyrimidinol (XXVII).—Treatment of 255 mg. (1 mmole) of XXV (8) with 372 mg. of XXIII (prepared in 81% yield as described for the methyl ester) (11) as described for preparation of XXVI, gave 369 mg. (94%) of crude product, m.p. 220–223°. Recrystallization from 85% ethanol afforded 319 mg. (81%) of white crystals, m.p. 223–224°. $\nu_{max.}$ 3300, 3100 (NH); 1710 (ester C=O); 1645, 1600 (sh), 1540 (NH, C=O, C=C, C=N); 1385, 1160 (SO_2); 1270, 1100 cm.$^{-1}$ (ester C—O—C); $\lambda_{max.}$ (pH 13) 279 mμ.

Anal.—Calcd. for $C_{17}H_{22}N_4O_5S$: C, 51.8; H, 5.62; N, 14.2. Found: C, 51.5; H, 5.68; N, 14.1.

2 - Amino - 5 - (p - carboxybenzenesulfonamidopropyl) - 6 - methyl - 4 - pyrimidinol (XV).—A suspension of 394 mg. (1 mmole) of XXVII in 10 ml. of 1 *N* aqueous sodium hydroxide was magnetically stirred at room temperature for 2 days, during which time solution was complete. The product was isolated and reprecipitated from sodium bicarbonate solution as described for XIII; yield, 293 mg. (80%) of white solid, m.p. 310–315° dec.; $\nu_{max.}$ 3400, 3250 (NH); 1680, 1650 (C=N, C=C, C=O, NH); 1385, 1150 (SO_2); no 1280 or 1100 cm.$^{-1}$ ester C—O—C bands; $\lambda_{max.}$ (pH 13) 278 mμ.

Anal.—Calcd. for $C_{15}H_{18}N_4O_5S$: C, 49.2; H, 4.95; N, 15.3. Found: C, 49.1; H, 5.03; N, 15.4.

2 - Acetamido - 5 - (N - acetyl - p - carboxyanilinopropyl) - 6 - methyl - 4 - pyrimidinol (XXVIII).—A solution of 250 mg. (0.83 mmole) of VII (14) in 5 ml. of reagent pyridine and 5 ml. of acetic anhydride was heated in a bath at 95° for 2 hr., then spin-evaporated *in vacuo*. Crystallization from aqueous ethanol with the aid of decolorizing carbon gave 275 mg. (86%) of white crystals, m.p. 225–226°. $\lambda_{max.}$ (pH 1) 247, 265 mμ (inflect.) (*A* 260/280, 1.89); (pH 13) 245, 273 mμ (inflect.) (*A* 260/280, 1.45).

Anal.—Calcd. for $C_{19}H_{22}N_4O_5$: C, 59.1; H, 5.74; N, 14.5. Found: C, 58.8; H, 5.61; N, 14.4.

2 - Amino - 5 - (N - acetyl - p - carboxyanilinopropyl)-6-methyl-4-pyrimidinol (XVI).—A solution of 230 mg. (0.6 mmole) of XXVIII and 0.1 ml. of *n*-butylamine in 8 ml. of methanol was refluxed for 3 hr. when an aliquot no longer showed any change in the absorbance at 247 mμ in 0.1 *N* hydrochloric acid. The solution was spin-evaporated *in vacuo*. The residue was dissolved in 4 ml. of hot methanol, 8.0 ml. of 0.2 *N* aqueous acetic acid was added, then the solution was chilled. The product was collected on a filter; yield, 148 mg. (74%), m.p.

239–240°. $\lambda_{max.}$ (pH 1) 234, 263 mμ (*A* 260/280, 1.80); (pH 13) 235, 278 mμ (*A* 260/280, 1.04).

Anal.—Calcd. for $C_{17}H_{20}N_4O_4$: C, 59.3; H, 5.85; N, 16.3. Found: C, 59.0; H, 5.98; N, 16.0.

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Analogs of Tetrahydrofolic Acid XXXIV

Hydrophobic Bonding to Dihydrofolic Reductase VI. Mode of Phenyl Binding of Some 6-Arylpyrimidines

By B. R. BAKER* and HOWARD S. SHAPIRO

New data are presented which strongly support the concept that the increment in better binding observed by substituting a phenyl group on the 6-position of 4-pyrimidinol is due to hydrophobic bonding of the phenyl group. Furthermore, 11 6-phenylpyrimidines with various substituents at the 2,4, and 5-positions were compared with 18 5-aryl and 5-arylalkylpyrimidines as inhibitors of dihydrofolic reductase. The results cannot be explained by a single conformation of the pyrimidine being complexed to the enzyme; therefore, a number of rotational conformers for the pyrimidine ring are proposed where the strong hydrophobic bonding by the phenyl or phenylalkyl substituent is the determining factor for the particular preferred conformation of a given inhibitor. Such a hypothesis has previously been invoked to explain the inhibitor and substrate binding to chymotrypsin by Niemann *et al.*

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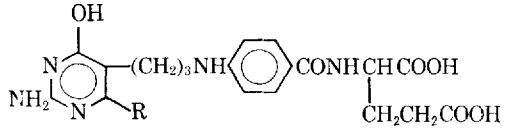

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with folic acid as substrate at pH 6.1, I was found to be a twentyfold better inhibitor of the reductase than II (1, 4). Three possible explanations for this increased binding were proposed (1)—namely, (*a*) a charge-transfer complex, (*b*) the phenyl influences the binding of the pyrimidine ring, and (*c*) hydrophobic bonding. Since inductive effects by substitutents on the phenyl ring could not be completely correlated (5), explanations (*a*) and (*b*) can now be considered unlikely. The emergence of strong hydrophobic bonding by 5-alkylpyrimidines (6) and the further experimental evidence (7, 8) for hydrophobic bonding by the aryl group of 1-aryl-1,2-dihydro-*s*-triazines, 2-aryl-*s*-triazines, and 5-arylpyrimidines suggested that the mode of phenyl binding of 6-phenylpyrimidines be further investigated from the standpoint of hydrophobic bonding.

Rather than proceeding chronologically on the development of the possible hydrophobic bonding of the phenyl group of 6-phenylpyrimidines—as is customarily done with papers from this laboratory—it is somewhat easier to follow the evidence if the assumptions on binding are presented in logical order rather than by order arrived at from the tortuous trail of chronology.

DISCUSSION

The following recent developments on binding of inhibitors to dihydrofolic reductase are pertinent to the arguments to follow.

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239–240°. $\lambda_{max.}$ (pH 1) 234, 263 mμ (*A* 260/280, 1.80); (pH 13) 235, 278 mμ (*A* 260/280, 1.04).

Anal.—Calcd. for $C_{17}H_{20}N_4O_4$: C, 59.3; H, 5.85; N, 16.3. Found: C, 59.0; H, 5.98; N, 16.0.

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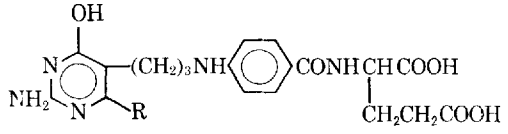

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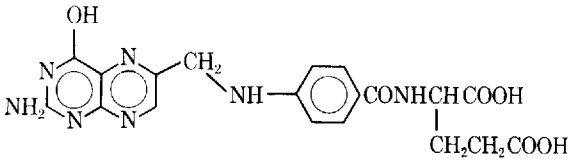


III

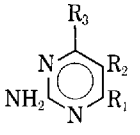
(*a*) The pH profiles for some strongly basic and some weakly basic pyrimidine-type inhibitors were determined (9, 10). These results gave experimental evidence to support the hypothesis that the pyrimidines had only 2 binding points to the enzyme and that a variety of conformations of the pyrimidines were possible for complexing to the enzyme; the points of binding of the pyrimidine proposed were: first, a general bonding to the electron-rich π-cloud of the pyrimidine ring by a weakly acidic group on the enzyme, and second, 1 additional hydrogen bond.

(*b*) Experimental evidence has been presented (11, 12) that the hydrophobic bonding region most probably is not between the binding regions for the pyrimidyl and *p*-aminobenzoyl moieties of inhibitors such as II or folic acid.

Since folic acid (III) and aminopterin are good inhibitors of dihydrofolic reductase (9, 13), both inhibitors and the substrate, dihydrofolate, must have a definite conformation when complexed to the enzyme. The conformation of the pteridin moiety of folic acid (III) and aminopterin can be assigned as in III and the enzyme can then take a conformation in space that will fit the pteridine as written. The conformation of the various pyrimidines will then be written with relationship to the assigned conformation of the pteridine moiety of III. The assumption has already been made that pyrimidines and pteridines have 2 binding points to the enzyme and that the pyrimidines may have any one of a number of conformations when complexed to the enzyme, depending upon the substituents attached to the pyrimidine (9, 10).

A single conformation for pyrimidine binding cannot explain all the inconsistencies on binding

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASE BY



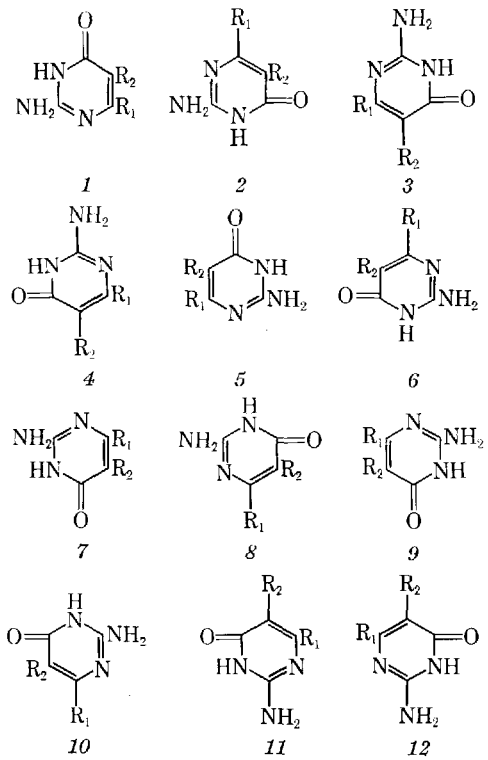
Compd.	R_3	R_2	R_1	μM Concn. for 50% Inhibition[a]	Suggested Conformation for Binding[b]
IV	OH	$C_6H_5NH(CH_2)_3$—	CH_3	800[c,d]	*11*
V	SH	$C_6H_5NH(CH_2)_3$—	CH_3	44	*11*
VI	OH	$C_6H_5NH(CH_2)_3$—	C_6H_5	Insol.[e]	*2*
VII	SH	$C_6H_5NH(CH_2)_3$—	C_6H_5	>1000[d,e,f]	*2*
VIII	H	$C_6H_5NH(CH_2)_3$—	CH_3	480[c]	*11*
IX	H	$C_6H_5NH(CH_2)_3$—	C_6H_5	>3000[d,f]	*2*
X	NH_2	$C_6H_5(CH_2)_4$—	CH_3	0.027[g]	*11, 12*
XI	NH_2	$C_6H_5NH(CH_2)_3$—	CH_3	2.2[c]	*11, 12*
XII	NH_2	$C_6H_5(CH_2)_4$—	C_6H_5	1.1	*2*
XIII	NH_2	$C_6H_5NH(CH_2)_3$—	C_6H_5	0.88	*2*
XIV	NH_2	H	CH_3	1100[h]	*2, 3, 5, 6, 9–12*
XV	NH_2	H	C_6H_5	160[d,i]	*2*
XVI	NH_2	n-C_4H_9	CH_3	2.0[d,h]	*11, 12*
XVII	NH_2	n-C_4H_9	C_6H_5	29[d]	*2*
XVIII	NH_2	H	p-$C_6H_5C_6H_4$	29[d]	*2*
XIX	NH_2	$C_6H_5(CH_2)_4$—	n-C_3H_7—	0.021	*12*
XX	OH	$C_6H_5(CH_2)_4$—	n-C_3H_7—	900[d]	*11*
XXI	OH	$C_6H_5(CH_2)_4$—	CH_3	30[d,j,k]	*11*
XXII	SH	$C_6H_5(CH_2)_4$—	C_6H_5	320[d]	*2*
XXIII	H	$C_6H_5(CH_2)_3$—	C_6H_5	3800[d,l]	*2*
XXIV	NH_2	$C_6H_5NH(CH_2)_3$—	$C_6H_5CH_2$—	3.8	*12*
XXV	NH_2	$C_6H_5(CH_2)_4$—	$C_6H_5CH_2$—	0.34	*12*
XXVI	OH	$C_6H_5(CH_2)_4$—	C_6H_5—	Insol.[e]	*2*

[a] Dihydrofolic reductase was a 45–95% saturated ammonium sulfate fraction prepared from pigeon liver acetone powder and assayed with 6 μM dihydrofolate, and 12 μM TPNH in 0.05 M Tris buffer (pH 7.4) containing 10 mM mercaptoethanol and 1 mM Versene as previously described (14). The 50% inhibition points were determined by plotting V_0/V_I against I, where V_0 = velocity without inhibitor, V_I = velocity with inhibitor, and I = inhibitor concentration; the 50% inhibition point occurs at the intercept of $V_0/V_I = 2$ (35, 36). The technical assistance of Miss Maureen Baker, Mrs. Shirley Humphrey, Mrs. Gail Salomon, and Miss Karen Smith is acknowledged. [b] See Table II. [c] Data from *Reference 14*. [d] Cell contained 10% *N,N*-dimethylformamide. [e] Too insoluble to determine 50% inhibition point with this assay. However, with folic acid as a substrate at pH 6.0, VI had $Ki = 1.8 \times 10^{-6}$, XXVI had $Ki = 0.58 \times 10^{-6}$, VII had $Ki = 9.8 \times 10^{-6}$, IV had $Ki = 63 \times 10^{-6}$, and V had $Ki = 4.5 \times 10^{-6}$ (5). [f] Since 20% inhibition is readily detectable, the concentration for 50% inhibition is at least 4 times greater than the concentration measured. [g] Data from *Reference 15*. [h] Data from *Reference 3*. [i] Data from *Reference 8*. [j] Same result obtained with or without *N,N*-dimethylformamide present. [k] Data from *Reference 37*. [l] Estimated from $V_0/V_I = 1.30$, and the error is larger than when $V_0/V_I = 2$.

previously observed, such as (*a*) 2-amino-5-(anilinopropyl)-6-methyl-4-pyrimidinethiol (V) is a better inhibitor than the 4-pyrimidinol (IV) (14), but in the 6-phenyl series (VI and VII) the reverse is true (5). (*b*) 2-Amino-5-(anilinopropyl)-6-methyl-4-pyrimidinol (IV) still binds as well to the enzyme when the 4-oxo group is removed (VIII) (14), but in the 6-phenyl series, removal of the oxo group of VI gives an inhibitor (IX) that has a large loss in binding compared to IV (Table I). (*c*) 2,4-Diamino-6-methyl-5-(phenylbutyl)pyrimidine (X) is a considerably better inhibitor than the corresponding 5-anilinopropyl pyrimidine (XI) (15) (Table I), but in the 6-phenyl series, the 2 compounds (XII and XIII) give about the same inhibition (5). (*d*) In contrast to (*b*), 2,4-diamino-6-phenylpyrimidine (XV) is a better inhibitor than 2,4-diamino-6-methylpyrimidine (XIV) (8), but when an *n*-butyl or a phenylbutyl side chain is introduced, the 6-methyl series (XVI and X) is better than the 6-phenyl series (XVII and XII) (Table I). There are numerous other examples. By the use of several conformations for pyrimidine binding, but using only a 2-point attachment of the pyrimidine ring, all of these inconsistencies can be suitably rationalized.

If no assumptions on binding are made, then there are 12 major possible conformations for pyrimidine binding (Table II) including the *1* conformation given for folic acid and aminopterin.

TABLE II.—POSSIBLE BINDING CONFORMATIONS FOR 2-AMINO-5,6-DISUBSTITUTED-4-PYRIMIDINOLS TO DIHYDROFOLIC REDUCTASE



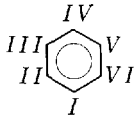
1 *2* *3*

4 *5* *6*

7 *8* *9*

10 *11* *12*

If one considers that 60°-twists between the 12 conformations could be further split into fraction of degrees of rotation, there are an infinite number of conformations; for example, the difference between *conformation 1* and *conformation 11* is a 60° twist. It might be argued that other conformers with smaller rotations between *1* and *11* should be considered, but as a first approximation, these 60° *rotational conformers* listed in Table II can be used to rationalize most of the inhibitor data. The designation of pyrimidine numbering cannot be used to signify the position of a group with respect to the given conformation of folic acid, *1*, with other conformers unless the particular conformer is also named; therefore the binding areas in Table II are numbered, based on conformer *1*, as follows:



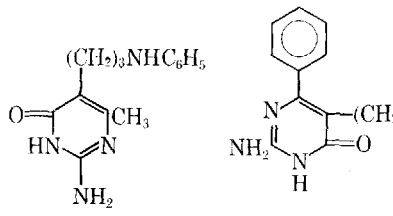
As pointed out previously, the pyrimidine probably has 2 binding points: (*a*) between a weakly acidic group on the enzyme to the electron-rich π-cloud of the pyrimidine ring and (*b*) a hydrogen bond to the enzyme where the pyrimidine group is an electron donor to the enzyme (9). The first bond (*a*) would be fairly independent of conformation since it is somewhat centralized, but the second bond must be in one of the areas *I* to *VI*.

Assume that the hydrogen bond (*b*) is at area *VI*. It should be noted among the 12 conformers that an NH_2, ring N, or C=O can be an electron donor for a hydrogen bond, but the acidic ring NH cannot and a C=S can donate for a hydrogen bond only poorly. On this basis the following conformers are possible for the 2-aminopyrimidines depending upon the 4-substituent. (*a*) 2-Amino-4-oxo: *2, 3, 5, 6, 10, 11*. (*b*) 2,4-Diamino: *2, 3, 5, 6, 9, 10, 11, 12*. (*c*) Among the conformations possible for (*a*) and (*b*), the 2-amino-4-H and 2-amino-4-thione can have conformations *5, 6, 9, 10, 11*, if binding is as good as 2-amino-4-oxo, but *2*, or *3*, if binding is poorer than 2-amino-4-oxo.

If *additional binding* occurs at the *p*-aminobenzoyl locus (area *V*) by R_1 or R_2, then the following conformations of those cited still remain possible. (*a*) 2-Amino-4-oxo: *2, 11*. (*b*) 2,4-Diamino: *2, 11*. (*c*) 2-Amino-4-H or 2-amino-4-thione: *11* if binding is as good as 2-amino-4-oxo, but *2*, if binding is poorer than 2-amino-4-oxo.

Assume that additional hydrophobic bonding occurs in area *IV*. Then the following conformations are still possible if either R_1 or R_2 bonds to the hydrophobic region, regardless of whether there is binding at the *p*-aminobenzoyl locus. (*a*) 2-Amino-4-oxo: *2, 6, 11*. (*b*) 2,4-Diamino: *2, 6, 11, 12*. (*c*) Among the conformations for (*a*) and (*b*), the 2-amino-4-H and 2-amino-4-thione can have conformations *6* and *11* if binding is as good as 2-amino-4-oxo, but only *2* if binding is poorer than 4-oxo.

Consider the specific case of 2-amino-5-(anilinopropyl)-6-methylpyrimidine with a 4-substituent. Since R_2 = anilinopropyl is the only group that can hydrophobically bond at region *IV* or complex to the *p*-aminobenzoyl locus (region *V*), and since both the 4-mercapto and 4-H compounds bind better than the 4-oxo compound, the following conforma-



IV–*11* VI–*2*

tions are possible if the anilinopropyl group is complexed to the hydrophobic region. (*a*) 2-Amino-4-oxo: *2* and *11*, but not *6*. (*b*) 2,4-Diamino: *2*, *11*, and *12*, but not *6*. (*c*) 2-Amino-4-H and 2-amino-4-SH: *11* since these compounds bind better than the 2-amino-4-oxo, but not conformation *2*.

The binding of the anilinopropyl group to the *p*-aminobenzoyl locus can only be by conformation *2* since *11* has the R_2 group placed incorrectly. Since the replacement of the 4-oxo group by 4-thione or 4-H gave better inhibitors, conformation *2* is not possible. Therefore, the anilinopropyl group cannot complex with the *p*-aminobenzoyl region *V*, but can only be hydrophobically bonded to the *IV* region. Either conformation *11* or *12* is satisfactory for 2,4-diamino and 2-amino-4-H, but only *11* is satisfactory for the 2-amino-4-oxo and 4-thione derivatives. Again it should be pointed out that *12* is not satisfactory when an acidic NH is present at the *VI* region needed for hydrogen bonding, but *12* is satisfactory with the 4-amino or 4-H since the acidic hydrogen of *12* has been removed. Evidence that *12* is the preferred conformation for the 2,4-diamino compound will be presented later in the fine points on interpretation.

Now consider the specific case of 2-amino-5-(anilinopropyl)-6-phenylpyrimidine with various 4-substituents. This case differs from the 6-methyl case in that either the 5- or 6-side chain of the pyrimidine could be complexed to the hydrophobic region. The following conformations are possible. (*a*) 2-amino-4-oxo: *2*, *6*, *11*. (*b*) 2,4-Diamino: *2*, *6*, *11*, *12*. (*c*) Of the conformations possible in (*a*) and (*b*), the 2-amino-4-H gives poorer binding than 2-amino-4-oxo only in conformation *2*.

Therefore, conformation *2* will explain all the binding of the 2-amino-5-(anilinopropyl)-6-phenylpyrimidines with 4-substituents. Conformation *2* places the anilinopropyl group at the *p*-aminobenzoyl locus and the 6-phenyl group in the hydrophobic region *IV*. There is additional information which supports the suggestion that the 6-phenyl group is hydrophobically bonded and that will be discussed later.

Although all of the data can be explained by the assumptions that there are 2 bonds from the enzyme to a pyrimidine—one which is complexed with the electron-rich π-cloud of the pyrimidine and the other is at the *VI*-region—are there other sets of assumptions which can or cannot explain the observed inhibition data?

First, is it necessary to assume that one of the 2 bonds is to the electron-rich π-cloud of the pyrimidine? Cannot there be 2 bonds to 2 definite groups in regions *I–VI*? In such a consideration, one can eliminate immediately any bonding by the hydrogen of an acidic NH, otherwise 2-amino-4-pyrimidinols would be better inhibitors than 2,4-diaminopyrimidines rather than vice versa. Assume that there are 2 hydrogen bonds to the *II* and *VI* regions; then the following conformations in Table II are possible. (*a*) 2-Amino-4-oxo: *2*, *6*. (*b*) 2,4-Diamino: *2*, *6*, *11*, *12*. (*c*) Of the possible conformations in (*a*) and (*b*), the 2-amino-4-H can have conformation *11* or *12* if it binds as good as 2-amino-4-oxo.

Therefore, the assumption of 2 hydrogen bonds to areas *II* and *VI* is invalid, since the 4-oxo derivative cannot bind in conformation *11* or *12* and the 4-H derivative cannot bind in conformation *2* or *6*. Similarly, 2 hydrogen bonds to areas *II* and *IV*, or areas *IV* and *VI*, or areas *I* and *IV* can be eliminated.

Assume that there are 2 hydrogen bonds to areas *I* and *II*, then the following conformations in Table II are possible. (*a*) 2-Amino-4-oxo: *1*, *12*. (*b*) 2,4-Diamino: *1*, *2*, *6*, *7*, *11*, *12*. (*c*) Of the possible conformations in (*a*) and (*b*), the 2-amino-4-H can have conformations *1* and *12* if it binds as good as 2-amino-4-oxo.

With these 2 particular hydrogen bonds it is not possible to account for 2-amino-5-(3-anilinopropyl)-6-phenylpyrimidine (IX) being a poorer inhibitor than the corresponding 4-oxopyrimidine (VI), since both allowable 4-oxo conformations, *11* and *12*, would not lose a binding group if the 4-oxo group were removed. Therefore, the assumption that pyrimidine binding occurs through 2 hydrogen bonds at the *I* and *II* regions is invalid. Similarly, hydrogen bonds to areas *I* and *VI* can be eliminated. Thus, all 10 combinations of 2 hydrogen bonds to discrete areas are eliminated. Similarly, 3 or 4 hydrogen bonds can be eliminated, the 4 hydrogen bond theory (16) having been eliminated by other means (9, 10).

With the assumption that 1 of the bonds to the pyrimidine is a general one depending on the electron-rich π-cloud (general basicity) of the pyrimidine, how much can the other two assumptions made earlier be varied and explain the data? These other 2 assumptions agreeing with the data were that (*a*) a hydrogen bond to the *VI* region was present, and (*b*) hydrophobic bonding was in the *IV* region.

First, keep (*a*) constant and vary (*b*). If the hydrophobic bonding region were at the *III* area, then the following conformations are possible. (*a*) 2-Amino-4-oxo: *5*, *6*. (*b*) 2,4-Diamino: *5*, *6*, *9*, *12*. (*c*) Of the possible conformations in (*a*) and (*b*), the 2-amino-4-H can have conformations *5*, *6*, *9*, and *12* if binding is as good as 4-oxo, but there is no possible conformation for the 4-H being poorer than 4-oxo.

Therefore, these parameters will not allow for a conformation that will bind 2-amino-5-(anilinopropyl)-6-phenylpyrimidine with a 4-oxo group (VI), but that will not bind to the corresponding 4-H pyrimidine (IX). Thus, the assumption of hydrophobic bonding at the *III* region is invalid. By similar arguments, hydrophobic bonding at regions *I*, *II*, and *V* can be eliminated.

There is 1 other set of assumptions which will explain all the data—namely, (*a*) 1 general bond due to the electron-rich π-cloud of the pyrimidine, (*b*) a hydrogen bond to the *IV* region, and (*c*) hydrophobic bonding at the *VI* region. Conformation *1* will accommodate the 6-phenyl series and conformation *7* will accommodate the 6-methyl series; note that *1* is a "flipped-over" conformation of *2*, and *7* is a "flipped-over" conformation of *11*. To dis-

tinguish this mode of binding from a hydrogen bond to the *VI* area and hydrophobic bonding to the *IV* area is not possible with the compounds in Table I; such a useful differentiation will require, and is worthy of, further study. Whether there are other combinations of these 3 basic assumptions that will explain all the data would probably require computer techniques to make sure that no possibilities have been overlooked.

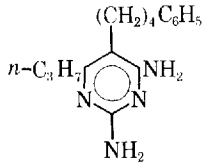
Additional evidence that the 6-phenyl group of 6-phenylpyrimidines is hydrophobically bonded to dihydrofolic reductase can be gleaned from Table I. (*a*) 2,4-Diamino-6-phenylpyrimidine (XV) is a sevenfold better inhibitor than 2,4-diamino-6-methylpyrimidine (XIV) (Table I). (*b*) The hydrophobic region is already complexed by the 6-phenyl of XV, since introduction of a *n*-butyl group on XV gives an inhibitor (XVII) that is only fivefold better. In contrast, the 5-*n*-butyl-6-methylpyrimidine (XVI) is a 550-fold better inhibitor than the corresponding 5-H pyrimidine (XIV). (*c*) Further change of the *n*-butyl group of the 6-phenylpyrimidine (XVII) to anilinopropyl (XIII) or phenylbutyl (XII) gives about the same thirtyfold increment in binding indicating that the aryl 5-side-chain group of XII and XIII is not complexed in the hydrophobic region but more likely is complexed to the *p*-aminobenzoyl locus. In contrast, change of the butyl group of the 6-methylpyrimidine (XVI) to anilinopropyl (XI) gives no increment, but change to phenylbutyl (X) gives an 85-fold increment in binding, indicating that this terminal aryl group in the 6-methyl series is complexed to the hydrophobic region where phenylbutyl would be expected to bind better than anilinopropyl.

These 3 basic assumptions explain the following points.

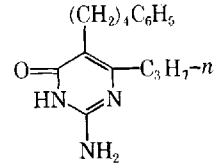
(*a*) In the 6-phenyl series with binding in conformation *2*, replacement of the 4-oxo group (VI) by 4-H (IX) or 4-thione (VII) decreases the binding, whereas in the 6-methyl series with conformation *11*, replacement of the 4-oxo group (IV) by 4-H (VIII) or 4-thione (V) gave better binding.

(*b*) The 4-thione group of V in conformation *11* is adjacent to the hydrophobic region and could lead to better binding than the 4-oxo group of IV, since the former is better tolerated in a hydrophobic region. In the 4-H series (VIII), one might expect VIII to be even less repulsed in the *III* region; however, it is possible that the highly polar 3-N of VIII is water solvated which could then make VIII about the same as IV as an inhibitor.

(*c*) A new point that is now explainable is in the comparison of the 6-methyl series (X, XXI) and 6-propyl series (XIX, XX). Note that with 2,4-diamino substituents, X and XIX are equal in effectiveness, but the 2-amino-4-oxo derivative in the 6-methyl series (XXI) is a thirtyfold better inhibitor than in the 6-propyl series (XX). The conformational binding assignments for 2,4-diamino-6-methylpyrimidines with a 5-side chain were *11* and *12*, whereas the 2-amino-4-oxo-6-methyl pyrimidines could only have conformation *11*. Conformation *11* forces the *n*-propyl group into region *V*, a probable hydrophilic region, whereas conformation *12* allows the propyl group to be in a hydrophobic region *III*. Thus, some repulsion of a propyl group in conformation *11* could occur which would explain the difference between XX and XXI. The opposite type of repulsion has been



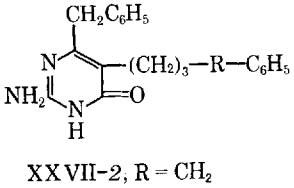
XIX-*12*



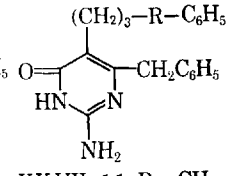
XX-*11*

observed with 5-phenylbutyl-2,4,6-triaminopyrimidine (6, 17); since conformations *11* and *12* are equivalent with the 4,6-diaminopyrimidines, 1 amino group must project into the hydrophobic *III* region with resultant repulsion of the polar amino group. One would expect the order of repulsion in the *III* region to be $NH_2 > =O > =S$.

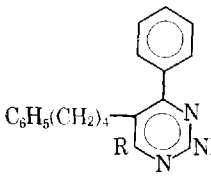
(*d*) The fact that 2,4-diamino-5-phenylbutyl-6-benzyl pyrimidine (XXV) is an elevenfold better inhibitor than the corresponding 5-anilinopropyl pyrimidine (XXIV) indicates that XXIV and XXV have conformation *12*, where the 5-side chain is hydrophobically bonded to area *IV*; conformation *2* is unlikely with the 6-benzyl hydrophobically bonded since one would expect less difference between the anilinopropyl and phenylbutyl side chains as previously noted with the comparison of XII and XIII in the 6-phenyl series. Note, however, that in the 2-amino-4-oxo-6-benzyl series, conformation *12* is not allowable due to the acidic NH in the *VI* region. Therefore, in this series, conformations *2* and *11* must be considered. It was previously reported (5) that in the 6-benzyl series the 5-anilinopropylpyrimidine (XXVIII) was about twice as effective as the corresponding 5-phenylbutylpyrimidine (XXVII). Since there is so little difference in binding between these two 5-side chains, conformation *2* is preferred which places the 5-side chain at the *p*-aminobenzoyl locus where the phenylbutyl and anilinopropyl side chains could be expected to have a similar amount of binding (note the similar comparison of XII and XIII in Table I where conformation *2* is favored). Even though the phenylbutyl side chain could be expected to give much stronger hydrophobic bonding than a benzyl side chain (19), the combination of probable hydrophilic repulsion of the benzyl group in conformation *11* could be sufficient to give the best net binding in conformation *2*. Where the balance between hydrophobic bonding in region *IV* and *p*-aminobenzoyl locus binding in region *V* is close, it is quite difficult to differentiate the preferred conformations. For example, these results could also be explained by XXVIII having conformation *2* and XXVII conformation *11*; if such were the case, then the 6-methyl 5-(phenylbutyl)-4-pyrimidinol (XXI) should bind the same as XXVII-*11*—since there is no hydrophobic bonding by the benzyl group in XXVII-*11*. With the anilinopropyl side chain, conformation XXVIII-*2* could bind better in the 6-benzyl series—with its benzyl hydropho-



XXVII-*2*, R = CH_2
XXVIII-*2*, R = NH

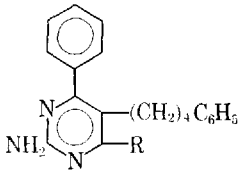


XXVII-*11*, R = CH_2
XXVIII-*11*, R = NH



XII–*6*, R = NH_2

XXIII–*6*, R = H



XII–*2*, R = NH_2

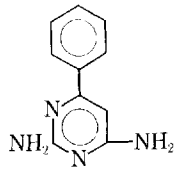
XXIII–*2*, R = H

bically bonded and the anilino bonded at the *p*-aminobenzoyl locus in region *V*—than can the 6-methyl pyrimidine (IV) which can have hydrophobic bonding in conformation *11*, but with no additional binding at the *p*-aminobenzoyl locus.

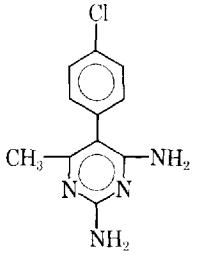
(*e*) It might be possible that 2,4-diamino-5-phenylbutyl-6-phenylpyrimidine (XII) is complexed in the *6*-conformation if the added increment in binding between XII and the *n*-butylpyrimidine (XVII) is due to hydrophobic bonding by the ω-phenyl in the *III–IV* region. If such were the case, then the corresponding 4-H pyrimidine (XXIII) should be as good an inhibitor, complexed in the conformation *6*, as the 4-oxopyrimidine (XXVI). Unfortunately, XXVI was too insoluble to determine the concentration necessary for 50% inhibition of dihydrofolic reductase; therefore, a less direct comparison was made. In the 6-methyl series, removal of the 4-amino group of XI to give VIII led to a 220-fold decrease in binding—due primarily to a loss of basicity (9, 10)—but VIII and IV were similar in binding. In the 6-phenyl series, removal of the 4-amino group of XII to give XXIII led to a 3500-fold decrease in binding. The sixteen-fold difference in these 2 increments is about what could be expected if 1 binding point were missing in XXIII, the remaining 220-fold decrease between XII and XXIII being presumably due to decreased basicity (9, 10). Therefore, this quite tenuous interpretation indicates that XII and XXIII bind in conformation *2*, and not in conformation *6*.

An important difference remains in rationalizing why a 6-phenylpyrimidine such as XV with conformation *2* gives only about a sixfold increment by hydrophobic bonding, but a 5-phenyl pyrimidine such as XXIX or a 1-phenyl-dihydro-*s*-triazine (XXX) in conformation *12* gives about a 1000-fold increment in binding (6). There are some obvious possibilities, such as (*a*) the 6-phenyl pyrimidine (XV) may be a weaker base than XXIX (pKa 7.7) or XXX (pKa 11.2) (21); (*b*) the 3-point attachment of pyrimidine π-cloud, hydrogen bond, and hydrophobic region are juxtapositioned somewhat differently in the case of XV *versus* the case XXIX or XXX, that is, a lateral movement of XV-*2* to the left by 1 atom distance compared to XXIX or XXX would be necessary for the same hydrogen bonding in the *VI* region; (*c*) the π-cloud overlaps between the phenyl and pyrimidine rings may be more favorable for π-cloud and hydrophobic interaction for XXIX with the enzyme than XV; (*d*) if the 4-NH_2 group of XV-*2* is less basic than the 3-N of XXIX-*12* or the 5-N of XXX-*12*, then XV-*2* will bind less effectively, but not so much as the 150-fold less effectiveness noted.

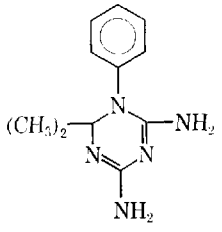
Spectrophotometric determination of the pKa of XV afforded a value of 6.23. Thus, XV is 6.4% protonated at the pH 7.4 of the assay, XXIX is 67% protonated, and XXXII is 99.9%.



XV-*2*



XXIX-*12*

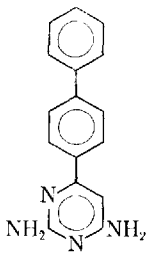


XXX-*12*

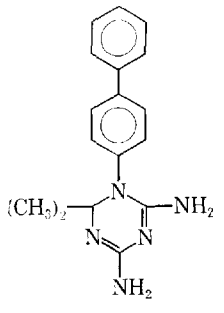
The difference in binding observed between XXIX and the totally unprotonated 6-trifluoromethyl analog of XXIX was only 250-fold (10). Thus, the 800-fold difference in binding between XV and XXIX is only partially accounted for by the difference in their respective basicities.

That the aryl group of XV-*2* was not placed in the hydrophobic region in exactly the same manner as the aryl group of XXX-*12* was clearly shown by comparison of the *p*-biphenyl analog (XVIII-*2*) with the *p*-biphenyl-*s*-triazine (XXXI-*12*). The 6-(*p*-biphenyl)pyrimidine (XVIII-*2*) was a five-fold better inhibitor than the corresponding 6-phenylpyrimidine (XV-*2*), indicating some additional hydrophobic bonding compared to XV-*2*; in contrast, the *p*-biphenyl-*s*-triazine (XXXI-*12*) was 1400-fold less effective than the corresponding phenyl-*s*-triazine (XXX-*12*) (7). It should be noted that the steric interference of the *p*-phenyl group of XXXI-*12* with enzyme binding was greatly reduced in the corresponding *m*-biphenyl-*s*-triazine (XXXII-*12*) which was only elevenfold less effective than XXX-*12* (7).

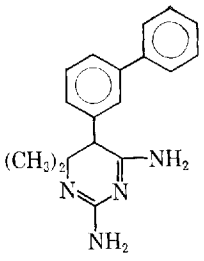
Another important difference exists between 2,4-diamino-6-phenyl-5-(phenylbutyl)pyrimidine (XII) and the corresponding 6-methyl (X) and 6-propyl analogs (XIX). If a 5-phenylbutyl group in conformation *11* or *12* can give so much stronger hydrophobic bonding than 6-phenyl in conformation *2*, why does not the 6-phenyl analog (XII) assume



XVIII-*2*



XXXI-*12*



XXXII-*12*

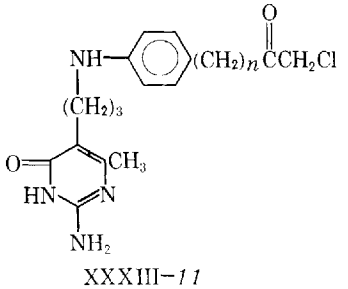
conformation *11* or *12* in order to get maximum hydrophobic bonding from the phenylbutyl group? If such were the case, then X, XII, and XIX would be expected to be equally effective. Since XII is so much less effective, it can only be concluded that XII cannot complex to the enzyme in conformation *12* due to steric interaction with the enzyme when the flat coplanar benzene ring is placed in the *III* region. If XII assumed conformation *11*, this would place the 4-amino group in the *III* region, a position believed to be the cause of a 130-fold repulsion as noted previously with 2,4,6-triamino-5-(phenylbutyl)pyrimidine compared to X (6, 17). Since this 130-fold repulsion is even larger than the 41-fold observed difference between the 6-methyl (X) and 6-phenyl pyrimidine (XII), conformation *2* might be preferred for XII. Although conformation *11* could be a possibility, conformation *11* would predict that XII would be a ten to thirty-fold better inhibitor than the anilinopropyl pyrimidine (XIII) which it is not. It is also possible that the preferred conformation for XII is *11*, but for XIII it is *2*. Ultimately, it should be possible to differentiate the conformational preference for *2* or *11* with a particular 5-side chain by comparison of active-site-directed irreversible inhibitors (18) which vary only in the hydrophobic or hydrophilic nature of the bridge between the 5-position of the pyrimidine and the terminal aryl group.

In summary, the inhibition of dihydrofolic reductase observed with a variety of phenyl and phenylalkyl pyrimidines cannot be rationalized with a single rotational conformer; therefore, a number of rotational conformers are proposed and the strong hydrophobic bonding is the determining factor for the particular preferred conformation of a given inhibitor. Such a concept for enzyme binding is not new. Complexing to chromotrypsin can occur in a number of rotational conformations where an aryl or large alkyl group on the substrate or inhibitor is determinant (22, 23); this bonding by aryl or alkyl groups to chymotrypsin is probably of a hydrophobic nature (24, 25). Similarly, 3-(β-D-ribofuranosyl)adenine and its nucleotide analogs can bind to some enzymes normally requiring adenosine or its corresponding nucleotide derivatives. Since the strong binding by the sugar moiety is apparently determinant, it has been proposed (26, 27) that a "flip" conformation for the adenine of the 3-ribosyl derivatives can account for the binding data.

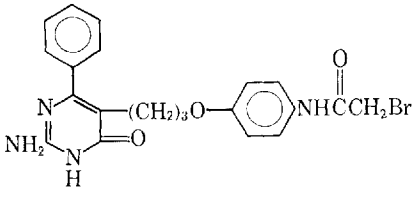
The most important deduction that arises from the derivations presented here is that if only 1 hydrophobic group is present on an inhibitor of dihydrofolic reductase, then this group will be complexed in a particular conformer that allows the hydrophobic bonding to be determinant. Therefore, a potential active-site-directed irreversible inhibitor (18) of dihydrofolic reductase such as XXIIII (3) would have its alkylating function in the hydrophobic area; by definition, the hydrophobic area of the enzyme has no polar groups that could be attacked by XXXIII in its favored conformation *11*. However, if 2 side chains are present, at least 1 of which is hydrophobic, then the hydrophobic group will complex in the hydrophobic region in a conformation that will project the second side chain in a hydrophilic region of the enzyme. Thus, XXXIV in conformation *2* is an active-site-directed irreversible inhibitor (18) of dihydrofolic reductase (28). By similar reasoning, XXXV, which should complex in conformation *11*, was synthesized and was also an active-site-directed irreversible inhibitor (29). These results will be presented in future papers.

EXPERIMENTAL

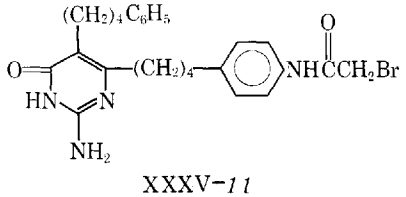
Methods.—The required 2-amino-5,6-disubstituted-4-pyrimidinols (XX, XXIX, and XL) were synthesized by alkylation of the appropriate β-keto ester followed by condensation with guanidine (30). The 2,4-diaminopyrimidines (XVII–XIX) were synthesized from the 4-pyrimidinols *via* the 4-chloro-



XXXIII-*11*



XXXIV-*2*



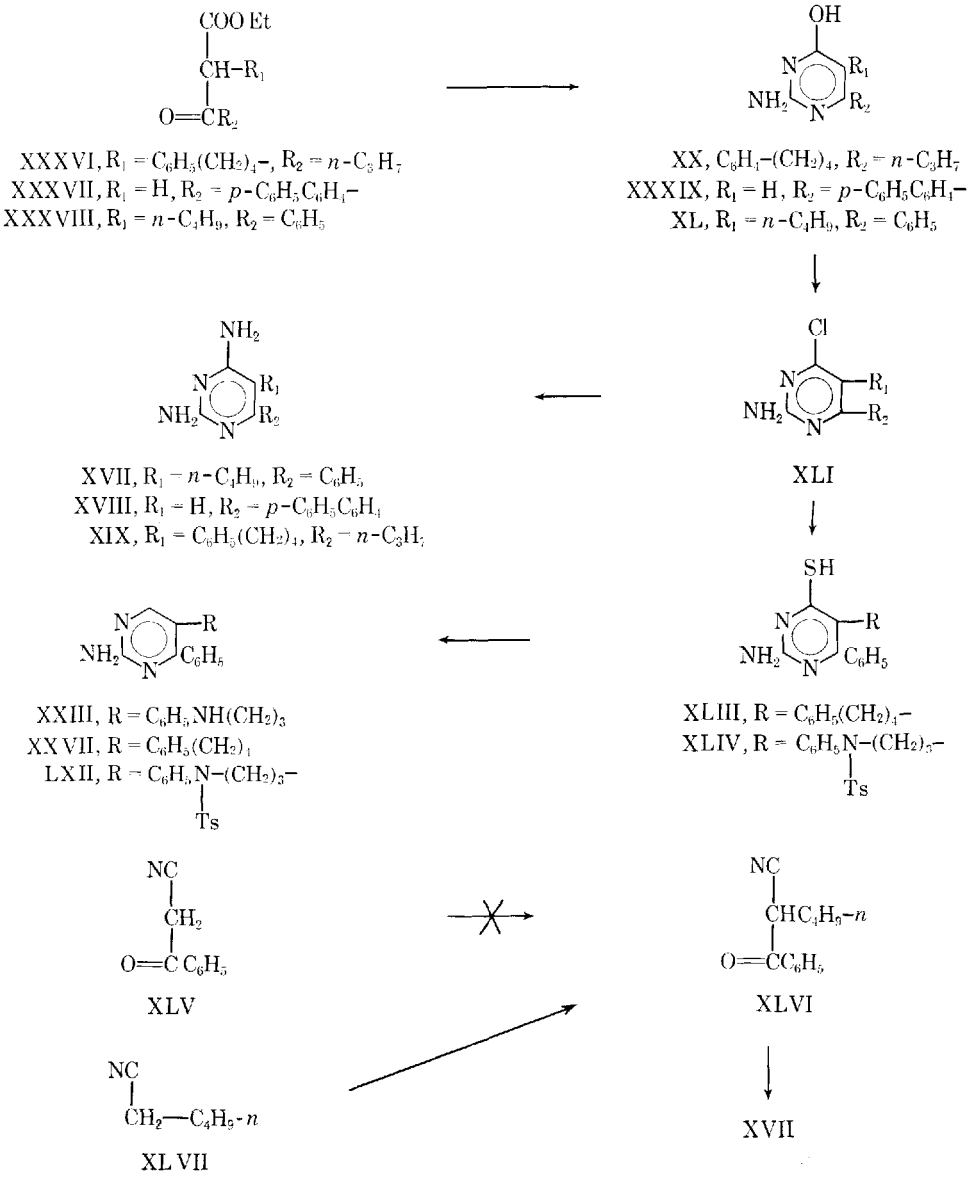
XXXV-*11*

pyrimidines (XLI) by treatment with ammonia (31). Reaction of the appropriate 4-chloro-6-phenylpyrimidine (XLI) with thiourea (5) afforded the 4-mercaptopyrimidines (XLIII and XLIV) which were desulfurized with Raney nickel (31) to the 2-amino-4-H-pyrimidines (XXVII and LXII). Treatment of LXII with hydrogen bromide in acetic acid (30, 32) removed the *N*-tosyl blocking group to give the required XXIII. (Scheme I.)

An alternate route to 5-alkyl-2,4-diamino-6-phenylpyrimidines such as XVII *via* α-butylbenzoylacetonitrile (XLVI) was investigated since it would be of general utility. Attempts to prepare XLVI by alkylation of benzoylacetonitrile (XLV) with sodium hydride and *n*-butyl bromide in such diverse solvents as dimethylsulfoxide or benzene gave only *O*-alkylation. However, XLVI could be prepared by Claisen condensation of hexanonitrile (XLVII) with ethyl benzoate (33). Condensation of XLVI with guanidine carbonate by fusion at 180° gave the 2,4-diaminopyrimidine (XVII) directly in 21% yield. (Scheme I.)

Synthesis.—Melting points were determined in capillary tubes on a Mel-Temp block and those below 230° are corrected. Infrared spectra were determined in KBr disk (unless otherwise indicated) with a Perkin-Elmer model 137B spectrophotometer; ultraviolet spectra were determined with a Perkin-Elmer model 202 spectrophotometer. Thin-layer chromatograms (TLC) were run on silica gel GF_{254} (Brinkmann), and spots were detected under ultraviolet light.

2 - Amino - 5 - phenylbutyl - 6 - n - propyl - 4-pyrimidinol (XX).—To a magnetically stirred solution of 3.1 Gm. (20 mmoles) of ethyl *n*-butyroylacetate in 10 ml. of reagent dimethylsulfoxide pro-



Scheme I

tected from moisture was added portionwise 0.867 Gm. (20 mmoles) of 55.6% dispersion of sodium hydride in mineral oil. When the evolution of hydrogen had ceased, 3.85 Gm. (18 mmoles) of 4-phenylbutyl bromide (34) was added. After being stirred for about 18 hr., the mixture was warmed on a steam bath for 30 min. and then neutralized with acetic acid. The warm mixture was poured into a stirred mixture of 50 ml. of benzene and 30 ml. of water. The separated aqueous phase was extracted once more with benzene. The combined benzene solutions were washed with several portions of ice cold 3% aqueous sodium hydroxide, then water. Dried with magnesium sulfate, the benzene solution was spin-evaporated *in vacuo* leaving 5 Gm. of crude XXXVI as an oil.

The crude XXXVI was dissolved in 25 ml. of absolute ethanol, then refluxed with 1.62 Gm. (9 mmoles) of guanidine carbonate for 19 hr. The cooled reaction mixture was neutralized to near pH 7 with 3 *N* aqueous hydrochloric acid. The product was collected on a filter and washed with ethanol; yield, 1.54 Gm., m.p. 209–215°. By concentration of the filtrate an additional 1.17 Gm. (total 53%) was obtained, m.p. 209–215°. Recrystallization of a portion from aqueous ethanol gave white crystals, m.p. 209–215°, which moved as one spot on TLC in 3:1 benzene–methanol. The compound had $\lambda_{max.}$ 2.98 (NH); 6.05, 6.10, 6.50, 6.70, (NH, C=O, C=C, C=N); 13.5, 14.3 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 269 mμ (ϵ 7800); (pH 7): 275 mμ (ϵ 4400); (pH 13): 282 mμ (ϵ 9800).

Anal.—Calcd. for $C_{17}H_{23}N_3O$: C, 71.6; H, 8.07; N, 14.7. Found: C, 71.6; H, 8.20; N, 14.7.

2 - Amino - 5 - n - butyl - 6 - phenyl - 4 - pyrimidinol (XL).—Alkylation of 5 Gm. of ethyl benzoylacetate with *n*-butyl bromide in *tert*-butyl alcohol followed by condensation with guanidine carbonate in the same solvent, as described for the preparation of a related 6-phenylpyrimidinol (30), gave 2.24 Gm. (39%) of product, m.p. 309–314°. Recrystallization from ethanol–toluene gave white crystals, m.p. 310–315° dec. $\lambda_{max.}$ 2.83 (NH); 6.05, 6.12, 6.35 (NH, C=O, C=N, C=C); 14.3 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 233 (ϵ 14,400), 280 mμ (ϵ 10,900); (pH 7): 235 (ϵ 17,400), 300 mμ (ϵ 8100); (pH 13): 289 mμ (ϵ 9600).

Anal.—Calcd. for $C_{14}H_{17}N_3O$: C, 69.1; H, 6.99; N, 17.3. Found: C, 69.4; H, 7.18; N, 16.9.

2 - Amino - 6 - (4 - biphenylyl) - 4 - pyrimidinol (XXXIX).—To a solution of 5.3 Gm. (23.4 mmoles) of ethyl 4-biphenylylcarboxylate in 7.2 Gm. (82 mmoles) of reagent ethyl acetate was added 3.16 Gm. (58.5 mmoles) of sodium methoxide. The mixture was heated in a bath at 80° under a condenser with stirring and protected from moisture for 20 hr. The cooled reaction mixture was diluted with several volumes of benzene, then neutralized with glacial acetic acid. The sodium acetate was removed by filtration and washed with benzene. The combined filtrate and washings were spin-evaporated *in vacuo;* ethyl acetoacetate was then removed by continued spin-evaporation in a hot water bath in high vacuum leaving 6.23 Gm. of crude XXXVII as an oil.

A solution of 4.82 Gm. (18 mmoles) of crude XXXVII in 40 ml. of absolute ethanol was refluxed with 1.8 Gm. (10 mmoles) of guanidine carbonate with magnetic stirring for 17 hr. The cooled reaction mixture was filtered and the product washed with alcohol, then water; yield, 1.57 Gm., m.p. 370–372° dec. From the filtrate was isolated an additional 0.87 Gm. (total, 52% over-all), m.p. 373–376° dec. Two recrystallizations from aqueous 2-methoxyethanol afforded white crystals, m.p. 378–380° dec. $\lambda_{max.}$ 2.95 (NH); 5.85, 6.02, 6.08 (NH, C=O, C=C, C=N); 12.2, 14.5 μ (phenyl); $\lambda_{max.}$ (pH 1): 308 mμ (ϵ 25,300); (pH 7): 285 mμ (ϵ 32,100); (pH 13): 295 mμ (ϵ 16,700).

Anal.—Calcd. for $C_{16}H_{13}N_3O$: C, 73.0; H, 4.94; N, 15.9. Found: C, 72.7; H, 5.07; N, 15.6.

2,4 - Diamino - 5 - phenylbutyl - 6 - n - propylpyrimidine (XIX).—A mixture of 500 mg. (1.76 mmoles) of XX and 4 ml. of phosphorus oxychloride was heated for 45 min. under a reflux condenser in a bath preheated and maintained at 110°. The cooled mixture was poured into 35 Gm. of ice and 40 ml. of ether with stirring. After 15 min., the layers were separated. The ether layer was washed with 5% aqueous sodium bicarbonate (2 × 30 ml.) and water (2 × 30 ml.), then dried with magnesium sulfate. Spin-evaporation *in vacuo* left XLI (R_1 = C_6H_5—$(CH_2)_4$—, R_2 = *n*-C_3H_7) as a gum which could not be crystallized, but had $\lambda_{max.}$ (pH 1): 315 mμ (*cf. Reference 31*).

The crude gum was dissolved in 40 ml. of methanol saturated with ammonia, then heated in a steel bomb at 150° for 24 hr. The solution was clarified by filtration through a Celite pad, then spin-evaporated *in vacuo* to a small volume. The solution was made strongly alkaline with 10% aqueous sodium hydroxide, then diluted with water to turbidity and cooled at −4°. The crude product was collected on a filter and recrystallized twice from aqueous methanol; yield, 115 mg. (23%), m.p. 121–127°. For analysis, the material was dissolved in warm 10% aqueous acetic acid. The solution was clarified by filtration, then the product was precipitated by addition of excess 10% aqueous sodium hydroxide. Recrystallization from aqueous ethanol gave 70 mg. (14%) of analytically pure product, m.p. 126–128°. $\lambda_{max.}$ 2.80, 3.00 (NH); 6.05, 6.39 (NH, C=C, C=N); 13.03, 14.43 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 280 mμ (ϵ 7400); (pH 7): 289 mμ (ϵ 7300); (pH 13): 301 mμ (ϵ 7900).

Anal.—Calcd. for $C_{17}H_{24}N_3$: C, 71.8; H, 8.45; N, 19.7. Found: C, 71.8; H, 8.60; N, 19.4.

5 - n - Butyl - 2,4 - diamino - 6 - phenylpyrimidine (XVII).—*Preparation A.*—Conversion of 500 mg. (2.06 mmoles) of XL to XVII *via* LXI, as described for the preparation of XIX, gave 143 mg. (29%) of analytically pure product, m.p. 149–152°. $\lambda_{max.}$ 2.80, 2.95 (NH); 6.05, 6.20, 6.40 (NH, C=C, C=N); 13.05, 14.25 μ (C_6H_5).

Anal.—Calcd. for $C_{14}H_{18}N_4$: C, 69.4; H, 7.44; N, 23.1. Found: C, 69.6; H, 7.57; N, 23.3.

Preparation B.—α-Benzoylhexanonitrile (XLVI) was prepared by Claisen condensation of hexanonitrile and ethyl benzoate; a yield of 60% has been recorded (33). A mixture of 1 Gm. (5 mmoles) of XLVI and 450 mg. (2.5 mmoles) of guanidine carbonate was placed in a bath preheated to 120°, then the temperature was raised to 180° over a period of 30 min. After an additional 10 min. at 180°, gas evolution was complete. The cooled residue was extracted with hot ethanol. The filtered solution was spin-evaporated *in vacuo*. The residue was extracted with hot 10% aqueous acetic acid

and separated from some insoluble gum. The cooled extract was poured into an excess of cold 10% aqueous sodium hydroxide. The product was collected and recrystallized from aqueous ethanol with the aid of charcoal; yield, 256 mg. (21%) of white crystals, m.p. 151–152°, that were identical with *Preparation A* as shown by mixed melting point, infrared spectra, and TLC in 3:1 benzene–methanol.

6 - (4 - Biphenylyl) - 2,4 - diaminopyrimidine (XVIII).—A mixture of 1.00 Gm. (3.8 mmoles) of XXXIX, 10 ml. of phosphorus oxychloride, and 1 ml. of triethylamine was refluxed for 75 min., then processed and treated with ammonia as described for the preparation of XIX; the yield of analytically pure white crystals after recrystallization from 2-methoxyethanol by addition of water was 145 mg. (15%), m.p. 273–275°. $\lambda_{max.}$ 2.92, 3.00 (NH); 6.25, 6.35, 6.50 (NH, C=C, C=N); 12.3, 13.71, 14.50 μ (phenyl); $\lambda_{max.}$ (pH 1): 313 mμ (ϵ 25,000); (pH 7, 13): 278 mμ (ϵ 28,000).

Anal.—Calcd. for $C_{16}H_{14}N_4$: C, 73.3; H, 5.34; N, 21.4. Found: C, 73.7; H, 5.43; N, 21.2.

2 - Amino - 6 - phenyl - 5 - phenylbutyl - 4 - pyrimidinethiol (XLIII).—To a solution of 675 mg. (2 mmoles) of XLI [R_1 = $C_6H_5(CH_2)_4$—, R_2 = C_6H_5] (5) in 20 ml. of *tert*-butyl alcohol was added 160 mg. (2.1 mmoles) of thiourea. After being refluxed for 2 hr., the solution was treated with 12 ml. of 10% aqueous sodium hydroxide, then was refluxed for 15 min. more. The mixture was diluted with 20 ml. of water and then acidified to about pH 5 with 3 *N* hydrochloric acid. The yellow precipitate was collected on a filter and washed with water; yield, 448 mg., m.p. 186–198°. The solid was dissolved in hot ethanol, filtered from some insoluble material, then the solution was diluted to turbidity with water; yield, 239 mg. (36%) of analytical sample, m.p. 225–227°, which moved as one spot on TLC in 3:1 benzene–methanol. $\lambda_{max.}$ 2.90, 2.98 (NH); 6.08, 6.40, 6.49 (NH, C=C, C=N); 13.3, 14.4 μ (C_6H_5).

Anal.—Calcd. for $C_{20}H_{21}N_3S$: C, 71.6; H, 6.27; N, 12.5. Found: C, 71.6; H, 6.32; N, 12.3.

2 - Amino - 6 - phenyl - 5 - phenylbutylpyrimidine (XXVII) Hydrochloride.—To a solution of 100 mg. (0.30 mmole) of XLIII in 10 ml. of ethanol was added about 200 mg. of Raney nickel. The mixture was refluxed with magnetic stirring for 1 hr., then filtered through a Celite pad. The ethanol solution was spin-evaporated *in vacuo* leaving 50 mg. of a glass. A solution of this glass in ether was treated with excess hydrogen chloride gas. The gummy hydrochloride was crystallized from ethyl acetate–petroleum ether (b.p. 60–110°); yield, 30 mg. (30%) of white crystals, m.p. 147–149°, that moved as a single spot on TLC in 3:1 benzene–methanol. $\lambda_{max.}$ 5.88 ($C=NH^+$), 5.99, 6.25 (NH, C=C, C=N); 13.5, 14.1, 14.4 μ (C_6H_5).

Anal.—Calcd. for $C_{20}H_{21}N_3 \cdot HCl$: C, 70.5; H, 6.47; N, 12.4. Found: C, 70.4; H, 6.60; N, 12.5.

2 - Amino - 5 - (3 - anilinopropyl) - 6 - phenylpyrimidine (XXIII).—A solution of 500 mg. of XLIV (5) in 20 ml. of ethanol was refluxed with about 600 mg. of Raney nickel for 2 hr. The clarified solution was evaporated *in vacuo*. The residue was dissolved in chloroform; the solution was clarified by filtration, then spin-evaporated *in vacuo* leaving 200 mg. (43%) of crude LXII as a glass.

To the crude LXII was added 86 mg. of phenol and 4 ml. of 30% hydrogen bromide in acetic acid. The mixture was magnetically stirred for 18 hr. protected from moisture, then poured into several volumes of ether. The solid which separated was triturated with fresh ether, then collected on a filter. The hydrobromide salt was dissolved in 25 ml. of water, then the solution was made strongly alkaline with 10% sodium hydroxide. The product was collected on a filter, washed with water, then recrystallized from aqueous ethanol with the aid of charcoal; yield, 86 mg. (64%) of analytically pure material, m.p. 131–132°. $\lambda_{max.}$ 2.85 (NH); 6.20, 6.25, 6.39 (NH, C=C, C=N); 13.41, 14.45 μ (C_6H_5); $\lambda_{max.}$ (pH 1): 233 (ϵ 17,400), 285 (ϵ 6400), 325 mμ (ϵ 6900); (pH 7, 13): 313 mμ (ϵ 6600).

Anal.—Calcd. for $C_{19}H_{20}N_4$: C, 75.0; H, 6.56; N, 18.4. Found: C, 74.7; H, 6.53; N, 18.4.

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Water/Oil Emulsions Prepared by Low Pressure Capillary Homogenization I

Effects of Emulsator and Composition Variables on Mannide Mono-oleate Stabilized Systems

By C. DAVID FOX* and RALPH F. SHANGRAW

An automated reciprocating capillary emulsator is described which duplicates the low-pressure homogenization principle of the interconnected glass syringe method used to prepare repository w/o emulsions. The w/o emulsions produced by this emulsator were evaluated by rotational viscometry and optical microscopy. Rheological flow curves of concentrated emulsions could be correlated with the extent of dispersion and degree of coalescence. Coalescence rates were found to be an inverse function of the surfactant concentration, with 20 per cent mannide mono-oleate furnishing the best relative stability for water-in-mineral oil emulsions. The rate of shear was found to govern the degree of emulsification, with a short capillary of large diameter producing the finest dispersion.

IT HAS been shown that antibody production in animals can be enhanced and prolonged when the aqueous immunologic agent is emulsified in mineral oil and injected intramuscularly to provide a depot of slowly released antigen (1). Since the work of Henle and Henle in 1945 (2), many clinicians have developed their own w/o emulsions of various agents in efforts to achieve a sustained immunologic response in humans (3–6). The best clinical results to date have been obtained by using light mineral oil as the external phase, since it cannot be metabolized by the human body. It is theorized that the mineral oil is removed by a slow phagocytosic process which appears to be the rate-limiting step in the release of antigen from the internal phase (7).

Although numerous reports have confirmed the efficacy of this innovation in hyposensitization therapy for influenza and pollinosis, there is considerable diversity of opinion among clinicians as to which emulsification method and formulation will yield the most stable emulsion with a uniform small particle size. Standardized w/o emulsions of various antigens for annual repository injection are not commercially available. Thus, the physician has had to develop his own individual formulation and emulsification techniques, determine the degree of dispersion, and estimate the appropriate dosage. The most widely used method for preparing these emulsions consists of placing the components to be emulsified in a 10-ml. glass hypodermic syringe and then connecting this syringe to a second 10-ml. syringe by means of a double-hubbed hypodermic needle. The shearing force required for emulsification is achieved by repeatedly forcing the components from one syringe to the other, either manually (4), or by means of an automated device[1] that pneumatically reciprocates the syringe pistons (8).

This low-pressure capillary homogenization project was designed to study the effect of variation in certain operating conditions on emulsification efficiency and to determine how various factors affect the stability of the w/o emulsions prepared. The variables studied were phase volume ratio, concentration of the emulsifying agent, homogenization pressure, capillary hypodermic needle diameter and length, and the number of processing cycles. The definite interrelationship that exists among the numerous variables was found to be reflected in the emulsion flow properties or rheologic behavior. For this reason, the evaluation of the experimental emulsions was based on particle size determination and rheologic flow curves, with an attempt to correlate these 2 parameters.

Mannide mono-oleate[2] was chosen as the emulsifying agent since it has received extensive toxicity evaluation and is generally accepted for use in w/o repository emulsions (9, 10). A purified grade of light mineral oil[3] was selected as the oil

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[1] Brown Emulsor, Andonian Associates, Inc., Waltham, Mass.
[2] Marketed as Arlacel A by Atlas Chemical Industries, Wilmington, Del.
[3] Marketed as Drakeol 6VR by Pennsylvania Refining Co., Butler, Pa.

phase since its suitability and safety is well documented (11).

The maximum volume of 10 ml. of emulsion produced by the glass syringe method is obviously inadequate for extensive evaluation. It was therefore decided to construct an automated capillary homogenizer based on the interconnected glass syringe principle, but capable of producing sufficient emulsion for rheological and stability studies (12).

Description of the Capillary Emulsator.—The emulsator designed and constructed in this laboratory consists of 2 modified hydraulic cylinders interconnected by a double-hubbed hypodermic needle. The emulsion components are introduced into 1 of the cylinders, and the system is then closed. Under a constant preset pneumatic force, the cylinder pistons alternately force the components repeatedly through the capillary hypodermic needle, producing the shearing action required for emulsification. A front view of the emulsator is shown in Fig. 1. The emulsator constructed has a maximum capacity of 114 ml., but larger volumes of emulsion could be produced with cylinders of increased bore or stroke.

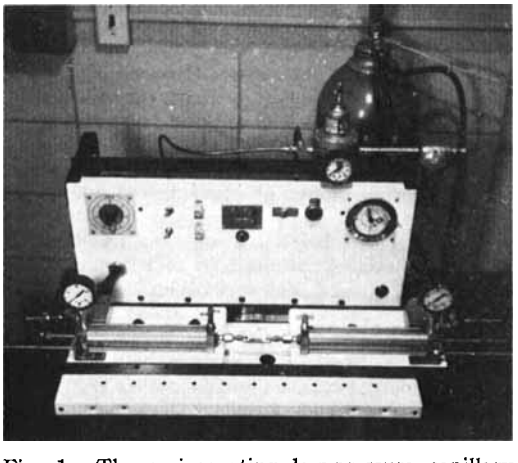

Fig. 1.—The reciprocating low-pressure capillary emulsator.

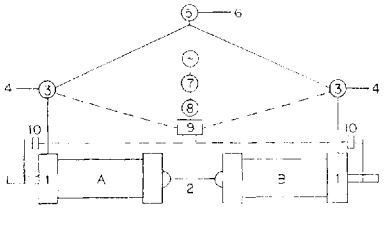

Fig. 2.—The reciprocating low-pressure capillary emulsator. Electrical system (– – –), pneumatic system (——). Key: 1, hydraulic cylinders, 1⅛ in. bore, 7 in. stroke; 2, double-hubbed hypodermic needle; 3, solenoid valves; 4, exhaust lines; 5, air pressure regulator; 6, compressed air source; 7, clock timer; 8, interval timer; 9, impulse relay; 10, microlimit switches.

The reciprocating action of the cylinder pistons is controlled by an impulse switching relay. This relay alternately activates 2 independent circuits—a pneumatic and an electrical circuit to each of two 3-way electromagnetic solenoid valves. A schematic diagram of the pneumatic and electrical system is shown in Fig. 2.

Air pressure on cylinder piston A forces the emulsion in cylinder A through the hypodermic needle into cylinder B. A micro-limit switch is tripped when cylinder piston A has forced all the emulsion into cylinder B. The limit switch directs a pulse of current to the impulse relay which switches current from the cylinder A solenoid to the cylinder B solenoid. This simultaneously exhausts cylinder A, and air pressure is directed into cylinder B. When cylinder piston B has forced the emulsion back into cylinder A, another limit switch is tripped, and the cycle commences again. An electrically operated digital counter is connected to the impulse relay. Each time the relay switches circuits, 1 count is recorded, with 2 counts indicating 1 complete cycle.

The emulsator can be set to commence and cease operation automatically at any desired time within a 12-hr. period, since a clock timer and an interval timer are included in the electrical circuit.

The machine parameters of operation time, capillary diameter, capillary length, and shearing stress can be held constant enabling a valid comparison among emulsions since the dispersions are produced under reproducible and well-defined conditions.

EXPERIMENTAL

Preparation of Emulsions.—In this study, the external phase of all emulsions is composed of light mineral oil and mannide mono-oleate, while the internal phase is distilled water. It should be noted that when all components were placed in 1 cylinder of the emulsator, a stable o/w emulsion is invariably produced when the ratio of oil phase to water phase is 1:1. This was unexpected since mannide mono-oleate is oil-soluble and should tend to produce w/o emulsions. It was therefore necessary to inject the aqueous phase into the oil phase on the first stroke to obtain the desired w/o emulsion. The ease with which mineral oil–mannide mono-oleate systems form o/w emulsions has not been reported in the literature.

Measurement of Flow Properties.—The instrument used in this study was the Haake Rotovisko rotational viscometer (Gebrüder Haake K.G., Berlin, Germany), with a rotor radius of 2.004 cm. and a cup radius of 2.10 cm., which gave a range of shear

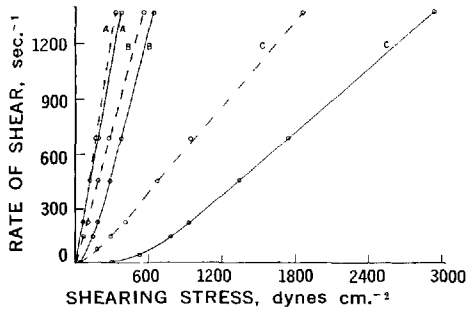

Fig. 3.—Effect of phase volume variation on w/o emulsion flow curves. Key: ———, initial; – – – –, 30 days. (Distilled water internal phase) A, 5%; B, 20%; C, 50%.

rate from 7×10^{-2} to 1.14×10^{3} sec.$^{-1}$ and a range of shear stress from 15 to 3×10^{3} dynes/cm.^{2} All determinations were made immediately after homogenization and after a 30-day storage period, at a temperature of 30°.

Particle Size Determination.—Dark-field microscopy was chosen as the method for particle size analysis. Although this method has inherent limitations in measuring submicron particles, it was still preferable to the problems of coping with sedimentation or light-scattering techniques when applied to w/o systems. A size–frequency analysis was performed on each emulsion immediately after homogenization and again after 30 days, with a Leitz Ortholux dark-field microscope (E. Leitz, G. m. b. H., Wetzlar, Germany) equipped with a 95/1.32 N.A. fluorite oil immersion objective, immersed substage condenser, and 25× oculars for a total magnification of 2375×. A small aliquot of each emulsion was diluted 1:400 with light mineral oil and a drop placed in a Petroff-Hauser bacteria counting chamber (Arthur H. Thomas Co.) of 0.02-mm. cell depth and covered with a cover glass of 0.25-mm. thickness, thus permitting critical focusing. Fields from 2 slides were examined with the diameters of 400 particles being measured with a screw micrometer eyepiece.

Storage of Emulsions.—The portion of each emulsion used for the initial rheological determination was discarded and the remainder stored in 4-oz. clear glass bottles for 30 days at room temperature.

Effect of Variation in Phase Volume.—Representative flow curves for w/o emulsions containing 5, 20, and 50 vol. % of distilled water are shown in Fig. 3, with size–frequency data presented in Table I. These emulsions all contain 20% mannide mono-oleate. Emulsions containing smaller amounts of emulsifying agent exhibited lower apparent viscosities, but their flow curves had the same general shape. Emulsator constants were: homogenizing pressure, 50 psig; capillary diameter, 18 gauge; capillary length, 1.25 cm.; and cycles, 25.

It can be seen that emulsions containing 5% internal phase possess Newtonian flow properties, and despite the high degree of coalescence that occurred in 30 days, little change is reflected in the flow curve. Emulsions containing 50% water in the internal phase, however, exhibit flow curves which show a high degree of correlation with the extent of droplet coalescence. Furthermore, this concentration is the one most commonly utilized by clinicians for their repository emulsions.

Effect of Variation in Mannide Mono-oleate Concentration.—The effect of 4 different concentrations of mannide mono-oleate on simple 50:50 w/o systems is shown by the size–frequency distribution data presented in Table II. Concentrations of 2, 5, 10, and 20 vol.% were used in the light mineral oil external phase replacing the oil, and each emulsion contained 50% distilled water internal phase. Emulsator constants were: homogenizing pressure, 50 psig; capillary diameter, 18 gauge; capillary length, 1.25 cm.; and cycles, 25. Varying each of the constants in turn for emulsions of identical composition yielded similar data. It is evident that a high percentage of mannide mono-oleate is required to furnish the best relative stability; hence, a concentration of 20% was used for the emulsions in the remainder of this study.

Effect of Variation in Homogenizing Pressure.—The first emulsator parameter to be investigated was that of homogenizing pressure, and the influence of pressures varying from 20 to 75 psig is shown in the flow curves of Fig. 4. Each emulsion is composed of mannide mono-oleate, light mineral oil, and distilled water in the volume ratio of 2:3:5, respectively. Emulsator constants were: capillary diameter, 18 gauge; capillary length, 1.25 cm.; and cycles, 25.

A microscopical examination of the freshly prepared emulsions revealed that droplet diameters in each emulsion were essentially all below 1 μ. Since a decreased particle size is the only factor which could have caused the increased apparent viscosities, it is assumed that the increase in pressure resulted in a small but significant decrease in droplet diameters. Previous work in this laboratory had shown that droplet diameters below 1 μ have a profound influence on flow curves of concentrated emulsions. However, in this study, significant differences in particle diameters below 1 μ could not be discerned due to the inherent limitations of the microscope technique employed. It was decided to keep the homogenizing pressure constant at 50 psig for all subsequent emulsions since this is the maximum pressure that 10-ml. glass syringes will withstand without leakage past the plungers.

Effect of Variation in Capillary Diameter.—Emulsions were prepared using hypodermic needles of 20, 18, and 15 gauge (nominal inside diameters of 0.58, 0.84, and 1.37 mm., respectively). An analysis of the rheological and micromeritical data for these emulsions showed that, among the gauges used, an

Table I.—Size–Frequency Distributions for Fig. 3 Emulsions,[a] Phase Volume Variation

Diam., μ	Emulsions A[b] Time, Days 0	A[b] Time, Days 30	B[c] Time, Days 0	B[c] Time, Days 30	C[d] Time, Days 0	C[d] Time, Days 30
<0.5	388	25	392	5	389	20
0.5–1	12	75	8	15	10	60
1–3	...	160	...	75	1	80
3–5	...	80	...	100	...	60
5–8	...	40	...	100	...	100
8–15	...	15	...	100	...	70
>15	...	5	...	5	...	10

[a] Each determination based on 400 measured droplets. [b] 5% aqueous internal phase. [c] 20% aqueous internal phase. [d] 50% aqueous internal phase.

TABLE II.—SIZE FREQUENCY DISTRIBUTIONS FOR 50:50 W/O EMULSIONS STABILIZED WITH MANNIDE MONO-OLEATE[a]

	Percentage (v/v) Mannide Mono-oleate in Light Mineral Oil External Phase							
	2%		5%		10%		20%	
	Time, Days		Time, Days		Time, Days		Time, Days	
Diam., μ	0	30	0	30	0	30	0	30
<0.5	235	...	229	...	256	20	389	30
0.5–1	100	...	117	...	109	40	10	80
1–3	65	...	54	40	35	80	1	80
3–5	...	10	...	32	...	53	...	60
5–8	...	32	...	48	...	67	...	70
8–15	...	78	...	100	...	60	...	70
>15	...	280	...	180	...	80	...	10

[a] Each determination based on 400 measured droplets.

increase in capillary diameter resulted in emulsions with slightly higher apparent viscosities and a more uniform particle size distribution.

Effect of Variation in Capillary Length.—The effect of variation in the capillary length on flow curves for w/o emulsions is shown in the flow curves of Fig. 5. Each emulsion is composed of mannide mono-oleate, light mineral oil, and distilled water in the volume ratio of 2:3:5, respectively. Emulsator constants were: homogenizing pressure, 50 psig; capillary diameter, 18 gauge; and cycles, 25. It is readily evident that as the needle length is decreased, apparent viscosities increase, again undoubtedly due to a decrease in droplet size and increased uniformity. The same effect is also noted in using either 20- or 15-gauge capillaries of various lengths. Thus, all subsequent emulsions were prepared with the largest diameter and shortest length double-hubbed capillary hypodermic needle, *i.e.*, 15 gauge and 0.625 cm., respectively.

Effect of Variation in Processing Cycles.—The final emulsator variable to be studied was the number of homogenization cycles to which the emulsion is subjected. The number of cycles was varied from 13 to 100. For any given set of conditions, essentially constant rheological values were obtained after 25 cycles, and no discernible improvement in particle size reduction could be observed by continuing the homogenization process for more than 25 cycles.

DISCUSSION

Since 1945, the formulation and production of w/o repository emulsions for human use has undergone extensive improvement. These improvements have, for the most part, resulted from subjective observations and appear to have been empirically derived. At present, most reported formulations are composed of mannide mono-oleate, light mineral oil, and aqueous antigenic extract in the volume ratio of 2:3:5, respectively, and it is generally recognized that a droplet diameter of less than 1 μ is an essential requirement for satisfactory *in vivo* sustained antibody production (13).

Previous workers have probably been misled by the limitations of their optical equipment. It is quite likely they have reported lower particle size limits above those which actually existed. No attempt is made in this work to claim accurate particle size distributions in the submicron range, but only to make comparisons of those particles which can be accurately determined, since, for the purpose of this research, it was of greater importance to know the size–frequency distribution of droplets greater than 1 μ. The measurement of the droplets in the experimental emulsions was facilitated by the fact that Brownian motion was negligible, an observation directly opposed to that reported by Silverman (14), who stated that Brownian motion was especially significant in his series of w/o emulsions, which are similar in composition to the ones used in this study.

In the area of particle size and its effect on viscosity, this investigation has confirmed the opinion of Sherman (15, 16), who reported that the observed increase in apparent viscosity of homogenized emulsions is due to a decrease in droplet size. This leads to increased interfacial area and interaction among droplets in concentrated emulsions, especially when the droplet diameters are below 2 μ.

The representative flow curves furnish additional evidence to establish the fact that particle size can be correlated with rheological data, *i.e.*, the relative extent of flow curve deviation after storage when

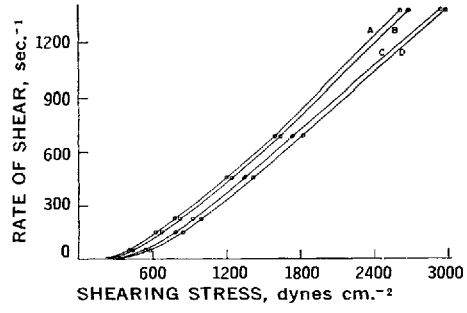

Fig. 4.—Effect of homogenizing pressure on flow curves of 50:50 w/o emulsions. Key: A, 20 psig; B, 25 psig; C, 50 psig; D, 75 psig.

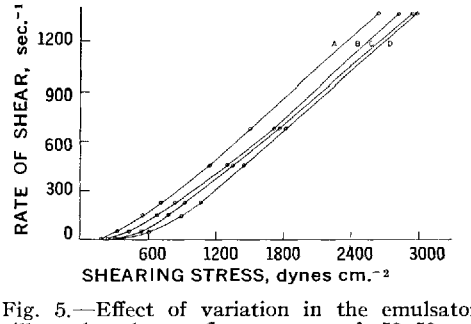

Fig. 5.—Effect of variation in the emulsator's capillary length on flow curves of 50:50 w/o emulsions. Key: A, 5.0 cm.; B, 2.50 cm.; C, 1.25 cm.; D, 0.625 cm.

compared with the initial flow curve is a direct indication of the extent of droplet coalescence. As shown in Fig. 3, the validity of the above statement increases as the volume per cent internal phase increases. When emulsions contain a low percentage of internal phase, the extent of particle–particle interaction is only a minor contributing factor to the over-all rheologic behavior of the system, and the flow curve is influenced primarily by the viscosity of the external phase. The anomalous flow behavior and high viscosity of concentrated homogenized emulsions is due primarily to the high resistance to flow offered by the tightly packed droplets and the increased adsorption of emulsifying agent at the greatly extended interface. As the droplets coalesce, the emulsion becomes polydisperse and cubic-packing decreases with a concomitant reduction in interfacial area and contact among droplets. The net result of these reductions will be reflected in the flow curve by a significant decrease in both apparent viscosities and shear dependence. This work confirms the rotational viscometric work of Saunders (17), who observed that when the internal phase volume becomes greater than 5%, shear dependence increases due to hydrodynamic interference. Sherman (18), however, has reported observing Newtonian flow in w/o emulsions at internal phase volumes of up to 50%, as determined by capillary viscometry, a method which possesses definite inherent limitations when used to obtain rheological data on shear dependent systems.

While o/w emulsions can generally be stabilized by low concentrations of surfactants, w/o repository emulsions require extremely high concentrations of mannide mono-oleate, with 1 report stating that 35% was required (19). This research has confirmed the fact that high percentages of mannide mono-oleate are required, but as repository w/o emulsions prepared by the interconnected glass syringe method are subjected to very low shear conditions, this would not be unexpected. The majority of emulsions in this study were homogenized at 50 psig to correlate the data with that reported by workers in the repository emulsion field.

The development of automatic devices for the production of small quantities of repository w/o emulsions has enabled the physician to achieve some degree of process standardization, but a review of the literature reveals that tremendous variations exist in methods of preparing essentially identical emulsions in interconnected glass syringes with pneumatically operated syringe plungers. The double-hubbed hypodermic needle length, in almost all cases where reported, is given as 5 cm., but the needle gauge and process time vary over an extremely wide range, as illustrated by 1 report which states emulsions were homogenized for 1 hr. through an 18-gauge needle and then for 3 hr. through a 22-gauge needle (20). Another author reports processing 50 min. through an 18-gauge needle, followed by 20 min. through a 22-gauge needle, and finally, 20 min. through a 25-gauge needle (6).

In reviewing the data, it was noted that an increase in capillary diameter, an increase in homogenizing pressure, and a decrease in capillary length were each associated with an increased emulsion velocity through the capillary. This is in agreement with Poiseuille's law for liquid flow through capillaries, which states that the volumetric flow rate is a direct function of the the capillary radius and pressure differential, and an inverse function of capillary length. The fact that an increased emulsion velocity through the capillary resulted in increased apparent viscosities with concomitant decreases in the mean diameter of the dispersed droplets would indicate that the degree and uniformity of dispersion is influenced by the rate of shear to which the emulsion is subjected. While commercial homogenizers operate in the range of 1000 to 5000 psig and can subject dispersions to shear rates of several hundred thousand reciprocal seconds, the highest approximate rates of shear encountered in this study, as calculated by the method of Henderson *et al.* (21), were 17,800, 20,00, and 20,900 sec.$^{-1}$ for 3 emulsions of identical composition prepared using 20, 18, and 15-gauge capillaries, respectively, of 0.625 cm. length at 100 psig. The majority of emulsions, however, were prepared under approximate shear rate conditions of 10,000 sec.$^{-1}$. In attempting to extend the calculations of rheologic parameters based directly and rigorously on capillary tube viscometry and Newtonian liquids to the highly viscoelastic non-Newtonian systems encountered in this study, it is realized that considerable error is introduced by the assumptions made and by failure to include additional parameters to account for the effects of viscoelasticity. However, approximate calculations of the Reynolds number for representative emulsions in this study yielded values less than 100, indicating that under the conditions employed, velocity profiles could be assumed laminar, thus permitting reasonable approximations of the mean shear rates.

On the basis of the data presented, and as noted by other authors (22, 23), the use of capillary needles 5 cm. in length and of gauges larger than 18, *i.e.*, smaller diameter, actually will hinder effective emulsification by reducing the rate of shear which consequently reduces the work done on the dispersion. However, the lengthy process time reported by the majority of clinicians undoubtedly offsets the decreased dispersion efficiency resulting from lower rates of shear. Thus, where repository emulsions are prepared by means of 2 interconnected glass syringes, the use of a very short, large-diameter doubled-hubbed capillary (0.625 cm., 15 gauge) for a short interval will result in a greater work input and subsequent particle size reduction than can be achieved by the use of a long and smaller diameter capillary for extended periods of time.

Although the interconnected glass syringe method of emulsification is extensively employed for the extemporaneous preparation of small quantities of repository w/o emulsions, the recently developed Mulsi-Churn unit (Mulsi-Jet Inc., Elmhurst, Ill.) (24) for the preparation of these emulsions would appear to be more suitable due to the extremely high shear rate produced by jet velocities of 300 to 800 in./sec. which would tend to insure a maximum dispersion of the aqueous antigenic extract in a minimum of time. However, this device was not evaluated in this study.

SUMMARY AND CONCLUSIONS

1. An automated reciprocating capillary emulsator has been developed which duplicates the low-pressure capillary homogenization principle of the 2 interconnected glass syringe method for preparing repository w/o emulsions.

2. The evaluation of w/o emulsions by the use of flow curves obtained with a concentric cylinder viscometer is demonstrated. Rheological flow curves were found to be an effective method for indicating the extent of dispersion and degree of droplet coalescence in concentrated emulsions, which behave as general plastic solids.

3. Under low shear conditions, concentrations of mannide mono-oleate in excess of 10% are required to prepare and furnish adequate relative stability to water-in-mineral oil emulsions, although absolute stability is poor.

4. The rate of shear governs the degree of emulsification where the emulsion components are repeatedly forced through a double-hubbed hypodermic needle, and a short capillary of large diameter produced the finest dispersion.

5. For any given set of preparative conditions, a maximum degree of particle size reduction is achieved quite rapidly, and emulsator operation for extended time periods appears unnecessary.

6. For the preparation of w/o repository emulsions, high shear rate emulsators would appear to offer substantial advantages over the low shear rate interconnected glass syringe method.

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Water/Oil Emulsions Prepared by Low Pressure Capillary Homogenization II

Stabilizing Influence of Inorganic Electrolytes, Secondary Emulsifiers, and Temperature

By C. DAVID FOX* and RALPH F. SHANGRAW

Aqueous dispersions in light mineral oil stabilized with mannide mono-oleate were prepared under a set of standard conditions and evaluated by rotational viscometry in conjunction with optical microscopy. Inorganic electrolytes in the aqueous internal phase, at concentrations as low as 0.01 *M*, increased apparent viscosity, retarded sedimentation, and had a marked stabilizing influence. The addition of small amounts of water-soluble surfactants to the internal phase yielded extremely fine dispersions, but these agents decreased stability and tended to cause inversion. Storage of w/o emulsions at 5° had a definite stabilizing influence when compared to room temperature storage.

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This investigation was supported by research career program award 1-F1-GM-16,652-01A1 from the Division of General Medical Sciences, U. S. Public Health Service, Bethesda, Md.
* Present address: Ayerst Laboratories, Rouses Point, N. Y.
Previous paper: Fox, C. D., and Shangraw, R. F., *J. Pharm. Sci.*, **55**, 318(1966).
[1] Brown Emulsor, Andonian Associates, Inc., Waltham, Mass.
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absolute stability was poor and droplet coalescence progressed to an appreciable extent in 30 days. In the present paper, the same technique of preparation and evaluation as previously reported has been used to study more complex dispersion systems in efforts directed toward reducing the rate of droplet coalescence. The major objective in this study was a determination of factors contributing to the objective formulation and preparation of repository antigenic emulsions.

EXPERIMENTAL

Apparatus.—All w/o emulsions in this study were prepared by means of the reciprocating capillary emulsator described earlier in detail (1). Each emulsion was homogenized by means of 50 passes through a 15-gauge (1.37-mm. i.d.) double-hubbed hypodermic needle, 0.625 cm. in length, at a constant pressure of 50 psig.

Materials.—Mannide mono-oleate was used as the primary oil-soluble emulsifying agent, and a purified grade of light mineral oil[3] made up the remainder of the external oil phase. Three water-soluble surface-active agents, polyoxyethylene sorbitan mono-oleate,[4] alkyl phenoxy polyethoxy ethanol,[5] and dioctyl sodium sulfosuccinate,[6] were selected for evaluation as secondary emulsifying agents. All inorganic electrolytes used were of the purest grade commercially available.

Procedure.—The rheological behavior of each emulsion at 30° was determined at the time of manufacture, and after a 30-day storage period with the Haake Rotovisko rotational viscometer (Gebrüder Haake K. G., Berlin, Germany) using the same cup and bob as in the earlier study (1). Size–frequency determinations were also performed on each emulsion by an optical method previously described (1). Unless otherwise stated, emulsions were stored at room temperature.

Influence of Inorganic Electrolytes.—In his later publications, Brown (2, 3) has stated that the use of a "truly" isotonic fluid as a vehicle for repository emulsified antigenic extracts will eliminate any local irritation that results when physiological saline or Coca's fluid is used. The formula for the isotonic fluid recommended by Brown is as follows:

Calcium chloride, hydrate	0.367 Gm.
Magnesium chloride, hydrate	0.301 Gm.
Potassium chloride	0.373 Gm.
Glycine	1.201 Gm.
Sodium sulfate, anhydrous	0.071 Gm.
Sodium bicarbonate	2.270 Gm.
Sodium phosphate, dibasic	0.142 Gm.
Lactic acid, 85%	0.636 mg.
Hydrochloric acid, 0.5 *N*	180 ml.
Sodium hydroxide, 0.5 *N*	224 ml.
Distilled water *q.s. ad*	1000 ml.

Although the rationale behind adjusting tonicity of w/o emulsions is questionable, dramatic effects on emulsion stability were noted when the Brown isotonic fluid was utilized for the internal phase of w/o systems. Emulsions exhibited sharply increased apparent viscosities, diminished sedimentation rates, and a marked improvement in stability as evidenced by a reduced coalescence rate. These effects are shown by the flow curves of Fig. 1 for emulsions containing mannide mono-oleate, light mineral oil, and Brown's isotonic fluid in the volume ratio of 2:3:5, respectively.

On the basis of these preliminary results, emulsions containing different concentrations of various electrolytes were prepared to determine the cause and extent of this influence as functions of concentration and valency. Each emulsion was composed of mannide mono-oleate, light mineral oil, and aqueous phase in the volume ratio of 2:3:5, respectively.

The first studies utilized sodium chloride in molar concentrations of 0.01, 0.10, 0.154, 0.25, and 0.50. Some flow curves for the freshly prepared emulsions are presented in Fig. 2.

Concentrations of sodium chloride greater than 0.10 *M* produced no further change in the initial flow curves and are not shown in Fig. 2. A concentration of 0.154 *M* sodium chloride, however, did influence the stability as confirmed by the 30-day size–frequency data. This stabilizing influence can be seen in the flow curves of Fig. 3, which reflect the extent of droplet coalescence. The marked retardation of sedimentation is illustrated in Fig. 4.

Additional emulsions were prepared to determine if this electrolytic effect could be modified by polyvalent cationic electrolytes, and w/o systems were produced containing calcium chloride, ferric chloride, and aluminum chloride, in concentrations of 0.01 and

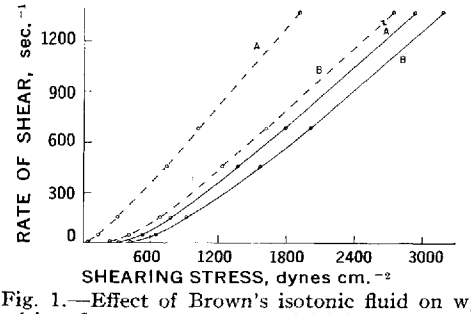

Fig. 1.—Effect of Brown's isotonic fluid on w/o emulsion flow curves. Key: initial, ———; 30 days, – – – –; A, control (50% distilled water); B, 50% Brown's isotonic fluid.

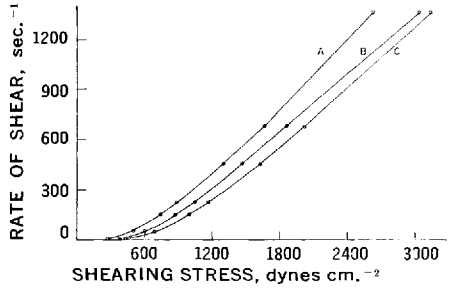

Fig. 2.—Effect of sodium chloride in the internal phase on initial flow curves of 50:50 w/o emulsions. Key: A, control (distilled water); B, 0.01 *M* sodium chloride; C, 0.10 *M* sodium chloride.

[3] Marketed as Drakeol 6VR by Pennsylvania Refining Co., Butler, Pa.
[4] Marketed as Tween 80 by Atlas Chemical Industries, Wilmington, Del.
[5] Marketed as Triton X-100 by Rohm and Haas Co., Philadelphia, Pa.
[6] Marketed as Aerosol OT by American Cyanamid Co., New York, N. Y.

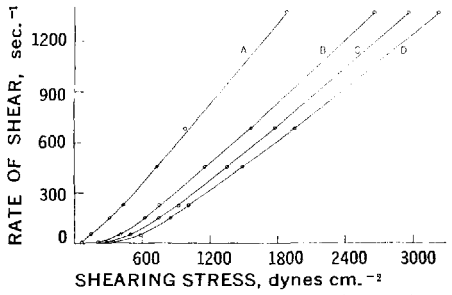

Fig. 3.—Effect of sodium chloride in the internal phase on flow curves of 50:50 w/o emulsions after storage for 30 days. Key: A, control (distilled water); B, 0.01 *M* sodium chloride; C, 0.10 *M* sodium chloride; D, 0.154 *M* sodium chloride.

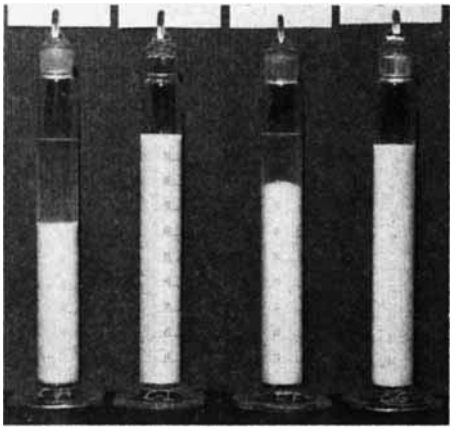

Fig. 4.—The influence of 0.54 *M* sodium chloride in the internal phase of 50:50 w/o emulsions after 30 days' storage. Key: A, external phase: Drakeol 6VR, 40 ml., Arlacel A, 10 ml. Internal phase: distilled water, 50 ml. B, external phase: Drakeol 6VR, 40 ml., Arlacel A, 10 ml. Internal phase: 0.154 *M* sodium cloride, 50 ml. C, external phase: Drakeol 6VR, 30 ml., Arlacel A, 20 ml. Internal phase: distilled water, 50 ml. D, external phase: Drakeol 6VR, 30 ml., Arlacel A, 20 ml. Internal phase: 0.154 *M* sodium chloride, 50 ml.

0.25 *M*. A review of the rheological and micromeritical data for these emulsions indicated that the divalent and trivalent cations exerted essentially the same influence as the monovalent sodium cation, both initially and after 30 days' storage.

Since an influence dependent on cationic valency could not be demonstrated, w/o emulsions containing 0.01 and 0.25 *M* sodium sulfate in the internal phase were prepared to determine if the existence of a relation dependent upon anionic valency could be shown. The results obtained indicated that the divalent sulfate anion exerted a much greater stabilizing influence than did the monovalent chloride anion. After 30 days, a microscopical examination gave no evidence of droplet coalescence in the emulsion containing 0.25 *M* sodium sulfate, with all droplet diameters being less than 1 μ. A flow curve was obtained identical to that of the freshly prepared emulsion, except for the 2 highest rates of shear where the apparent viscosities had actually increased after storage. These flow curves are shown in Fig. 5.

The emulsion containing 0.01 *M* sodium sulfate gave an initial flow curve with apparent viscosities greater than those obtained from the emulsion containing 0.25 *M* sodium sulfate. Furthermore, after 30 days the apparent viscosities were essentially identical to those of the freshly prepared emulsion at all except the 3 highest rates of shear, where again, as noted with the 0.25 *M* system at high rates of shear, apparent viscosities had increased after storage. Droplet coalescence was negligible and all particles were less than 1 μ in diameter. Flow curves for the 0.01 *M* sodium sulfate emulsion are shown in Fig. 6, with the 0.01 *M* sodium chloride system included for comparison. The decreased apparent viscosities noted with the 0.25 *M* sodium sulfate emulsion, when compared with the 0.01 *M* system, may possibly be the result of the extremely hydrophilic nature of this electrolyte. The higher concentration could conceivably cause a "salting out" effect on the adsorbed mannide mono-oleate, thereby weakening the interfacial film.

Effect of Secondary Surfactants.—An investigation was undertaken to determine what effect the inclusion of small amounts of water-soluble surfactants in the aqueous phase would have on the formation and stability of w/o emulsions.

Polyoxyethylene sorbitan mono-oleate in concentrations of 0.02, 0.2, 1, and 2% were prepared in physiological saline solution (0.154 *M* sodium chloride), and these solutions were emulsified in equal volumes of a solution containing light mineral oil (60%) and mannide mono-oleate (40%). Flow

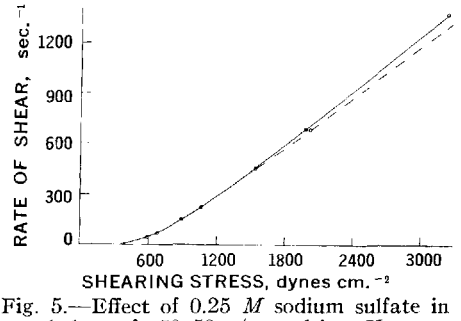

Fig. 5.—Effect of 0.25 *M* sodium sulfate in the internal phase of a 50:50 w/o emulsion. Key: ———, initial; – – – –, 30 days' storage.

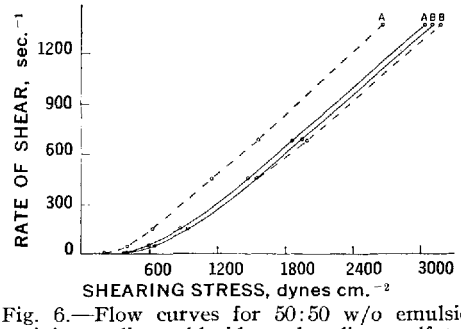

Fig. 6.—Flow curves for 50:50 w/o emulsions containing sodium chloride and sodium sulfate in the internal phase. Key: ———, initial; – – – –, 30 days; A, 0.01 *M* sodium chloride; B, 0.01 *M* sodium sulfate.

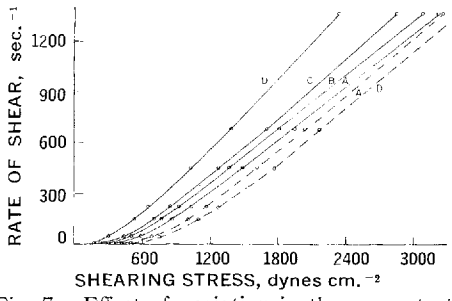

Fig. 7.—Effect of variation in the concentration of polysorbate 80 on flow curves of 50:50 w/o emulsions after 30 days' storage. Key: - - - -, initial; ———, 30 days; A, control (0.154 *M* sodium chloride); B, 0.02% in 0.154 *M* sodium chloride; C, 0.20% in 0.154 *M* sodium chloride; D, 1.0% in 0.154 *M* sodium chloride.

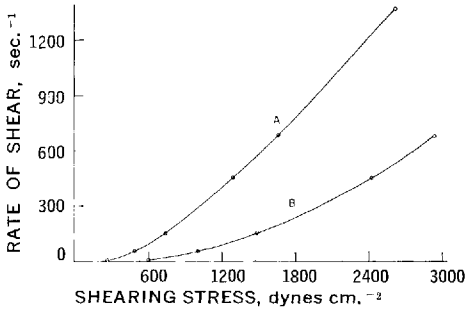

Fig. 8.—Effect of dioctyl sodium sulfosuccinate in the internal phase of a 50:50 w/o emulsion at zero time. Key: A, control (distilled water); B, 1.0% dioctyl sodium sulfosuccinate in distilled water.

curves for the freshly prepared emulsions containing 0.02 and 0.2% polyoxyethylene sorbitan mono-oleate were superimposable upon that of the control which contained only saline solution in the internal phase.

Emulsions containing 1 and 2% polyoxyethylene sorbitan mono-oleate exhibited progressive increases in apparent viscosities at all rates of shear.

After a 30-day storage period, however, marked differences were noted and the effect of the various concentrations of surfactant on emulsion stability is shown by the flow curves of Fig. 7. The emulsion containing 2% polyoxyethylene sorbitan mono-oleate had completely broken in 30 days.

Emulsions containing 1% alkyl phenoxy polyethoxy ethanol and 1% dioctyl sodium sulfosuccinate in the aqueous internal phase were also prepared. As noted with 1 and 2% polyoxyethylene sorbitan mono-oleate, the freshly prepared emulsions have the majority of droplet diameters below the limit of optical microscope resolution, *i.e.*, less than 0.2 μ. The apparent viscosities are correspondingly very high, as shown by Fig. 8, in which the initial flow curves for 50:50 w/o emulsions containing distilled water and 1% dioctyl sodium sulfosuccinate in the internal phase are compared. All emulsions contained 20% mannide mono-oleate in the external oil phase. After 30 days' storage at room temperature, however, the emulsions containing 1% alkyl phenoxy polyethoxy ethanol and 1% dioctyl sodium sulfosuccinate had completely broken.

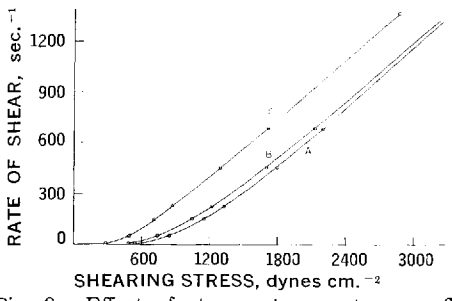

Fig. 9.—Effect of storage temperature on flow curves of a 50:50 w/o emulsion. Key: A, initial flow curve; B, 30 days' storage at 5°; C, 30 days' storage at 25°.

It should be noted that the w/o emulsions containing 1% or more of the previously mentioned water-soluble surfactants were extremely difficult to prepare in the usual manner. The aqueous phase had to be added dropwise to the oil phase with stirring prior to capillary homogenization to prevent an almost immediate inversion to an o/w system.

Effect of Storage Temperature.—Emulsions containing giant ragweed extract in the internal phase were also prepared. The giant ragweed antigen used for this study was a commercial extract[7] containing 5% w/v antigen in Coca's fluid. For each experimental emulsion, 30 ml. of the aqueous antigenic extract was diluted to 50 ml. with physiological saline solution and emulsified in 50 ml. of oil phase containing 30 ml. of light mineral oil and 20 ml. of mannide mono-oleate. The resultant w/o emulsion contained 10,000 pollen units/ml. of emulsion, the highest concentration usually employed for the repository hyposensitization treatment of hay fever.

Since all antigenic extracts must be stored under refrigeration for therapeutic stability, experiments were carried out to determine the effect of refrigeration storage on w/o emulsions. In comparing the rheological and micromeritical data for portions of the same emulsion stored at 25 and 5°, it was evident that storage at 5° resulted in a flow curve almost identical with that of the freshly prepared emulsion and only a slight change in droplet size and distribution was noted. The flow curves shown in Fig. 9, for a representative w/o emulsion, demonstrate the stabilizing influence of storage at 5°. Regardless of storage temperature, all flow curve determinations were made at a constant temperature of 30°.

DISCUSSION

Previous studies have shown that the apparent viscosities of w/o emulsions can be markedly increased by a reduction in the size of the dispersed droplets. However, the addition of inorganic electrolytes to the aqueous phase did not result in a particle size reduction greater than that observed with distilled water. Therefore, it is obvious that the observed rheological effect must result from some other factor which, in all likelihood, is electrical in nature, with the negative charge in the predominant role.

The conclusion, based on this research, that electric charges have an important stabilizing effect on

[7] Supplied through the courtesy of Abbott Laboratories, North Chicago, Ill.

w/o emulsions is contradictory to the work of Albers and Overbeek (4, 5), who have stated that electric charges cannot be expected to stabilize w/o emulsions of more than extremely low concentrations, and that no correlation exists between zeta potential and coalescence rate. Schulman and Cockbain (6) have stated that the droplets in w/o emulsions cannot possess a charge and hence cannot be electrically stabilized. On the other hand, Verwey (7) has suggested that in w/o emulsions, the major part of the potential drop occurs in the oil phase, as this has the lower dielectric constant, and that the interaction of the double layers of the droplets will determine the stability of an emulsion.

Several authors have indicated that the electrical double layer surrounding the water droplets is several microns in thickness for w/o emulsions. This thickness would therefore be greater than the distance between the dispersed phase droplets of the emulsions prepared for this investigation, where diameters were usually less than 1 μ. If the diffuse double layer is several microns in thickness, as appears reasonable, then obviously these double layers surrounding the droplets must overlap in concentrated emulsions, with a concomitant reduction in the potential energy of repulsion. The occurrence of rapid flocculation and sedimentation in those experimental emulsions that contained only distilled water would indicate that a substantial energy barrier is absent and may only be on the order of $1kT$ or $2kT$ (k, Boltzmann's constant; T, absolute temperature), depending on the droplet size and volume of the internal phase. The thermal agitation supplied by room temperature would be sufficient to overcome this energy barrier. However, the fact that inorganic electrolytes effectively prevented flocculation would imply that the presence of potential-determining ions in the systems resulted in a significant increase in the height of the potential energy barrier. This increased energy barrier could well result from a marked increase in the zeta potential by a compact charge density of counter-ions, which will increase the energy of repulsion. The extent of this increased zeta potential appears to be directly related to the valency of the electrolyte anion. Thus, the emulsion droplets are stabilized by the formation of an electrokinetic charge to a degree considerably in excess of the mechanical stabilization achieved by the use of nonionic emulsifiers alone.

In addition to the significant electrokinetic stabilization, the observed increases in apparent viscosities after storage, noted only in those emulsions containing sodium sulfate, could well be the result of an increased adsorption of emulsifying agent at the oil/water interface with the formation of a semirigid film which would contribute considerable mechanical stability. With coalescence effectively hindered, the film of mannide mono-oleate surrounding each water droplet would grow thicker and stronger with age, creating an interfacial condition that would be reflected in the flow behavior by increased apparent viscosities only at higher rates of shear where mechanical resistance to droplet deformation would become more evident.

It is well recognized that sometimes combinations of nonionic emulsifiers may be more effective than a single emulsifier in stabilizing an emulsion. The inclusion of small amounts of the water-soluble surfactant, polyoxyethylene sorbitan mono-oleate, in concentrations ranging from 0.02 to 2% in the aqueous phase, has been reported as being effective for w/o repository emulsions (8–10), but the authors present no evidence to substantiate this view. As indicated by the flow curves in Fig. 7 and confirmed by optical size–frequency analyses, the adverse effect of polyoxyethylene sorbitan mono-oleate on the stability of w/o emulsions is a direct function of its concentration in the internal phase. Increasing the concentration of this surfactant results in an increased coalescence rate and degree of polydispersity, the extent of which is reflected in the flow curves.

Emulsions containing dioctyl sodium sulfosuccinate in the internal phase exhibited a degree of anomalous flow behavior far greater than any dispersion investigated in this study, as shown by Fig. 8. Although the systems were extremely unstable, the apparent viscosity at the time of preparation could not be calculated at 1370 sec.$^{-1}$, since the shearing stress exceeded 3600 dynes cm.$^{-2}$, the maximum stress possible with the 500 Gm.-cm. torsion spring and an annular gap of 0.96 mm. in the viscometer. All droplets were below the limit of optical resolution, a condition undoubtedly responsible for the high viscosity. Unfortunately, methods for obtaining an initial size–frequency distribution in the submicron range were not available. The majority of physicians have reported the preparation of emulsions with droplet diameters of 0.1 to 1 μ, although Brown (11) has reported a much smaller size as evidenced by electron microscopic examination of films of flash-frozen emulsion. However, the electron microscopic work by Brown is questionable since he states that the frozen emulsion film (−35°) is less than 200 Å. (0.02 μ) in thickness, and then reports observing a droplet diameter distribution extending from 0.02 up to 0.1 μ, a condition difficult to reconcile with such a thin film.

The fact that water-soluble surfactants have an adverse effect on the stability of w/o emulsions definitely precludes their use in repository emulsions. Concentrations greater than 0.2% appear to result in an initial finer dispersion of the aqueous phase, but this advantage is offset by the increased coalescence rate that results. These observations may help to explain the results of Gaillard *et al.* (12, 13), who noted that their w/o repository emulsions of aqueous insect venom could not be stabilized for longer than 21 days, even with concentrations of mannide mono-oleate as high as 35%. The authors attributed the rapid cracking to an alteration of the mannide mono-oleate interfacial film by some component in the insect venom. Since it is known that insect venom contains an appreciable quantity of water-soluble surface-active compounds, these surfactants may have been the causative agents responsible for the instability.

It is a well recognized fact that solutions of allergenic materials must be stored under refrigeration at 5° to retain their effectiveness, although it is not known what changes occur at room temperature which result in decreased potency. Since any w/o repository emulsion containing an allergen would also require refrigeration, the temperature studies were undertaken and as indicated by Fig. 9, a storage temperature of 5° has a definite influence in retarding the rate of droplet coalescence. As a further confirmation of the adverse effect of water-soluble

surfactants discussed previously, emulsions stored at 5° containing polyoxyethylene sorbitan mono-oleate, when compared to those without it, were found to have a greater degree of droplet coalescence and lower apparent viscosities in spite of the stabilizing influence of refrigeration temperature.

It is interesting to note that although allergenic materials theoretically require refrigeration to maintain their potency, repository w/o emulsions after intramuscular injection are maintained at body temperature for periods of up to 1 year with no reported decrease in effectiveness.

The unqualified success of repository emulsion therapy may well depend upon elucidation of the specific agent responsible for individual allergic reactions, since the formulation of a stable w/o emulsion, consistent with the desired objectives, certainly is not insurmountable when the exact chemical composition of the components is known. Difficulties arise from the introduction of the unknown variables that undoubtedly exist in current antigenic extracts. The production methods for the extracts are a consideration since they are not standardized. The units of potency measurement constitute another variable since 3 different systems are in common use among allergists. The product may also vary as a result of variation in the amount of active principle in the raw material.

Considering the tremendous number of variables involved in the techniques of preparing and standardizing extracts of pollen and the extremely wide variations reported by physicians in the emulsification and administration of the extracts in repository form, the success that has been achieved is remarkable.

SUMMARY

This investigation has been concerned primarily with an evaluation of aqueous dispersions in light mineral oil stabilized with mannide mono-oleate and prepared under a set of standard conditions. The results obtained indicate the following.

1. Inorganic electrolytes, at concentrations as low as 0.01 *M*, in the aqueous internal phase of w/o emulsions, increase the apparent viscosities, hinder sedimentation, and have a marked stabilizing influence. This electrokinetic effect appears to be directly related to the valency of the anion.

2. The addition of water-soluble surfactants to the internal phase of w/o emulsions facilitates emulsification. However, these agents decrease stability and tend to cause inversion.

3. The storage of w/o emulsions at 5° has a definite stabilizing influence when compared to room temperature storage.

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Technical Articles

Critical Evaluation of the Compactor

By R. COHN, H. HEILIG, and A. DELORIMIER

With the assistance of a suitably prepared experimental design a compactor was optimized to prepare a basic granulation to which other drugs could be added and directly compressed into tablets. An IBM 1620 computor was employed to evaluate the data and to extrapolate additional information for the preparation of contour charts. The contour charts permitted a simple and accurate interpretation of the data and allowed selection of a set of optimum processing conditions. Additional trials confirmed the reliability of this technique.

A NUMBER OF investigations (1–3) in the chemical processing industry have employed continuous compacting equipment for the unit operation of particle size enlargement. They have shown the compaction process to be useful in the conversion of fine powders into larger agglomerated units. These particulates generally exhibited a reduced tendency to cake, improved flowability, increased bulk density, less dust, and

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a modified dissolution rate. On the basis of these earlier studies, it was expected that the compaction process would be applicable for the preparation of pharmaceutical granulations in large volumes.

A study was undertaken to utilize a compactor[1] for the preparation of a basic granulation to which other drugs would be added and directly compressed into tablets. Potassium chloride, which is a major component of a number of this company's marketed products, was selected as the test material.

The facilities of the Squibb Scientific Computing Center were employed for the preparation of an experimental design and the interpretation of the resultant data.

A photograph of the compactor utilized is presented in Fig. 1.

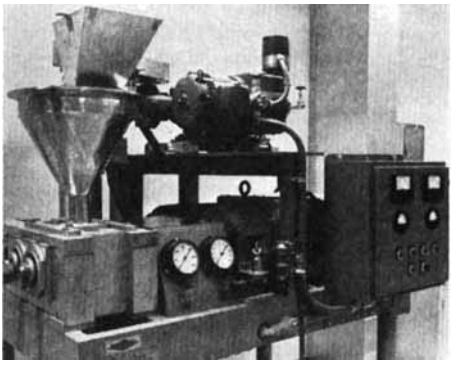

Fig. 1.—Photograph of the compactor.

EXPERIMENTAL

The experimental design permitted controlled modifications to be made on the 4 primary processing variables shown in Table I.

TABLE I.—PHASE *A* PROCESSING VARIABLES AND THEIR RANGES FOR THE COMPACTOR

Variable	Ranges
Oil pressure	220–800 p.s.i.
Roll speed	20–80% (10–40 r.p.m.)
Feed screw speed	20–80 (23–92 r.p.m.)
Added moisture	0–1%

Moisture evaluation of the test material was limited to the range of 0–1%, since it was observed that the addition of more than 1% moisture impeded material flow from the hopper of the compactor to the compacting rolls.

The criteria that were used to evaluate the quality of the granulation and the compressed tablets subsequently produced in the first phase study are shown in Table II.

The flow properties of the granulations were measured as a function of their angle of repose (4). Particle size distributions were obtained by sieve analysis. Loss on drying after 3 hr. at 160° F. under vacuum was used to define the moisture content of the granulation. The term "per cent fines" refers to the amount of potassium chloride that sifted through the compactor without being compacted.

TABLE II.—CRITERIA FOR EVALUATING PHASE *A* RESPONSES

Granulation	Tablet
Particle flow	Weight uniformity
Particle size distribution	Individual tablet assay
Moisture content	Binding
Per cent fines	Picking
	Capping
	Hardness
	Friability
	Disintegration time

A Colton 216 tablet machine was employed for the preparation of the tablet cores. The criteria referred to as binding, picking, capping, hardness, friability, and disintegration time were determined by standard procedures. Statistically sampled tablets were separately weighed on an analytical balance. To determine drug distribution, individual tablet assays were performed on 10 tablets selected at random from each trial.

On the basis of the data obtained from the full replicate factorial design of 18 runs shown in Table III, the IBM 1620 computer was able to extrapolate a total of approximately 2000 responses. Satisfactory correlations were obtained between the theoretical computer responses and the actual trial results.

A statistical analysis of the data obtained from these first 18 trials indicated that, aside from the moisture content of the granulations, the per cent fines, and the tablet hardness, all responses listed in Table II met the proposed specifications over the entire range of the experiment. Hence, these were not considered to be of primary importance in planning the second phase of the study.

During the course of the experiment, it was noted that under certain processing conditions the

TABLE III.—PHASE *A* EXPERIMENTAL DESIGN FOR THE COMPACTOR EVALUATION

Trial	Oil Pressure, p.s.i.	Roll Speed, %	Feed Screw, %	Moisture, %
1	800	80	80	0
2	220	80	20	0
3	220	20	80	1.0
4	800	80	80	1.0
5	800	80	20	0
6	220	80	20	1.0
7	510	50	50	0.5
8	800	20	80	0
9	800	20	20	0
10	800	20	80	1.0
11	220	20	20	0
12	220	80	80	0
13	220	20	20	1.0
14	800	80	20	1.0
15	220	20	80	0
16	510	50	50	0.5
17	220	80	80	1.0
18	800	20	20	1.0

[1] Model SN Chilsonator, manufactured by the Fitzpatrick Co.

amperage sensor on the drive motor indicated that the motor was being overloaded. It thus became apparent that the motor amperage response would become a very significant factor in the selection of an optimum processing condition. Hence, the responses for this factor were recorded for each trial and were used in the preparation of the contour charts.

Plotting of several contour charts from the tabulation of phase *A* responses led to the design for phase *B*. It was determined from analysis of phase *A* data that satisfactory responses for moisture, fines, hardness, and motor amperage could be obtained within the limits of the processing variables as shown in Table IV.

TABLE IV.—PHASE *B* PROCESSING VARIABLES AND THEIR RANGES

Variable	Range
Oil pressure	300–500 p.s.i.
Roll speed	70–90% (35–45 r.p.m.)
Feed screw speed	50–70% (57–80 r.p.m.)
Added moisture	0 %

RESULTS AND DISCUSSION

Data from the phase *B* trials were combined with those from phase *A* for a comprehensive analysis and determination of an optimum operating condition. The selected data are listed in Table V.

The perusal of the initial trial responses indicated that the factors of primary concern were *per cent fines, motor amperage,* and tablet hardness. Hence, these responses were evaluated for the preparation of a contour chart.

In order to prepare this type of descriptive diagram it was necessary to fix 2 of the 4 processing variables listed in Table I. Due to the inherent simplicity of processing dry powders and the lack of any significantly enhanced responses, moisture was held at a level of 0%. With added moisture fixed at 0%, roll speed could be set at 80% of the maximum. The results indicated that at these settings the most satisfactory set of responses would be attained and in addition, maximum throughput of material could be achieved in the shortest period of time.

Figures 2–4 illustrate the preparation of a contour chart and its use in determining optimum processing conditions. The contour lines are drawn by connecting responses of equal magnitude. For clarity the numerical responses that were obtained from the computer were removed from Figs. 3 and 4.

It can be observed from Fig. 2 that there was a relatively simple relationship between amperage data and the processing variables. It was observed that as the effective throughput of material or the forces acting on the compression rollers were increased, more power had to be expended. Since this work was measured as a function of motor amperage, any increase in either or both of these factors causes a corresponding increase in amperage levels. The maximum amperage at which the drive motor could be satisfactorily operated was 17. Hence, any set of processing conditions which would result in an amperage reading of less than 17 was satisfactory.

TABLE V.—SELECTED DATA EMPLOYED FOR THE PREPARATION OF CONTOUR CHARTS

Moisture..... 0%
Roll Speed.....80%

Oil Pressure	Feed Screw Speed	Amperage	Hardness	Fines
100	40	7.36	15.00	34.67
200	40	8.92	15.20	34.43
300	40	10.60	15.23	34.11
400	40	12.40	15.10	33.72
500	40	14.31	14.80	33.26
600	40	16.34	14.34	32.72
100	50	7.71	14.93	33.43
200	50	9.32	15.15	33.06
300	50	11.03	15.20	32.61
400	50	12.87	15.09	32.09
500	50	14.82	14.82	31.50
600	50	16.88	14.38	30.84
100	60	8.22	14.97	33.26
200	60	9.86	15.21	32.76
300	60	11.61	15.29	32.18
400	60	13.49	15.20	31.53
500	60	15.48	14.95	30.81
600	60	17.58	14.53	30.01
100	70	8.88	15.14	34.15
200	70	10.56	15.40	33.52
300	70	12.35	15.50	32.81
400	70	14.26	15.43	32.03
500	70	16.29	15.20	31.18
600	70	18.43	14.80	30.26
100	80	9.69	15.43	36.11
200	80	11.41	15.71	35.35
300	80	13.25	15.83	34.51
400	80	15.20	15.78	33.60
500	80	17.26	15.57	32.62
600	80	19.44	15.19	31.56
100	90	10.66	15.83	39.14
200	90	12.42	16.14	38.24
300	90	14.29	16.18	37.27
400	90	16.28	16.25	36.23
500	90	18.38	16.06	35.12
600	90	20.60	15.71	33.94

Hardness values have been superimposed on the amperage graph in Fig. 3.

The hardness data were obtained by measuring 20 individual tablets for each trial on a pneumatically

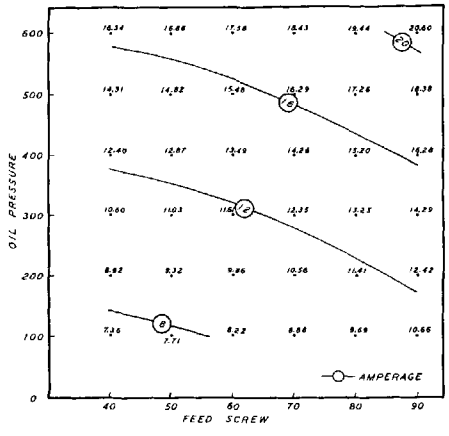

Fig. 2.—Amperage responses plotted as a function of feed screw speed and oil pressure. Moisture, 0%; roll speed, 80%.

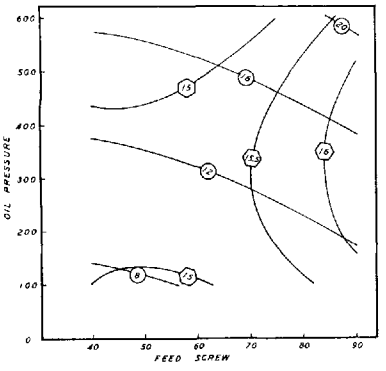

Fig. 3.—Tablet hardness values superimposed on the amperage plot. Moisture, 0%; roll speed, 80%. Key: O, amperage; ○, hardness.

operated Strong-Cobb hardness tester. Although one would hardly expect a significant difference in tablet quality for hardness values of 15.5 and 16.0, due to the relatively large number of samples (approximately 400), a noticeable trend was observed. That is, although the individually reported results did not appear to be significantly different, the data that were obtained to arrive at these numbers were different.

A perusal of this figure indicated that a simple relationship between the variables and the responses

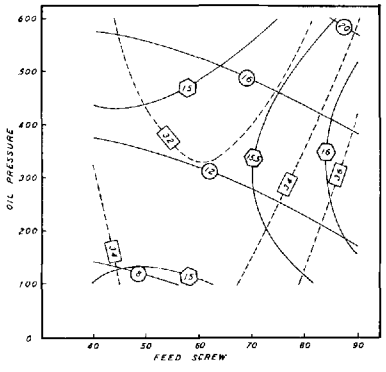

Fig. 4.—Per cent fines, amperage, and tablet hardness values displayed as functions of roll speed and oil pressure. Moisture, 0%; roll speed, 80%. Key: O, amperage; ○, hardness; □, fines.

did not exist, although a review of the tables of data confirmed that maximum feed screw speed was important to maximize this response.

The responses measured as functions of the processing variables were fairly difficult to interpret for the analysis of the amount of fines that sifted through the compactor without being compacted. It was observed that reduced feed screw speed tended to reduce the magnitude of this result. Since one of the major difficulties of the compactor was leakage of powders between the roller seals, as less material was brought in contact with the seals, less was forced through without being compacted. The assumption that increased pressure prevented or reduced the tendency of material to pass between the rollers without compression is probably incorrect. It was the authors' experience that a better fit of the compactor roller and seals was obtained under increased pressure, as less fines were observed under conditions of higher oil pressure. In actual usage, the fines were recycled back to the compactor and did not effect later processing elements.

Thus, as can be observed from Fig. 4, for the following fixed levels of moisture, 0%, and roll speed, 80%, the variable processing conditions of feed screw speed, 65%, and oil pressure, 400 p.s.i., yielded a product which manifested the maximum number of satisfactory responses.

A second and third series of contour charts were drawn with moisture fixed at 0%, but with roll speed fixed at 70 and 90%, respectively, at maximum conditions. The resultant data indicated that at optimum conditions the responses were slightly inferior to those obtained at a fixed roll speed of 80%.

A confirmatory trial was performed at the machine settings suggested by an analysis of the contour charts at moisture, 0%, roll speed, 80%, feed screw, 65%, and oil pressure, 400 p.s.i., with the result that satisfactory correlations of the actual responses to theoretical values were obtained. Excellent tablets were prepared when portions of the aforementioned compacted material were employed for the preparation of other potassium chloride-containing products.

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Comparative Study of Selected Disintegrating Agents

By WILLIAM FEINSTEIN* and ANDREW J. BARTILUCCI

Five tablet disintegrants were evaluated using 2 especially formulated bases alone and with active ingredients. The test tablet bases included a highly soluble formulation and an insoluble one. The materials evaluated were Veegum WG, Solka Floc BW-200, Jaguar A-20-B, Purity 825 cornstarch, and Landalgine P. An evaluation of the latter 2 previously was unreported. Purity 825 cornstarch and Jaguar A-20-B at 10 per cent levels were found to be the most effective. No significant change in disintegration time was noted upon the incorporation of a low concentration of an active ingredient, or upon the incorporation of a high concentration of a soluble medicament.

COMPRESSED tablets afford a convenient method for administering drugs. Most compressed tablets are formulated so as to contain fillers, binding agents, lubricants, and disintegrating agents, in addition to the active medicament. Excluding the disintegrating agent, the aforementioned components contribute chiefly to the smooth machine operation in the preparation of the finished compressed tablet. Fillers provide for a convenient tablet size and/or carrier for small amounts of active ingredients. Binding agents bring about a cohesive bond between the particles. Lubricants reduce friction and thereby permit the free flow of the granulation through the hopper and the ready ejection of the tablet from the die. It is the function of the disintegrating agent to cause the compressed tablet to break apart or disintegrate when in the presence of fluids to allow for a more favorable condition for the absorption of the contained medicament.

The literature is replete with reports pertaining to the evaluation of disintegrating agents under a variety of conditions. Factors influencing disintegration time include mechanical apparatus, materials, formulations, and techniques employed (1, 2). Several investigators (1, 3, 4) used cornstarch in the base formulation as a filler and/or binder. Kwan *et al.* (4) and Krebs (5) reported that cornstarch, when used as a filler and binder, caused significant changes in tablet disintegration times. Other investigators (6–8) reported the mutually potentiating effect of cornstarch when combined with other disintegrating agents or with substances that are capable of influencing the disintegration time of compressed tablets. Therefore, in an evaluation of disintegrating agents, the disintegration time values obtained when cornstarch is used as a binder or filler are not truly comparative representations of the disintegrating agent's ability. A more valid approach in evaluating disintegrating agents would involve the formulation of a granulation or base formula containing ingredients that did not in themselves act as disintegrating agents.

Employing a variety of active ingredients, the amounts of which were selected because they produced satisfactory tablets, many investigators (3, 7, 9–17) reported that these had an effect on disintegration time.

One objective of this investigation was the evaluation of representative disintegrating agents from each of the most commonly used chemical categories of disintegrants utilizing base formulations containing ingredients that did not themselves possess disintegrating properties. Disintegrating agents may be chemically classified as starches, clays, celluloses, algins, and gums. Two substances from the starch and algin categories, Purity 825 cornstarch and Landalgine P, respectively, not reported previously were included. Furthermore, the effect on disintegration time of 3 representative types of active ingredients present in realistic quantities was to be determined.

EXPERIMENTAL

Materials Used.—The chemicals employed were of U.S.P. or N.F. quality, or were offered by the manufacturer for drug use. The representative disintegrators were selected from several in each of the chemical categories on the basis of superior performance in preliminary experimental batches utilizing an aspirin granulation. The agents tested included: cornstarch U.S.P. and Purity 825[1] (a low moisture cornstarch) as representative of starches; Solka Floc[2] BW-40 and BW-200, Avicel,[3] and Methocel[4] MC 400 cps. as representative of celluloses; Jaguar[5] (guar gum) A-20-B and A-20-D as representative of gums; Landalgine[6] P and Kelacid[7] as representative of algins; and Veegum[8] (magnesium

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[3] American Viscose Corp., Pa.
[4] Dow Chemical Corp., N. Y.
[5] Stein, Hall and Co., N. Y.
[6] E. Mendell Co., N. Y.
[7] Kelco Co., N. J.
[8] R. T. Vanderbilt Co., N. Y.

TABLE I.—BASE FORMULATIONS

Ingredients	Wt., Gm. Base LS			Base HS		
Calcium sulfate, dihydrate	778.5	756.0	711.0	...	...	...
Lactose	...	...	...	508.5	486.0	441.0
Sucrose	...	...	...	270.0	270.0	270.0
Acacia	90.0	90.0	90.0	90.0	90.0	90.0
Calcium stearate	9.0	9.0	9.0	9.0	9.0	9.0
Disintegrating agent	22.5	45.0	90.0	22.5	45.0	90.0
(%)	(2.5)	(5.0)	(10.0)	(2.5)	(5.0)	(10.0)

TABLE II.—AVERAGE DISINTEGRATION TIME (min.) OF TABLETS OF BASE FORMULATIONS AND DISINTEGRANTS

Disintegrants	Base LS[a] 2.5%	5.0%	10.0%	Base HS[a] 2.5%	5.0%	10.0%
Purity 825 cornstarch	24.5	19.0	16.0	11.5	10.0	9.5
Jaguar A-20-B	22.5	19.0	17.0	14.0	11.0	9.0
Veegum WG	39.0	29.0	37.0	12.5	10.5	15.0
Solka Floc BW-200	35.5	30.0	32.0	12.0	11.0	16.0
Landalgine P	30.0	28.5	29.5	11.0	9.5	10.5

[a] Tablets prepared from the base formulations without the addition of disintegrating agents yielded an average disintegrating time of 44 min. for base LS and an average time of 14.5 min. for base HS.

TABLE III.—FORMULATIONS OF PREDNISONE AND BASE FORMULATION MATERIALS

Ingredients	Wt., Gm. Base LS		Base HS	
Prednisone	10.0	10.0	10.0	10.0
Calcium sulfate, dihydrate	746.0	701.0	...	...
Lactose	...	...	476.0	431.0
Sucrose	...	...	270.0	270.0
Acacia	90.0	90.0	90.0	90.0
Calcium stearate	9.0	9.0	9.0	9.0
Disintegrating agent	45.0	90.0	45.0	90.0
(%)	(5.0)	(10.0)	(5.0)	(10.0)

TABLE IV.—FORMULATIONS OF SODIUM SULFADIAZINE AND SULFADIAZINE AND BASE FORMULATION MATERIALS

Ingredients	Wt., Gm. Base LS		Base HS	
Sulfa drug	600.0	600.0	600.0	600.0
Calcium sulfate, dihydrate	156.0	111.0	...	...
Lactose	...	...	106.0	81.0
Sucrose	...	...	50.0	30.0
Acacia	90.0	90.0	90.0	90.0
Calcium stearate	9.0	9.0	9.0	9.0
Disintegrating agent	45.0	90.0	45.0	90.0
(%)	(5.0)	(10.0)	(5.0)	(10.0)

aluminum silicate) F and WG as representative of clays. Those included in the study of this report appear in Table II. The tableting properties of Purity 825 cornstarch and Landalgine P do not appear in the literature.

Development of Base and Test Formulations.—Two base formulations were sought that would yield a uniformly granular material of sufficient hardness that could be readily tableted, have significantly different disintegrating times and solubilities, and be free of materials that would contribute to the disintegration of the tablet by swelling when moist. Experimental batches of tablet granulations were prepared and tested. The 2 base formulations finally selected appear in Table I, with the concentration of each ingredient and the concentration of disintegrating agent utilized at each percentage level. The formulations produced uniform granulations which had a minimum of fines and tableted readily. Base LS formulations contained ingredients of low solubility while base HS formulations contained more soluble ingredients. The disintegrating agents were incorporated with the base formulations at concentrations of 2.5, 5.0, and 10.0%. Disintegrating agents at the 5.0 or 10.0% level were selected for formulation with the active medicaments, prednisone, sulfadiazine, and sodium sulfadiazine (Tables III and IV). The concentration of disintegrant represented the most effective level from Table II. The concentration of active ingredient incorporated in the formulation represented a realistic therapeutically prescribed amount. Thus, prednisone tablets were studied at the 5 mg. level and the sulfa drugs at the 300 mg. level. Disintegration times of tablets containing active ingredients appear in Table V.

TABLE V.—AVERAGE DISINTEGRATION TIME (min.) OF TABLETS CONTAINING ACTIVE MEDICAMENTS, BASE FORMULATION MATERIALS, AND DISINTEGRANTS

Disintegrants	Prednisone Base LS	Prednisone Base HS	Sulfadiazine Base LS	Sulfadiazine Base HS	Sodium Sulfadiazine Base LS	Sodium Sulfadiazine Base HS
Purity 825, 10%	15.5	9.0	78.0	69.0	9.5	9.0
Jaguar A-20-B, 10%	15.0	8.5	41.0	36.0	9.0	8.0
Veegum WG, 5%	27.5	11.0	90.0	88.0	10.5	9.5
Solka Floc BW-200, 5%	29.0	13.0	>100	98.0	11.5	10.0
Landalgine P, 5%	26.0	9.5	>100	95.0	15.0	12.5

Table VI.—Gross Appearance[a] of Tablet Surface

	Purity 825	Jaguar A-20-B	Veegum WG	Solka Floc BW-200	Landalgine P
Base LS—2.5% dis. agent	+/+	+/+	+/+	+/+	+/+
5.0% dis. agent	+/+	+/+	+/+	+/+	+/+
10.0% dis. agent	+/+	−/+	+/+	+/+	+/+
Base HS—2.5% dis. agent	+/+	+/+	+/+	+/+	+/+
5.0% dis. agent	+/+	+/+	+/+	+/+	+/+
10.0% dis. agent	+/+	−/+	+/+	+/+	+/+
Prednisone–base LS	+/+	+/+	+/+	+/+	+/+
Prednisone–base HS	+/+	+/+	+/+	+/+	+/+
Sulfadiazine–base LS	+/+	−/+	−/+	+/+	+/+
Sulfadiazine–base HS	+/+	−/+	−/+	−/+	−/+
Sodium sulfadiazine–base LS	+/+	−/+	+/+	+/+	+/+
Sodium sulfadiazine–base HS	+/+	−/+	+/+	+/+	+/+

[a] Whiteness/uniformity of appearance: −, off-white or mottled; +, white or nonmottled.

Manufacturing and Testing Procedures.—The powders were blended and passed through a No. 40 mesh screen. Purified water was added, and the mass was granulated by hand. The wet mass was forced through a No. 8 mesh screen, spread on trays, and dried in an oven at 38° for 14 hr. The dried granulation was forced through a No. 14 mesh screen. The fines smaller than No. 40 mesh were separated. The lubricating and disintegrating agents were mixed and passed through a No. 80 mesh screen and then blended with the granulation fines. These fines were incorporated in the granulation, and the granulation was compressed.

In the preparation of the prednisone tablets, the medicament was mixed with the fines, and these were incorporated into the granulation. The sulfadiazine tablets and the sodium sulfadiazine tablets were prepared by mixing the medicament with the filler before wet granulating. Two thousand tablets per batch were compressed on a Colton 216 rotary tablet machine utilizing $^{13}/_{32}$-in. standard concave punches and dies. The weight of each tablet was maintained at 450 ± 5 mg., and the hardness, as measured on the Stokes hardness tester, at 5 to 7 ± 0.5 Kg.

The disintegration tests were performed soon after compression using the U.S.P. (18) apparatus and method for uncoated tablets. In addition, formulations containing the active medicaments were reexamined after a minimum of 5 months' storage at room temperature. Test tablets were selected at random from each batch. Three determinations were made for each batch.

RESULTS AND DISCUSSION

It is often assumed that the disintegration time may be decreased by increasing the concentration of disintegrant. The results in Table II show this to be the case with Purity 825 cornstarch and Jaguar A-20-B. A concentration level for optimum effectiveness appears to apply with Veegum WG, Solka Floc BW-200, and Landalgine P, albeit the data are too limited to be definitive. The most effective disintegrants in the formulations studied were Purity 825 cornstarch and Jaguar A-20-B at 10% levels.

A study of Tables II and V reveals that no significant change in disintegration time occurred upon the incorporation of a low concentration of the active ingredient, prednisone, nor upon the incorporation of a high concentration of the soluble active ingredient, sodium sulfadiazine. The inclusion of a high concentration of the insoluble active ingredient, sulfadiazine, markedly increased the disintegration time of the tablets.

No significant change in disintegration time or tablet hardness was observed after storage at room temperature for 5 months.

To provide additional comparative information on the previously unreported Landalgine P and Purity 825 cornstarch, a description of the gross appearance of finished tablets appears in Table VI.

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New Approach to Development and Manufacture of Enteric Compression Coatings

By RAGHUNATH SRINIVAS, H. GEORGE DEKAY, and GILBERT S. BANKER

Five commercially available polymers were evaluated for the preparation of enteric compression coatings utilizing a unique polymer incorporation method, which was part of the granulation process, and which was applicable to all of the polymers with slight modifications. The effect of polymer plasticization, the various methods of polymer incorporation, diluent ingredient properties, and physical factors of the compression coating operation were related wherever possible to the enteric disintegration properties of the manufactured tablets. The compression coating formulations developed of 4 polymers were indicated to be enteric by *in vitro* disintegration tests, but only 3 of the polymer formulations were clearly enteric according to preliminary *in vivo* tests using an X-ray technique and human volunteers.

THE CONVENTIONAL method of enteric coating in a coating pan is time consuming, empirical, difficult to reproduce, and cumbersome. For large-scale production, newer methods of enteric coating have been developed. These include film coating and compression coating. The problem of developing highly satisfactory enteric film coatings, which will be stable in the gastric environment and at the same time will be impermeable to water and prevent leaching of the drug, is difficult to resolve. In the enteric coating field, compression coating appears to have the most potential based on greater precision and reliability of fabrication, in comparison to the pan-coating operation which is the other primary enteric coating technique.

Zapapas *et al.* (1) developed an enteric coating formulation of triethanolamine, lactose, and magnesium stearate. James *et al.* (2) developed an enteric compression coating formulation with a carboxylated polymer of vinyl acetate. Swintosky obtained a U. S. patent (3) for an enteric compression coating formulation consisting of 50 to 90% of a pharmaceutically available organic acid and a pharmaceutical binder. Miller and Lindner (4) patented an enteric compression coating formulation of penicillin, a water-soluble sulfonamide, and ammoniated polyvinyl acetate phthalate.

In this study a new method of enteric granulation preparation was utilized which may be more rapid and economical than other conventional compression coating granulation manufacturing procedures. The factors of formulation, granulation manufacture, compression properties, and probable mechanisms of enteric action were studied for their effects on the enteric characteristics of the final tablet coatings.

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EXPERIMENTAL

Polymeric Materials

The polymers studied (Table I) were selected for evaluation on the basis of meeting the following criteria as completely as possible: (*a*) water insolubility or substantial insolubility at pH values of 1.2 to 5, with increasing solubility at higher pH values, (*b*) resistance to moisture and water, (*c*) solubility in common organic solvents, (*d*) stability to heat and light under extreme conditions of pharmaceutical storage, (*e*) white or light in color and free from objectionable taste or odor, (*f*) chemical and physiological inertness, and (*g*) compressibility alone or in combination with other ingredients in the formulation. Cellulose acetate hydrogen phthalate was selected as a standard based on its wide use as an enteric coating.

Granulation Preparation Procedures

Core Formulations.—Cores of 2 dissimilar inert commonly used diluents, lactose and calcium sulfate dihydrate, as well as a radiopaque core of barium sulfate (Table II) were prepared. Lactose represented an organic soluble diluent and calcium sulfate an inorganic insoluble diluent.

Twelve-mesh core granulations of lactose and calcium sulfate and 16-mesh granulations of barium sulfate were prepared by wet granulation using a Stokes oscillator[1] and drying at 45° for 12 hr. The other formula ingredients were added to the sized granulations. Amaranth was incorporated in the cores to indicate leaching of a soluble ingredient through the coat.

Coat Granulations.—The coat formulations were prepared using the basic components and composition ranges given in Table III.

The granulations were prepared by mixing the dry polymer, coat diluent, polyethylene glycol 6000; massing the mixed powder with the granulating solvent containing the plasticizer (Table IV), if any, for 15 min. in a planary mixer to produce a very wet mass; reduction of the wet mass to pieces of about 1-in. diameter; and oven drying at 45° for 12 hr. (38° for the CAP granulations). The dried pieces were coarse-sized through a Fitzpatrick comminutor[2] operated at high speed with knives forward using a punched plate screen with 0.5-in.

[1] F. J. Stokes Corp., Philadelphia, Pa.
[2] W. J. Fitzpatrick Co., Chicago, Ill.

TABLE I.—POLYMERS STUDIED AS ENTERIC COMPRESSION COATING COMPONENTS

Polymer Designation	Chemical Designation	Commercial Name	Physical Form
CAP	Cellulose acetate hydrogen phthalate[a]	C-A-P	Flakes
PVM/MA 169	Poly(methyl vinyl ether/maleic anhydride)[b]	Gantrez AN-169	Powder
PVAc-C-H	Carboxylated copolymer of vinyl acetate[c]	Gelva C-3 V-30	Beads
PVAc-C-L	Same as above but lower viscosity grade[c]	Gelva C-3 V-10	Beads
PVAc	Polyvinyl acetate[d]	Vinac ASB-10	Beads

[a] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y. [b] General Aniline & Film Corp., N. Y. [c] Shawinigan Resins Corp., N. Y. [d] Colton Chemical Co., N. Y.

TABLE II.—CORE FORMULAS FOR *In Vitro* AND *In Vivo* TESTING

	Lactose Diluent, %	Calcium Sulfate Diluent, %	Barium Sulfate Radiopaque Core, %
Diluent	88.0	88.0	66.0
Lactose	...	...	19.0
Polyethylene glycol (40 mesh)	2.0	2.0	...
Cornstarch (dry)	10.0	10.0	15.0
Amaranth	0.5	0.5	0.3
Magnesium stearate	0.1	0.1	0.7
Granulating agent *q.s.*	12% Gelatin–4% acacia soln.	10% Starch paste	10% Starch paste

diameter openings. The coarse granules so obtained were further sized through a 12-mesh screen with a Stokes oscillator.

All compression-coated tablets were prepared with a Manesty DryCota series 500[3] compression coating machine with $^{5}/_{16}$ in. and $^{7}/_{16}$ in. s. c. punches on the core side and coat side, respectively. The cores and coated tablets had the following average specifications:

	Core	Coated Tablet
Weight	185.0 mg.	650.0 mg.
Hardness	0.5 Kg.	17–25 Kg.
Diameter	0.795 cm.	1.114 cm.
Crown thickness	0.355 cm.	0.634 cm.

RESULTS

The following factors were systematically investigated for their effect on the *in vitro* enteric disintegration, core centering, and other properties of the tablets: (*a*) the concentration of the polymer required in the coat composition, (*b*) the coat diluent materials employed, (*c*) the granulating solvent system employed, (*d*) the necessity for a plasticizer, (*e*) the addition of lubricant–intergranular enteric bonding agent and granule binder, and (*f*) the particle size and particle size distribution of the granulation.

In Vitro Enteric Properties

Each polymer (Table I) was prepared in a series of coating granulations according to the basic coat formula in the range of component concentrations shown in Table III. Based on *in vitro* enteric disintegration testing, the following results were obtained.

None of the initial CAP formulations tested, using polymer concentrations up to 30%, produced a product with enteric protection in gastric fluid. Such enteric protection was achieved when the plasticizers (Table III) were added in a compatible solvent system. Actual plasticization of the polymer in the granulations was reflected by the elastic nature of the granules. Drying the plasticized CAP granulations above 38° diminished the plasticization effect and produced brittle granules with a resultant loss of enteric properties. As with all the polymers studied, to achieve satisfactory enteric properties *in vitro*, an intergranular bonding agent (5% magnesium stearate) and a granule binder (5% PVP) were required. A 30% plasticized CAP formula (formula 1*b*, Table IV) thus prepared, produced a satisfactory enteric product by *in vitro* test. The resultant tablets were highly speckled. To produce a more elegant nonspeckled tablet, the 30% CAP granulation could be successfully combined with an insoluble granulation (formula 1*a*, Table IV) in combinations containing as little as 30% of the CAP formula. The tablets thus obtained were nonspeckled and resistant to darkening at elevated storage temperatures.

[3] Manesty Machines Ltd., Liverpool, England (Thomas Engineering, Skokie, Ill.).

TABLE III.—BASIC COATING FORMULA AND RANGE OF CONCENTRATIONS EVALUATED

Ingredients	Concn., % w/w
Polymer (40-mesh powder)	10.0–30.0
Polyethylene glycol 6000 (40-mesh powder)[a]	1.0–1.5
Diluent (lactose or calcium sulfate dihydrate)	46.5–87.0
Plasticizer (diethyl phthalate or triacetin)	0.0–10.0
Lubricant and intergranular enteric bonding agent[b]	2.0–5.0
Granule binder[c] (powdered acacia or PVP)[d]	1.0–5.0
Organic solvent (Table IV)	*q. s.*

[a] This material was present to promote core-coat bonding. In some combinations, the material used for lubricant-intergranular enteric bonding would also promote core-coat bonding. [b] Magnesium stearate, stearic acid, polyethylene glycol 4000, and calcium stearate were evaluated. [c] A substance which serves as a strong adhesive material and holds the granules together during exposure to gastric fluid. [d] Polyvinylpyrrolidone. Plasdone K-39, General Aniline & Film Corp., New York, N. Y.

PVM/MA 169, as the anhydride, was granulated with acetone to maintain the slowly soluble anhydride form. At a level of 10% polymer, using 3% magnesium stearate intergranular bonding agent and 3% PVP granule binder, satisfactory *in vitro* enteric properties were obtained when all the fines below 60 mesh were removed from the coat granulations (Table IV).

The 2 grades of carboxylated vinyl acetate copolymers, at a 30% concentration in the coat granulation produced tablets with a disintegration time in intestinal fluid which exceeded 2 hr. At a 20% polymer concentration, when granulation fines below 60 mesh were removed, satisfactory *in vitro* enteric properties were obtained with as little as 1% magnesium stearate and 2% PVP (Table IV).

PVAc, due to its swelling properties, could not be successfully developed as an enteric coat by the granulation method described here. All formulas studied prematurely liberated the soluble dye in gastric fluid. Plasticization did not improve this property.

Of the 4 lubricant-intergranular enteric bonding agents (Table III), at the range of concentrations studied, magnesium stearate was found to be the most efficient. However, none of the intergranular bonding agents when incorporated alone resisted the action of gastric fluid. It was also necessary to employ an efficient granule binder (PVP) to prevent tablet splitting during *in vitro* disintegration testing. The compositions of the 4 final enteric compression coating formulations are summarized in Table IV. When lactose was replaced with calcium sulfate dihydrate as the coating diluent in these formulations, the coated tablets split in simulated gastric fluid in 30 min.

Isopropanol alone did not prove to be a satisfactory granulating solvent for any of the polymer systems studied. It was necessary to use an intermediate polarity mixed organic solvent system (Table IV) as a granulating agent. Also, it was found advantageous to use a warm solvent system, with the exception of the CAP granulation.

The mechanism of *in vitro* disintegration of each formulation was studied by subjecting the coated tablets to the following disintegration tests in the U.S.P. apparatus: (*a*) 3 hr. in U.S.P. XVI simulated gastric fluid, (*b*) and (*c*) 1 hr. in U.S.P. XVI simulated gastric fluid followed by immersion in U.S.P. XVI simulated intestinal fluid with and without pancreatin. The CAP formulations were also subjected to disintegration test in intestinal fluids of pH 6.9 and 7.5. The tablets were evaluated during the above tests for leaching (release of soluble dye from the core through the coat), intergranular corrosion (erosion of the coat surface particularly along the granular boundaries), coat splitting, swelling, or disintegration.

The PVM/MA 169 compression coating formulations satisfactorily resisted gastric fluid *in vitro* for up to 1 hr. with slight swelling. The tablets compression coated with PVAc-C-H and PVAc-C-L formulations resisted gastric fluid *in vitro* for 2 hr. after which they demonstrated substantial coat erosion and slow leaching of the core ingredients. Tablets compression coated with CAP were intact after 3-hr. exposure to simulated gastric fluid. Pancreatin had no effect on the disintegration of tablets coated with any of the 4 formulations.

Physical and Mechanical Properties

Selected physical and mechanical properties of the 4 compression coating formulations having satisfactory enteric properties by *in vitro* tests, were evaluated as follows.

Particle Size Distribution.—The particle size distribution of the 4 coat formulations is given in Table V. To prevent or adequately retard intergranular corrosion in gastric media, it was found necessary to remove the fines below 60 mesh from the coat formulations *2*, *3*, and *4* (Table IV). The

TABLE IV.—FINAL ENTERIC COMPRESSION COATING FORMULATIONS

No.	Polymer and % Concn.	Diluent Lactose, %	Granulating Solvent *q.s.*	Triacetin, %	PEG 6000, %	Mag. Stearate, %	PVP, %	Disintegration Time, min.[a]
1a[b]	...	70	Gelatin–acacia soln.	...	...	30	...	72
1b[b]	CAP (30)	48.5	Ethyl acetate–isopropanol	10[c]	1.5	5	5	
2	PVM/MA (10)	84	Hot acetone (50°)	...	...	3	3	80
3	PVAc-C-H (20)	75.5	Ethyl acetate–isopropanol (1:1) 60°	...	1.5	1	2	90
4	PVAc-C-L (20)	75.5	Ethyl acetate–isopropanol (1:1) 60°	...	1.5	1	2	90

[a] Average disintegration time (in min.) in simulated intestinal fluid during U.S.P. XVI test. [b] Seventy per cent of *1a* and 30% of *1b* were mixed to give enteric compression coating formulation *1*. [c] The same concentration of diethyl phthalate was also an effective enteric plasticizer.

TABLE V.—SIEVE ANALYSIS[a] OF THE 4 COAT GRANULATIONS

Coat Formulations	On Screen, % No. 12	No. 16	No. 20	No. 40	No. 60	Through No. 60, %
CAP	...	20.57	31.20	24.11	8.51	15.60
PVM/MA 169[b]	0.23	2.03	10.78	53.91	33.02	...
PVAc-C-H[b]	...	0.43	11.84	59.20	28.53	...
PVAc-C-L[b]	...	0.39	3.91	50.78	44.92	...

[a] Determined with a Cenco-Meinzer sieve shaker at setting No. 1 for 5 min. [b] Coat formulations were freed of fines below 60 mesh.

percentage of fines removed and discarded averaged about 5% for granulations *2*, *3*, and *4*.

The selected coat formulations of each polymer, compressed over cores of calcium sulfate dihydrate and lactose, were evaluated by the following tests.

Hardness.—An average hardness of 10 coated tablets, randomly selected, was determined on a Dillon prototype direct force hardness tester.[4] In a comparison of Dillon, Monsanto, and Strong Cobb, the following relationships were found (5): 1 unit on the Dillon = 1 unit on the Monsanto, and 1.5 unit on the Dillon = 1 unit on the Strong Cobb.

Compression-coated tablet hardness was found to be a very important property affecting the enteric properties of the tablets. The approximate optimum hardness range to achieve *in vitro* enteric properties for the 4 coat formulations is summarized in Table VI. Tablets having a hardness below the optimum hardness range were subject to core composition leaching, and except for tablets coated with CAP, those which had a hardness above the optimum hardness range did not disintegrate in simulated intestinal fluid within 2 hr.

TABLE VI.—OPTIMUM HARDNESS RANGE FOR ENTERIC COMPRESSION COATED TABLETS

No.	Coat Formulation	Optimum Hardness Range, Kg. Calcium Sulfate Dihydrate and Lactose Cores[a]	Barium Sulfate Cores[a]
1	CAP	4–6	4–6
2	PVM/MA 169	24–25	15–18
3	PVAc-C-H	24–25	22–25
4	PVAc-C-L	24–25	22–25

[a] The compositions of the core tablets are given under *Experimental*.

Friability.—The friability of the tablets was determined in a 5.5-in. diameter baffled cylinder, revolving at 50 r.p.m. (6). A loss in tablet weight of less than 1% in 20 tablets for 100 rev. was not considered significant. At their optimum hardness range, none of the tablets were friable, according to this test.

Weight Variation.—The enteric compression-coated tablets as well as the cores were found to vary less than 1% in weight.

Horizontal Expansion and Centering of Cores.—At a machine speed of 250 tablets/min. and with the same die fill and pressure settings, 1500 tablets of the 4 successful coat formulations with lactose and calcium sulfate cores were collected and observed for visible off-centering. The percentage increase in diameter and decrease in crown thickness of a sample of the sectioned exposed cores of the coated tablets were compared with uncoated cores (Table VII). In every case, the calcium sulfate cores underwent less dislocation but demonstrated a greater decrease in crown thickness than did the lactose cores. Core composition appeared to have a much greater effect on centering properties than did the polymer coat formulation.

Minimum Coat Thickness.—Cores with an average weight of 120.0 mg., average diameter of 7.93 mm., and an average crown thickness of 3.20 mm. were compression coated with varying thicknesses of the 4 different coat formulations, keeping the hardness of the coated tablets within the optimum range for each coating. The minimum coat thickness required to give adequate enteric protection by *in vitro* standards was 1.3 mm. for all formulations.

[4] W. C. Dillon and Co., Inc., Van Nuys, Calif.

TABLE VII.—INFLUENCE OF CORE COMPOSITION ON THE OFF-CENTERING OF TABLETS COMPRESSION COATED WITH CAP, PVM/MA 169, PVAc-C-H, AND PVAc-C-L

Coat Formulation	Core Comp.	Av. % Off-Centering	Decrease in Crown Thickness, %	Increase in Tablet Core Diam., %
CAP	Lactose	7.2	24.03	2.14
CAP	$CaSO_4$	5.0	31.99	2.40
PVM/MA 169	Lactose	7.5	15.37	3.15
PVM/MA 169	$CaSO_4$	6.0	31.28	1.15
PVAc-C-H	Lactose	5.18	20.11	1.50
PVAc-C-H	$CaSO_4$	2.2	25.52	1.00
PVAc-C-L	Lactose	5.0	13.41	3.39
PVAc-C-L	$CaSO_4$	2.2	31.29	2.14

Storage Stability Tests.—The final tablet formulations of each polymer with the core compositions were stored in capped bottles at 50 ± 1°, 25 ± 2°, and 2 ± 1° for a period of 8 weeks. The *in vitro* disintegration properties of none of the 4 final tablet formulations were affected by any of the above storage conditions. The tablets, enteric compression-coated with PVAc-C-H and PVAc-C-L formulations progressively increased in hardness by 5 to 10 Kg. when stored for 8 weeks at 50°. This increase in hardness did not affect the disintegration properties of the formulations.

In Vivo Evaluation[5]

The preliminary *in vivo* evaluation of the enteric compression coated tablets was based on an X-ray examination of the tablets in the gastrointestinal tract of human volunteers, using 3 to 5 volunteers for each coat formulation.

Each volunteer was given 3 tablets. The second tablet was administered 2 to 3.5 hr. after the first tablet, and the third tablet was administered 1 to 3 hr. after the second tablet. The first and third tablets were of the same size and the second was of a different size ($^3/_8$-in. and $^7/_{16}$-in. tablets with 0.25-in. and $^5/_{16}$-in. barium sulfate cores, respectively). The radiographs were taken at calculated intervals after localization of the tablets using a Fluoricon image intensifier. Figures 1 to 4 are photographic reproductions of the X-ray films as they appear on a viewer.

CAP formulations were found to be intact in the stomach of human volunteers as long as 4 hr. and 15 min., while PVAc-C-L and PVAc-C-H tablets were found intact as long as 4 hr. and 35 min. and 4 hr. and 25 min., respectively, following administration. In the intestinal tract, CAP formulations were found to disintegrate within 6 hr. and 45 min. total time following administration, while PVAc-C-L and PVAc-C-H tablets were found to disintegrate within 6 hr. and 45 min. and 7 hr. and 30 min. or less, respectively.

[5] The authors are indebted to Dr. P. L. Webster, Radiologist, and Mr. James Barbee, Student Health Center, Purdue University, for their help in this study.

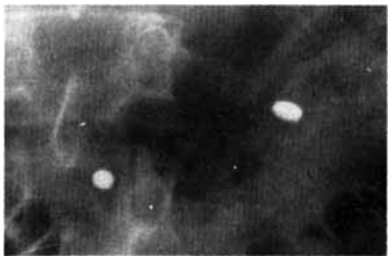

Fig. 1.—Film A_1 for cellulose acetate phthalate, illustrating second and third tablets intact in the stomach after 7 hr. and 10 min. and 3 hr. and 10 min., respectively.

Fig. 2.—Film A_4 for cellulose acetate phthalate, illustrating the third tablet disintegrating in the intestinal tract after 6 hr. and 50 min.

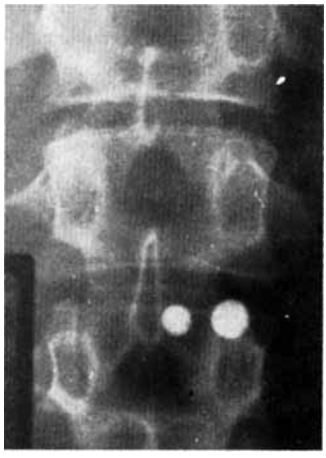

Fig. 3.—Film F_1 for PVAc-C-H formulation, illustrating second and third tablets intact in the stomach after 4 hr. and 27 min. and 3 hr. and 22 min., respectively.

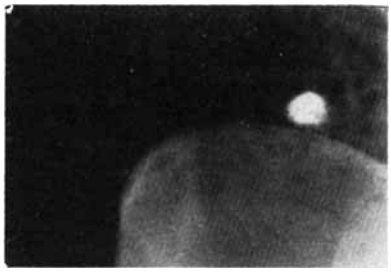

Fig. 4.—Film D_2 for PVAc-C-H formulation, illustrating the first tablet disintegrating in the intestinal tract after 6 hr. and 49 min.

In the case of tablets enteric compression-coated with PVM/MA 169, 1 tablet was found broken in the stomach after 6 hr. and 25 min. in 1 volunteer, but 4 tablets were found to be intact in the intestinal tract of other subjects where the disintegration time appeared to be 6 hr. and 35 min. or less. Thus, this formulation appears to have an enteric coating potential. The failure of 1 of the tablets in the stomach is due to the slow swelling rate of this polymer at gastric pH and the unduly long sojourn of the tablet in the stomach.

DISCUSSION

In this study, the respective polymers (Table I) were dry mixed with the coat diluent and granulated with an organic solvent system (Table IV) in which the polymer was soluble. This simple reproducible granulation–polymer incorporation method had the potential advantage, in addition to circumventing a formal coating step, of permitting the convenient uniform incorporation of higher proportions of polymers with much less solvent than would be feasible if the polymer was added in solution form.

The main objective of an enteric compression coating is to produce a strongly bonded coat layer over the core, without cracks or flaws, so that the coat will resist the penetration of gastric fluid and leaching of the core ingredients. This study indicated that this objective can be approached using the direct coat granulation method described and the following principles.

(*a*) Use of materials which weaken the interparticular bond. Shotten and Ganderton (7) refer to such materials as interparticular bonding agents. An interparticular bonding agent produces a strongly bonded tablet thereby minimizing premature intergranular corrosion and coat failure. In this application interparticular bonding agents were sought which were also enteric.

(*b*) Use of a "granule binder." This serves as a strong adhesive material and holds the granules together during exposure to gastric fluid.

(*c*) Removal of fines and fine granules. Petch (8) has reported that a coarser granule fraction gave a stronger bond.

One of the critical factors in this study, in addition to the selection of the polymeric material, was the role played by the nonpolymeric coating diluent in determining the enteric characteristics of the coat. To visualize gastric fluid permeability into the compression coatings, tablets were sectioned following

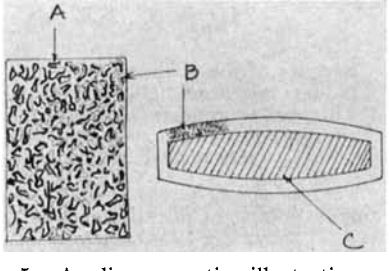

Fig. 5.—A diagrammatic illustration of the probable structure of the successful enteric compression coating layer. Key: A, matrix of partially coated granules; B, bed of intergranular enteric bonding agent—granule binder mixture; C, core.

varying periods of disintegration testing, and microscopically examined for soluble dye migration through the coat layer. Calcium sulfate dihydrate, due to its water absorbant character, drew test fluid into the coating resulting in coat failure. Lactose, though completely soluble, did not have the water-sorptive character of calcium sulfate, and was a satisfactory coat diluent in this study. Microscopic examinations of tablet cross sections also indicated that enteric compression coatings were produced when the matrix of partially coated granules is bound by the intergranular enteric bonding agent forming an impervious coat as illustrated in Fig. 5.

The greater off-centering found with the lactose cores in all of the compression-coated tablets, regardless of coat formulation (Table VII), is probably related to the greater expansion of the lactose cores during recovery which accentuates the core dislocation (9). Experiments by Kaplan and Wolff (10) have shown that calcium sulfate dihydrate is more compressible than lactose under the same conditions.

Of the 4 carboxyl-containing polymers evaluated in this study, 3 were subject to slow swelling or coat erosion in gastric fluid *in vitro*. The principle of enteric disintegration of PVM/MA 169, PVAc-C-H, and PVAc-C-L formulations appeared to be slow solubility at lower simulated gastric pH values and faster rates of solubility at higher simulated intestinal pH values. Bauer and Masucci (11) found that the disintegration of CAP coatings in intestinal contents of pH 6.9 is the result of the hydrolytic action of intestinal esterases. In this experiment pancreatin was found to have no influence on the disintegration of tablets compression coated with CAP in intestinal fluids of pH 6.9 and 7.5. This probably is due to the modification of the enteric properties of the polymer by the plasticizer, triacetin.

PVM/MA 169, which most quickly swelled *in vitro* was observed to fail in 1 subject *in vivo* as an enteric coating. A partially esterified derivative of the polymer (12, 13) with a slower dissolution rate would probably produce a more satisfactory enteric coating.

CONCLUSIONS

A method has been developed for the incorporation of slowly soluble polymer materials in a diluent by simple mixing and standard granulation procedures to produce enteric compression coatings. The granulations thus produced with each of 4 polymer materials when mixed with an intergranular enteric bonding agent, magnesium stearate, and an effective granule binder, polyvinylpyrrolidone, and compression coated at an optimum hardness range was found to be enteric by *in vitro* disintegration tests. The formulation factors, physical and tablet properties of the formulations, were investigated for their effect on *in vitro* enteric properties. *In vivo* evaluation using an X-ray technique and human volunteers indicated that 3 of the polymer systems studied were enteric and that the reported simplified method of polymer incorporation to produce enteric compression coatings is feasible.

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Evaluation of Amylose as a Dry Binder for Direct Compression

By K. C. KWAN* and GEORGE MILOSOVICH†

Because of the economies of direct compression, there exists a need for good dry binders which will effect compression of drugs at relatively low filler-to-drug ratios. This paper reports an evaluation of amylose for this purpose. The results on compression effects, physical properties, stability, and drug availability show that this material has the characteristics desired of the ideal binder.

THE DEVELOPMENT of forced-feed mechanisms for tableting presses and relatively free flowing tablet excipients has stimulated interest in commercial tableting by direct compression (1). The objective of direct compression is to produce pharmaceutically elegant tablets with a minimum of processing time and cost. Since many drugs cannot be compressed directly, 1 or more agents must be added to impart suitable compression properties. Unfortunately, many of these addi-

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TABLE I.—PROPERTIES OF AMYLOSE-BASED TABLETS

Drug	Amylose, % of Total	Other Additives	Disintegration[a]	Hardness[b]	Friability[c]	Wt. Variation,[d] Gm.
Aspirin	20	...	160 ± 47	4.8 ± 1.2	0.4	0.234 ± 0.004
Sodium PAS	55	5% Talc	>1800	5.9 ± 1.6	0.2	0.238 ± 0.004
Ascorbic acid	55	5% Talc	74 ± 36	5.7 ± 1.8	0.3	0.232 ± 0.006
Sulfathiazole	59	1% Stearic acid	43 ± 6	5.7 ± 1.6	0.3	0.220 ± 0.008
Sodium phenobarbital	62	3% Stearic acid	969 ± 336	4.1 ± 1.4	0.5	0.210 ± 0.004
Phenacetin	80	...	148 ± 22	4.3 ± 1.2	0.6	0.209 ± 0.004

[a] U.S.P. test without disks, sec. ± 2 S.E., 12 tablets. [b] Pfizer, Kg. ±2 S.E., 20 tablets. [c] Roche Friabulator, % on 6 Gm. of tablets. [d] Mean ±2 S.E., 20 tablets.

tives are either too costly or react with the active ingredients to decrease stability. Also, the ratio of these fillers to the drug necessary to effect compression may preclude use with higher dosage drugs.

The amylose used in this study[1] is unusual in that it can be tableted directly into pharmaceutically acceptable tablets. It is free-flowing, self-lubricating, and self-disintegrating so that it, by itself, functions as the filler, lubricant, and disintegrant. Since amylose is composed of large molecular weight polymers of glucose attached through 1–4 linkages, it has a minimum of reducing groups; thus, it should have very little, if any, reactivity with drug molecules. It does, however, require about 10–12% moisture for optimum compression and may be unsuitable for use with drugs subject to hydrolytic decomposition.

It was the purpose of this investigation to evaluate this amylose for its potential usefulness in direct compression applications.

EXPERIMENTAL

Six drugs known to be difficult to compress directly were selected for this study. Sulfathiazole, sodium *p*-aminosalicylate, ascorbic acid, sodium phenobarbital, phenacetin, and aspirin either do not form tablets of adequate hardness when compressed directly or form tablets with poor weight variation. Each of these materials, received from their respective suppliers as U.S.P. or N.F. grade, was mixed with an equal amount of amylose for 5 min. in a twin shell blender (Paterson-Kelly model LB2630). The resulting mixes then were tableted on a Colton 216 using $^5/_{16}$-in. standard concave punches and the standard feed frame. All tablets were prepared at maximum pressure obtainable with this press. Appropriate changes in the 1:1 formula were then made to establish the minimum amylose-to-drug ratio necessary for suitable tablets. The resulting tablets were tested for disintegration, hardness, friability, and weight variation.

To assess the possibility of classification and bridging in these powder mixes, weight variation and drug content were determined on samples removed periodically during tableting of a full hopper (2.0 Kg.) of mix. Aspirin, 200-mesh powder and 40-mesh crystals, and ascorbic acid, 200-mesh powder and 40/80-mesh crystals, were chosen to represent the range in particle sizes and amylose-to-drug ratio used in this investigation.

[1] Nepol amylose, pharmaceutical grade, a development product of the A. E. Staley Manufacturing Co., Decatur, Ill.

TABLE II.—WEIGHT AND DRUG CONTENT VARIATION

Sample Time, min.	Wt., Gm.	Drug, %
Ascorbic Acid Powder		
0–1	0.229 ± 0.006	40.4
16–17	0.232 ± 0.004	40.2
24–25	0.233 ± 0.005	40.3
32–33	0.233 ± 0.006	40.6
Ascorbic Acid Crystal		
0–1	0.254 ± 0.008	42.3
8–9	0.253 ± 0.007	41.7
16–17	0.253 ± 0.006	41.6
24–25	0.251 ± 0.008	42.5
Aspirin Powder		
0–1	0.232 ± 0.004	78.9
16–17	0.240 ± 0.004	77.8
32–33	0.239 ± 0.003	78.0
40–41	0.238 ± 0.003	79.5
Aspirin Crystal		
0–1	0.246 ± 0.007	78.1
12–13	0.252 ± 0.006	78.0
28–29	0.248 ± 0.007	78.1
36–37	0.242 ± 0.010	78.1

TABLE III.—RELATIVE STABILITY OF AMYLOSE AND COMMERCIAL ASCORBIC ACID TABLETS

Temp., °C.	$T_{.90}$ Values, Days — 75% R.H. Amylose	75% R.H. Control	45% R.H. Amylose	45% R.H. Control
55	37	20	6	6
45	15	15	5	5
37	43	43	16	16

Aspirin and ascorbic acid tablets were chosen also to test the effect of amylose on chemical stability. These and commercially available tablets were powdered using a mortar and pestle and were stored in loosely capped vials at 3 elevated temperatures and 2 relative humidities. The sample vials were arranged randomly in each desiccator and were withdrawn in a random manner to eliminate possible bias due to variation in storage conditions. Periodic assays were conducted using the U.S.P. method for acetylsalicylic acid tablets (2) and the method of Barakat *et al.* (3) for ascorbic acid.

To estimate the effect of aging on physical properties, tablets of each drug were stored in tightly capped amber bottles at 55° for 24 days, and their respective hardness, friability, disintegration time, and appearance were re-evaluated.

TABLE IV.—EFFECT OF AGING AT 55°C. FOR 24 DAYS ON PHYSICAL PROPERTIES

Drug	Hardness, Kg. ± 2 S.E. Before	Hardness, Kg. ± 2 S.E. After	Disintegration Time, sec. ± 2 S.E. Before	Disintegration Time, sec. ± 2 S.E. After	Friability, % Before	Friability, % After	Appearance after Storage
Aspirin	4.8 ± 1.2	7.2 ± 1.6	160 ± 47	32 ± 30	0.4	0.4	Needle crystals of salicylic acid on cap and neck of bottle
Ascorbic acid	5.7 ± 1.8	4.8 ± 1.8	74 ± 36	52 ± 26	0.3	0.5	Discolored
Sulfathiazole	5.7 ± 1.6	6.3 ± 1.6	43 ± 6	220 ± 132	0.3	0.4	No visible change
Sodium phenobarbital	4.1 ± 1.4	2.8 ± 1.2	969 ± 336	861 ± 56	0.5	1.0	Discolored
Phenacetin	3.4 ± 0.5	4.3 ± 1.4	333 ± 53	422 ± 80	0.6	0.8	No visible change

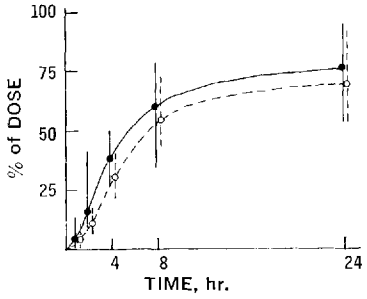

Fig. 1.—Cumulative urinary excretion of salicylates following ingestion of amylose and commercial aspirin tablets. Key: —●—, average of 8 subjects, amylose tablets; --○--, average of 8 subjects, commercial tablets; vertical lines, ranges of individual values obtained.

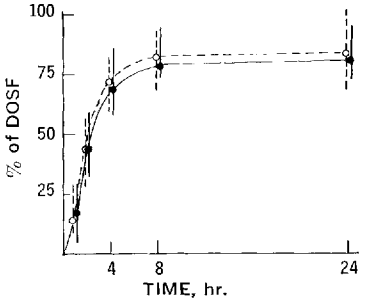

Fig. 2.—Cumulative urinary excretion of aromatic amines following ingestion of amylose and commercial sodium *p*-aminosalicylate tablets. Key: —●—, average of 8 subjects, amylose tablets; --○--, average of subjects, commercial tablets; vertical lines, ranges of individual values obtained.

Aspirin and sodium *p*-aminosalicylate tablets were chosen for investigation of drug release from amylose-based tablets. These tablets represented the 2 extremes in the *in vitro* disintegration test. Eight apparently healthy males were used in a crossover experiment, and urinary excretion data were obtained following controlled ingestion of test and commercially available control tablets. Prior to breakfast, each subject voided completely and was given 240 ml. of water. One hour later the subjects voided and were given their respective doses with an additional 240 ml. of water. They were then allowed to eat regular meals commencing 1 hr. after dosing. Urine samples were collected at 0, 1, 2, 4, 8, and 24 hr. for assay. Aspirin was determined on the basis of total salicylate content using Trinder's reagent, and sodium *p*-aminosalicylate by the method of Way *et al.* (4) for aromatic amines.

RESULTS

Amylose to Drug Ratios.—Table I gives the results obtained with the 6 test drugs. Acceptable tablets were obtained with each drug, although the proportion of amylose required varied from 20% for aspirin to 80% for phenacetin. The physical properties would be considered statisfactory for pharmaceutical application except for the relatively poor disintegration of sodium *p*-aminosalicylate. This was attributed to the basicity of this compound which may be sufficient to dissolve amylose to form a viscous gummy coating at the tablet surface retarding further penetration of water. From these results it appears that amylose can be used to effect successful direct compression of even the most difficult materials. It is expected that the amount of amylose required will depend on the inherent compression characteristics of a drug and that minimum amounts of amylose will suffice for drugs approaching aspirin in compression properties.

Weight and Drug Content in Extended Operation.—The data obtained from tablets made during extended operation in which the pressure and fill settings were held constant are given in Table II. The constancy of tablet weight and drug content during the time required to empty the hopper is a strong indication that classification was not prevalent for these mixes. Since these mixes represent wide ranges in both particle size and amylose-to-drug ratio, it is expected that classification will not be a problem with most amylose–drug mixes.

Stability.—Considering that amylose contains 10–12% water and can absorb considerably more at high humidity, it was expected that amylose would accelerate the hydrolytic degradation of aspirin. The results of this study showed, however, that even at 75% R.H. and 55°, amylose-based aspirin was equivalent in stability to the control. Since there was much more water in the amylose system, it appears that this water was bound tightly and not free for reaction.

The ascorbic acid powders exhibited considerably more decomposition than was evidenced with aspirin. Table III gives the $T_{.90}$ values for the various storage conditions. It is known that ascorbic acid degradation is very complex and no attempt is made to interpret these results. The important consideration is that there was no significant difference in

stability between amylose and the commercial control.

The results of accelerated aging on amylose-based tablets are shown in Table IV. It can be seen that physical stability was relatively good, even in cases where chemical decomposition was marked. Although changes in hardness, disintegration, and friability occurred, they were still well within the limits for acceptable tablets. Data on sodium *p*-aminosalicylate tablets are not included since this compound degraded to such an extent that the tablets were destroyed under the conditions of this test.

Drug Availability.—The results of the availability experiments are shown graphically in Figs. 1 and 2, where the mean and range of cumulative urinary excretion are plotted for each time period. It can be seen that even for the sodium *p*-aminosalicylate tablets showing poor *in vitro* disintegration the availability is essentially the same as for the control. Apparently the viscous surface film which retarded *in vitro* disintegration was mechanically eroded in the gastrointestinal tract.

CONCLUSIONS

It appears, from the results of this investigation, that amylose merits serious consideration for use as a direct compression tablet binder. In the form used in this study it can effect compression of problem drugs at relatively low concentration and yields tablets possessing the characteristics desired for pharmaceutical use. Although each drug formulation is unique and must be thoroughly tested, there is every indication from these results that successful application can be obtained. It should be pointed out that these results relate only to this particular amylose and may not extrapolate to other amyloses or amylose derivatives.

REFERENCES

(1) Milosovich, G., *Drug Cosmetic Ind.*, **92**, 557(1963).
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Notes

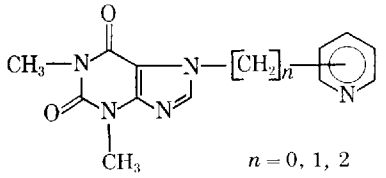
New Drugs in Xanthine Derivatives XXVI. Pyridylthioethyl Derivatives of Theobromine and Theophylline, and Products of Their Oxidation

By M. ECKSTEIN and J. SULKO

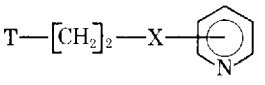
The synthesis of new 1- or 7-pyridylthioethyldimethylxanthines and their oxidation products (sulfoxides, *N*-oxides, and sulfones) are described.

SEVERAL, among previously synthesized theophylline (1) and theobromine (2) derivatives containing in the side-chain —S, —SO, —SO_2 groups, displayed interesting pharmacodynamic properties (3, 4). Compounds of the alkylthioether type are more hypotensive and less toxic than aminophylline. Water-soluble arylalkylsulfoxide derivatives appear to be active diuretics and their therapeutic index more favorable than aminophylline. The authors were interested in the synthesis of new sulfur derivatives of dimethylxanthines containing pyridyl rest in the side-chain. Among 7-substituted theophyllines, only a few compounds with a pyridine ring of type A are known.

Jucker *et al.* (5) patented 7-(pyridyl-4′)-theophylline (A, $n = 0$), and 7-(picolyl-3′ and 4′)-theophylline



A



B

T = 7-Theophyllinyl, resp. 1-theobrominyl rest.
X = —S, —SO, —SO_2

(A, $n = 1$) as the reaction products of sodium theophylline with 4-chloropyridine or picolyl chlorides, respectively. 7-β-(2′ and 4′-Pyridylethyl)-theophyllines were obtained in the reactions of pyridylethylation.

In this paper the synthesis of compounds of type B is described in which pyridine is coupled with the

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The synthesis of new 1- or 7-pyridylthioethyldimethylxanthines and their oxidation products (sulfoxides, *N*-oxides, and sulfones) are described.

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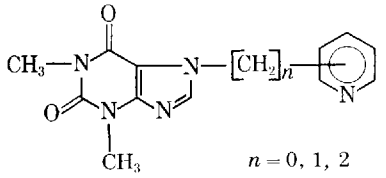
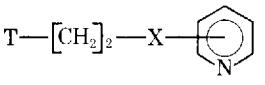


A



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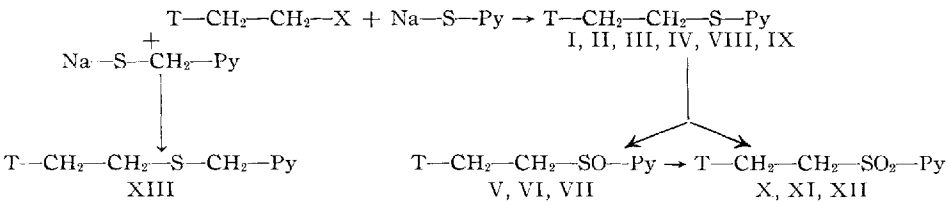

T = the 7-Theophyllinyl; resp. 1-theobrominyl rest.
X = Cl, Br

I, V, X, T = Tb, Py = 2-pyridyl
II, T = Tb, Py = 4-pyridyl
III, VI, XI, T = Th, Py = 2-pyridyl
IV, VII, XII, T = Th, Py = 4-pyridyl
VIII, T = Tb, Py = 2-pyridyl *N*-oxide
IX, T = Th, Py = 4-pyridyl *N*-oxide
XIII, T = Tb, Py = 3-pyridyl

Scheme I

xanthine system through an aliphatic chain containing sulfur as the thioether, sulfoxy, or sulfonyl group. The presence of a pyridyl group in these compounds intensifies their basic character in comparison with previously described sulfur-containing xanthine derivatives (1, 2).

The authors obtained 2- and 4-pyridylthioethyl derivatives of theobromine, and theophylline (I–IV) through condensation of 1-β-chloroethyltheobromine or 7-β-bromoethyltheophylline with sodium salts of 2- or 4-mercaptopyridines in anhydrous ethanol solution. Compounds I–IV are colorless, crystalline, easily soluble in alcohols, well soluble in dilute mineral acid solutions, and less soluble in water. They give stable picrates. By oxidation of I, III, and IV with an equimolar quantity of hydrogen peroxide in glacial acetic acid at room temperature, V, VI, and VII were formed. From comparison of physicochemical properties of V, VI, and VII with those of isomeric *N*-oxides (VIII and IX), it can be concluded that the former have a sulfoxide grouping. VIII and IX were prepared in an unambiguous way, in the reaction of halogentoethyl derivatives of theobromine resp. theophylline with the well-known 2-mercapto (6) or 4-mercapto-pyridine *N*-oxides (7). It is also in accordance with observations of other authors that in the oxidation conditions used, the pyridine *N*-oxides are formed only on heat (8–10) whereas the *S*-oxides are formed readily at room temperature. Alkyl-pyridyl sulfides give, by oxidation with hydrogen peroxide in acetic acid or with perbenzoic acid, the respective sulfoxides (6, 11). Taking into account the fact that purines are either degradated or unaffected by hydrogen peroxide (12), it may be assumed that purine *N*-oxides are not formed in the applied conditions of oxidation. Compound VI ($\lambda_{max.}$ 272 mμ, log ε 5.04) and IX ($\lambda_{max.}$ 291 mμ, log ε 5.71) showed no absorption band at ~230 mμ, which is characteristic for purine *N*-oxides (in neutral or alkaline solution) (13, 14). Similar results were obtained by U.V. examination of hydrogen peroxide oxidation products of sulfides I and IV. This supports the presence of a sulfoxide group in compounds V, VI, and VII. When, for the oxidation of I, III, and IV, an excess of hydrogen peroxide was used under analogous conditions the sulfonyl compounds (X, XI, XII) were obtained. The latter can be prepared also by oxidation of the corresponding sulfoxides. Sulfones (X, XI, XII) in the series of sulfide-sulfoxide-sulfone, have the highest melting points. X, XI, and XII, are readily soluble in ethanol and insoluble in water. Moreover, the condensation of pyridine-3-methanthiol (15) with 1-β-chloroethyltheobromine carried out in a manner analogous to compounds I–IV gives 1-β-(pyridyl-3′-methyl)-thioethyltheobromine (XIII), with a relatively low melting point.[1]

Analyses are reported in Table I.

EXPERIMENTAL[2]

1-β-(2′-Pyridyl)-thioethyltheobromine (I).—To a solution of 0.23 Gm. (0.001 mole) of sodium in 20 ml. of absolute ethanol, 1.11 Gm. (0.01 mole) of 2-mercaptopyridine (16) and 2.42 Gm. (0.01 mole) of 1-β-chloroethyltheobromine (17) were added, and the mixture was refluxed for 10 hr. The solvent was evaporated under reduced pressure, and the residue was crystallized from ethanol to obtain colorless needles, m.p. 197°. Picrate, m.p. 185° (from ethanol).

1-β-(4′-Pyridyl)-thioethyltheobromine (II), 7-β-(2′-pyridyl)-thioethyltheophylline (III), and 7-β-(4′-pyridyl)-thioethyltheophylline (IV) were obtained similarly.

1-β-(2′-Pyridyl)-sulfoxyethyltheobromine (V).—To a solution of 0.95 Gm. (0.003 mole) of 1-β-(2′-pyridyl)-thioethyltheobromine (I) in 10 ml. of glacial acetic acid, 1 drop of sulfuric acid solution and 0.34 ml. (0.003 mole) of 30% hydrogen peroxide were added. The mixture was allowed to stand for 3 days at room temperature. Acetic acid then was evaporated *in vacuo*, and the residue was crystallized from 70% ethanol to obtain colorless needles, m.p. 181°.

Similarly, the sulfoxides (VI and VII) were obtained from II and III, correspondingly.

1-β-(2′-Pyridyl)-sulfonylethyltheobromine (X).—*Method A.*—To a solution of 1.19 Gm. (0.006 mole) of 1-β-(2′-pyridyl)-thioethyltheobromine (I) in 20 ml. of glacial acetic acid, 1 drop of sulfuric acid in acetic acid solution (10 ml. of acetic acid + 2 drops of concentrated sulfuric acid), and 1.4 ml. (0.012 mole) of 30% hydrogen peroxide were added. The mixture was kept at room temperature for 3 days. After vacuum evaporation of the acetic acid, the residue was crystallized from 50% ethanol, and a colorless product, m.p. 216°, was obtained.

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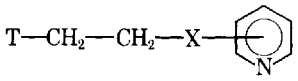
[1] It is probably due to the presence of a methylene bridge between the pyridine ring and sulfur. Similarly, 7-γ-picolyltheophylline has a lower melting point than 7-γ-pyridinetheophylline (5).

[2] Melting points were determined by the open capillary tube method and are not corrected. The analyses were done by T. Kulawik and Z. Pasternak from this laboratory.

TABLE I.—PYRIDYLTHIOETHYL DERIVATIVES OF THEOBROMINE AND THEOPHYLLINE



No.	T[a]	X	4-Pyridyl	M.p., °C., Solv. for Recrystn.	Formula, Mol. Wt.		Anal. C	H	N
I	Tb	S	2-Pyridyl[b]	195–197°	$C_{14}H_{15}N_5O_2S$	Calcd.	53.04	4.77	22.09
				Ethanol	317.36	Found	53.64	5.03	22.30
II	Tb	S	4-Pyridyl	174–175°	$C_{14}H_{15}N_5O_2S$	Calcd.	53.04	4.77	22.09
				Ethanol	317.36	Found	53.13	5.00	22.53
III	Th	S	2-Pyridyl	170–171°	$C_{14}H_{15}N_5O_2S$	Calcd.	53.04	4.77	22.09
				Ethanol	317.36	Found	52.86	4.90	22.26
IV	Th	S	4-Pyridyl	183–185°	$C_{14}H_{15}N_5O_2S$	Calcd.	53.04	4.77	22.09
				Ethanol	317.36	Found	53.15	5.21	22.40
V	Tb	SO	2-Pyridyl	181°	$C_{14}H_{15}N_5O_3S$	Calcd.	50.49	4.54	21.03
				Ethanol	333.36	Found	49.96	4.45	20.78
VI	Th	SO	2-Pyridyl	165–167°	$C_{14}H_{15}N_5O_3S$	Calcd.	50.49	4.54	21.03
				Ethanol	333.36	Found	50.31	4.73	20.91
VII	Th	SO	4-Pyridyl	204–206°	$C_{14}H_{15}N_5O_3S$	Calcd.	50.49	4.54	21.03
				80% Ethanol	333.36	Found	50.20	4.93	21.15
VIII	Tb	S	2-Pyridyl	200–201°	$C_{14}H_{15}N_5O_3S$	Calcd.	50.49	4.54	21.03
			N-oxide	Ethanol	333.36	Found	50.17	4.49	20.72
IX	Th	S	4-Pyridyl	220–221°	$C_{14}H_{15}N_5O_3S$	Calcd.	50.49	4.54	21.03
			N-oxide	Ethanol	333.36	Found	50.56	4.32	20.72
X	Tb	SO_2	2-Pyridyl	214–216°	$C_{14}H_{15}N_5O_4S$	Calcd.	48.18	4.33	20.07
				Ethanol	349.36	Found	48.18	4.52	20.15
XI	Th	SO_2	2-Pyridyl	193–194°	$C_{14}H_{15}N_5O_4S$	Calcd.	48.18	4.33	20.07
				50% Ethanol	349.36	Found	47.62	4.08	20.33
XII	Th	SO_2	4-Pyridyl	205–208°	$C_{14}H_{15}N_5O_4S$	Calcd.	48.18	4.33	20.07
				80% Ethanol	349.36	Found	48.63	4.01	20.53
XIII	Tb	S	3-Picolyl	123–124°	$C_{15}H_{17}N_5O_2S$	Calcd.			21.21
				Ethanol	331.38	Found			21.49

[a] T = the 7-theophyllinyl, resp. 1-theobrominyl rest. [b] Picrate m.p. 185° from ethanol. *Anal.*—Calcd. for $C_{20}H_{18}O_9N_8S$: C, 43.87; H, 3.33. Found: C, 43.97; H, 3.50.

The sulfones (XI and XII) were prepared similarly.

Method B.—To a solution of 0.5 Gm. (0.0015 mole) of 1-β-(2′-pyridyl)-sulfoxyethyltheobromine (V) in 20 ml. of glacial acetic acid with the catalytic amount of sulfuric acid, 0.2 ml. (0.0018 mole) of 30% hydrogen peroxide was added, and the mixture kept at room temperature for 2–3 days. After evaporation of acetic acid *in vacuo*, the residue crystallized from 50% ethanol yielded the product, m.p. 215°. No melting point depression was found for a mixture of the 2 products.

N-Oxide of 1-β-(2′-Pyridyl)-thioethyltheobromine (VIII).—A solution of 0.23 Gm. (0.01 mole) of sodium and 1.27 Gm. (0.01 mole) of 2-mercaptopyridine *N*-oxide (6) in 25 ml. of ethanol was added dropwise to a boiling solution of 2.42 Gm. (0.01 mole) of 1-β-chloroethyltheobromine in 25 ml. of ethanol. Heating was continued for 4 hr.; the hot solution was filtered and then cooled. The precipitate recrystallized from ethanol yielded the product, m.p. 201°. *N*-Oxide of 7-β-(4′-pyridyl)-thioethyltheophylline (IX) was obtained in the above manner by condensation of 7-β-bromoethyltheophylline (18) with 4-mercaptopyridine *N*-oxide (7) in ethanolic sodium ethoxide solution.

1 - β - (Pyridyl - 3′ - methyl) - thioethyltheobromine (XIII).—To the solution of 0.23 Gm. (0.01 mole) of sodium and 1.23 Gm. (0.01 mole) of 3-mercaptomethylpyridine (16) in 20 ml. of ethanol, 2.42 Gm. (0.01 mole) of 1-β-chloroethyltheobromine was added, and the mixture was heated under reflux for 8 hr. The solvent was evaporated under reduced pressure, and the residue was crystallized from ethanol to obtain a colorless product, m.p. 124°.

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Oxygen Flask Method for Iodine Determination in Thyroid Protein

By W. F. DEVLIN and SARAH P. ROSS

Nitrogenous oxidation products formed during the combustion of thyroid powder give rise to an appreciable error in the oxygen flask method for total iodine as described in the British Pharmacopoeia. This error can be reduced to negligible proportions by the use of a sulfamic acid reagent and an increased period of stirring of the combustion residue before the addition of excess iodide and titration with thiosulfate.

THE OXYGEN flask combustion method as applied to halogen determination in organic material (1) was modified by Vickers and Johnson (2) for the analysis of iodine in certain pharmaceuticals. More recently this method has been adopted by the British Pharmacopoeia (3) for iodine determination in thyroid. Vickers and Johnson mentioned that the presence of nitrogenous oxidation products in their method was undesirable. This communication describes the magnitude of the error thus caused in thyroid iodine measurements and assesses the effectiveness of sulfamic acid as used by Backer (4) to eliminate it.

EXPERIMENTAL

Equipment.—A Thomas-Lisk combustion flask, black wrappers with fuse, magnetic stirring apparatus with Teflon covered bar and ring as supplied by A. H. Thomas Co., Philadelphia, Pa., or similar equipment.

Reagents.—Prepare reagent solutions as described by Vickers and Johnson (2), using water which has been distilled over alkaline permanganate.

Bromine Solution.—Dissolve 100 Gm. of potassium acetate in glacial acetic acid, add 4 ml. of bromine, and dilute to 1 L. with glacial acetic acid.

Sulfamic Acid.—Dissolve 4 Gm. of sulfamic acid in 200 ml. of water.

Sodium Thiosulfate.—Standardize accurately to about 0.005 *N*.

Formic Acid.—88% H_2CO_2.

Starch Indicator.—0.5% in water.

Procedure.—Accurately weigh an amount of thyroid containing 0.2–0.5 mg. of iodine and enclose in a black paper wrapper. Use not more than 100 mg. of protein material when combustion is to be performed in a 1-L. flask. With larger amounts, combustion was often incomplete. Add 10 ml. of water and 2 ml. of *N* NaOH to the flask.[1] Place the magnetic stirring bar in the bottom of the flask and thoroughly flush the flask with 100% oxygen. After combustion of the thyroid powder, stir the residue for 15 min. rather than 5 min. as required in the B. P. procedure. Add 5 ml. of bromine reagent and let stand for 2 min. Add 0.5 ml. of formic acid and flush out the bromine vapors with air. Carefully wash down the sides of the flask with 15 to 20 ml. of water, add 2 ml. of sulfamic acid reagent, and allow to stand a further 2 min. Finally, add 1 Gm. of crystalline potassium iodide to the flask and titrate immediately with 0.005 *N* sodium thiosulfate using starch indicator as the end point is approached.

For combustion of standard amounts of *o*-monoiodobenzoic acid (MIB) accurately prepare alcoholic solutions containing about 10 mg. of MIB/ml. Apply carefully measured aliquots of these solutions directly on the black wrappers. Prepare dry mixes in mortars containing nicotinic acid and urea, respectively, and 80–100 mg. of MIB/10 Gm. total weight. For the preparation of combustion packets weigh the dry mixes or thyroid powders directly on the wrappers, enclose, and place in the platinum basket with wick protruding.

RESULTS AND DISCUSSION

Carbon Dioxide and Iodine Determination.—There was no appearance of free iodine or evidence of erroneous recoveries of added iodate when, following the usual procedure, 100 mg. of lactose was burned in a 120-mg. black paper wrapper. Carbon dioxide, and other oxidative residues which may result from the wrapper, did not therefore seem to produce an error.

Nitrogenous Oxidation Products of Urea Combustion.—Urea, if it is chemically pure, will yield only water, carbon dioxide, and nitrogenous oxides during combustion. Table I illustrates the extent of oxidation of iodide by nitrogenous products of urea combustion in this method. When an amount of urea containing 32 mg. of nitrogen (≡200 mg. of protein) was burned and the combustion residue stirred for 5 min. there appeared 0.20 meq. of free iodine following addition of excess iodide. When the stirring time was increased to 15 min. the amount of iodine appearing at the time of titration was reduced to 0.002 meq. Similarly, if sulfamate were added to the combustion residue after a 5-min. stirring period and the usual bromine treatment, the amount of free iodine appearing at the time of titration was also reduced to about 0.002 meq. When the longer stirring period was combined with the sulfamate treatment in the prescribed manner, there was no detectable iodine present at the time of titration.

It is reasonable to presume that at least some of the gaseous nitrogenous products of combustion will form nitrites in solution. Sulfamic acid reacts with nitrite in the manner indicated by the equation (5):

$$NH_2SO_3H + HNO_2 \rightarrow H_2O + N_2 + H_2SO_4$$

In view of the effectiveness of the sulfamic acid in prevention of the undesirable iodide oxidation, it seemed probable that the part of the nitrogenous oxidation products, which was present as, or could give rise to nitrites, caused most or all of the error. As confirmation of this hypothesis, a sensitive test

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[1] With the use of a magnetic stirring apparatus and a 1-L. round-bottom flask it has been found that the alkalinity and volume constituted by 10 ml. of water and 2 ml. of *N* NaOH is adequate to capture the iodine products of combustion. The B. P. procedure employs a 50-ml. volume of water in a 2-L. flask and 2 ml. of *N* NaOH. Excluding this minor departure, the term "B.P. method" in this text is as described in the "British Pharmacopoeia," 1963, p. 835.

TABLE I.—EFFICIENCY OF SULFAMIC ACID IN REMOVAL OF NITROGENOUS OXIDATION PRODUCTS WHICH OXIDIZE IODIDE TO FREE IODINE

Urea Nitrogen, mg.	Time Between Addition of KI and Titration, min.	Iodine, meq. — 5 min. Stirring After Combustion		15 min. Stirring After Combustion	
		No Sulfamate	Sulfamate	No Sulfamate	Sulfamate
32	2	0.20	<0.002	<0.002	No
32	5	0.21	0.002	0.002	detectable
32	10	0.21	0.002	0.002	iodine
32	30	0.21	0.002	0.003	
32	60	0.22	0.002	0.004	

TABLE II.—THE RECOVERY OF IODINE FOLLOWING COMBUSTION OF *o*-MONOIODOBENZOIC ACID (MIB., 51.17% IODINE)

MIB Combustion Prepn.	Mix Wt., mg.	Wt. MIB, mg.	Theoretical Iodine, mg.	Recovered Iodine, mg.	% Recovery
Alcoholic soln.,	...	0.101	0.0517	0.0497	96.0
1.01 mg. MIB/	...	0.202	0.1034	0.1025	99.2
ml.	...	0.404	0.2067	0.1870	90.4
	...	0.606	0.3102	0.2740	88.2
	...	0.808	0.4136	0.3760	91.0
	...	1.010	0.5170	0.4370	84.5
	...	5.050	2.5840	2.1450	83.0
Urea	53.7	0.430	0.2220	0.2210	99.5
MIB	71.4	0.572	0.2960	0.2835	95.8
Dry mix, 0.8055 mg. MIB/100 mg.	45.2	0.368	0.1905	0.1850	97.2
Nicotinic acid	101.2	1.068	0.552	0.551	99.8
MIB mix, 1.067 mg. MIB/100 mg.	97.2 99.1	1.037 1.058	0.531 0.541	0.507 0.527	95.6 97.6

for the presence of nitrite was performed on combustion residues. By this method, if nitrite is present in the test solution the diazonium derivative of sulfanilic acid is formed and couples with α-naphthylamine giving a red-colored solution (6). In this manner, nitrite was demonstrated to be present in urea and thyroid powder combustion residues both before and after treatment with bromine, notwithstanding a greatly diminished color intensity in the latter instance. After treatment of the combustion residues with sulfamic acid in the prescribed way, no nitrite could be detected by this method.

The recommended procedure, therefore, included the addition of sulfamate as well as the longer stirring period for the combustion residue. The longer stirring period evidently allowed the flask solution to reach a new chemical equilibrium which favored the disappearance of nitrite.

The Recovery of Iodine from Combustion of MIB.—When alcoholic solutions of MIB were applied to the black paper wrappers and the combustion and iodine measurement carried out in the recommended manner, it was possible to recover only about 90% of the theoretical amount (Table II). The recoveries of iodine under these conditions were observed when the iodine present on the wrapper ranged from 0.1 to 2.5 mg. The fluctuating recovery values suggested that combustion of the wrapper and MIB did not uniformly convert all the iodine to an inorganic form. When the combustion of MIB was carried out in the presence of organic nitrogenous material, as represented by urea and nicotinic acid, there was a distinct increase in recoverable iodine. Recoveries under these conditions ranged from about 96% to the theoretical value. Evidently, the slower burning, which would be expected with the greater bulk of organic material, allowed the iodine to reach an inorganic state before burning ceased.

Iodine Determination in Thyroid Powders.—Four different thyroid samples were analyzed for their iodine content by the (*a*) U.S.P., (*b*) B.P. (3) or Vickers-Johnson, and (*c*) B.P. modified procedures. The results are shown in Table III. The B.P. modified procedure included the 15-min. stirring period and the addition of sulfamic acid. The U.S.P. procedure (7) employs a carbonate ashing step at 675 to 700°, bromine treatment to convert all iodine to iodate, subsequent acidification with phosphoric acid, and expulsion of excess bromine by boiling. Traces of remaining bromine are organically bound by addition of a phenol solution, and the iodine is measured by a thiosulfate titration. With the proposed modifications in the oxygen flask procedure, the observed iodine levels are indistinguishable from those given by the U. S. P. method. These results indicate that, under the prescribed conditions, oxygen flask combustion of thyroid combined with bromine treatment of the residue, converts the organically bound iodine to iodate, and retains it in that form at least as efficiently as the U.S.P. method. Iodate is the required chemical state of iodine before addition of excess iodine and thiosulfate titration.

The B.P. method gave as much as 15% higher values than those obtained by the authors' modified

TABLE III.—A COMPARISON OF RESULTS OF IODINE ANALYSIS ON THYROID POWDERS AS DETERMINED BY THE U.S.P. AND OXYGEN FLASK PROCEDURES[a]

Thyroid Sample	U. S. P. XVII Procedure	Oxygen Flask: B. P.	Oxygen Flask: B. P. Modified
1	0.970	1.14	1.01
	0.977	1.16	1.02
	0.960	1.17	1.01
		1.08	1.00
2	0.730	0.864	0.740
	0.748	0.849	0.714
		0.812	0.718
		0.796	0.728
3	0.196	0.220	0.206
	0.206	0.226	0.207
	0.203	0.234	0.206
		0.240	0.209
4	0.516	0.632	0.549
	0.543	0.678	0.545
	0.560	0.644	0.543
		0.583	0.555

[a] All concentrations are expressed as per cent by weight iodine.

method. The difference, it is contended, is due to the spurious oxidation of iodide by nitrite. Furthermore, the noticeably greater variation between individual determinations may possibly be due to the recurring end point which is a characteristic of the B.P. method.

Recovery of Iodide from Thyroid Combustion Residues.—Iodine was added as potassium iodide to a thyroid powder combustion residue immediately after the 15-min. stirring period, and the iodine determination was carried out in the usual manner.

TABLE IV.—THE RECOVERY OF IODINE ADDED TO A THYROID POWDER COMBUSTION RESIDUE

Thyroid (0.548% I), mg.[a]	Iodine, mg.: From Thyroid	Added as KI	Recovered	% Recovery
50.8	0.278	0.200	0.476	99.5
48.7	0.266	0.200	0.466	100.0

[a] This iodine concentration is the average of the 4 determinations listed for sample *4* in Table III.

Table IV demonstrates that the recovery of the added iodine was quantitative, and, therefore, it appears that no combustion product of thyroid affected the titration conditions.

SUMMARY AND CONCLUSIONS

The British Pharmacopoeia method for the determination of iodine in thyroid was modified by the adoption of a 15-min. stirring period for the combustion residue and later addition of sulfamic acid to remove nitrites. With these modifications, the iodine levels obtained in thyroid powder analyses were comparable to those obtained by the U.S.P. procedure.

These measurements were performed on amounts of thyroid containing between 1.5×10^{-6} and 6×10^{-6} Gm. equivalent iodine. Tomlinson (8) has drawn attention to the error in halogen determination at this concentration due to high blank values caused by the wrapping material. It was demonstrated that the wrappers employed here do not give a blank value. He also pointed out that combustion may be incomplete by the very nature of the combustion operation. This was not in evidence in this investigation where sample size was restricted to 100 mg. or less and where the iodine constituted between 0.2 and 1.0% of the total organic substance burned. However, when amounts of MIB containing comparable quantities of iodine were burned with the wrappers only (120 mg.), the iodine could not be quantitatively recovered. The combination in thyroid powders of organic material and iodine in what appears to be fortuitous proportions, makes the oxygen flask method for iodine determination in thyroid most attractive. This procedure can be completed in 30 min. and there is no transfer of solutions.

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Synthesis of Certain 5-Nitropyridine Derivatives Structurally Related to Some Chemotherapeutic Agents

By K. M. GHONEIM, M. KHALIFA, and Y. M. ABOU-ZEID

The condensation of 2-chloro-5-nitropyridine with 1-aminohydantoin, sulfaguanidine, and semicarbazide is described. With sulfaguanidine the N^4 derivative was always obtained even under the conditions supposed to afford the N^1 derivative. With semicarbazide the condensation in all cases yielded a disubstitution product even when equimolecular amounts of the reactants were used. With aminohydantoin the condensation was effected in 1 per cent hydrochloric acid while in 10 per cent acid no condensation took place, and the haloheterocycle was hydrolyzed to the corresponding hydroxy compound. On the other hand, attempts to condense the halonitropyridine with 3-amino-2-oxazolidone were unsuccessful.

In recent years, heterocycles with a nitro group in position 5- gained success as antimicrobials, *e.g.*, nitrofurans (1), and as amebicides, *e.g.*, 2-diethanolamino-5-nitropyridine (2, 3). As a consequence the authors decided to synthesize compounds having the 5-nitropyridyl radical together with different side chains in position 2- in the hope that the new products would show superior amebicidal action[1] with lower toxicity. For the synthesis of such compounds 2-chloro-5-nitropyridine was condensed with certain amino derivatives.

2-Chloro-5-nitropyridine was prepared from 2-aminopyridine by a procedure derived from the method of Phillips (4), and that of Caldwell (5). The aminopyridine was first nitrated to the corresponding nitraminopyridine which was then rearranged with concentrated sulfuric acid to 2-amino-5-nitropyridine and its isomer the 3-nitro derivative, with the former predominating. The crude 5-nitro compound was then converted to the hydroxy derivative by treatment with sodium nitrite in presence of dilute sulfuric acid (4, 5). The chloronitropyridine was finally obtained from the hydroxy compound by reacting with phosphorus pentachloride in the presence of a small amount of the oxychloride.

Phillips reported that when sulfanilamide was condensed with 2-chloro-5-nitropyridine according to (*a*) Bobranski's (6) and (*b*) Ullmann's conditions (7), the reaction afforded the N^4 derivative in the former case and a mixture of the N^4 and N^1 derivatives in the latter case. However, when the sulfaguanidine was condensed with 2-chloro-5-nitropyridine adopting the two procedures mentioned above, the authors obtained the same product in both cases. This was shown qualitatively to be the N^4 derivative since it failed to diazotize and this conclusion was confirmed by ultraviolet analysis.[2] The absorption spectrum of the condensation product in methanol shows 2 maxima at 263.5 mμ (log ϵ 4.28), and 368 mμ (log ϵ 4.44). The former band is attributed to absorption of sulfanilamido chromophore ($\lambda_{max.}$ 262 mμ; log ϵ 4.25, in ethanol) (8), while the latter may be ascribed to the absorption of the *N*-substituted 2-amino-5-nitropyridine. The absorption in methanolic 2 *N* hydrochloric acid shows 2 maxima at 266.5 mμ (log ϵ 4.01) and 364 mμ (log ϵ 4.30). The hypsochromic shift and the hypochromic effect are due to salt formation. Such a phenomenon is observed in all aromatic amines (*cf.* the absorption spectra of *p*-nitroaniline in ethanol and in HCl). The absorption spectrum in 1 *N* sodium hydroxide shows 3 bands: 337.5 mμ (log ϵ 4.18), 295 mμ (log ϵ 3.92), and 465 mμ (log ϵ 4.46). The latter band which is indicative of a highly conjugated system may be ascribed to the aci-form I of the N^4 derivative which is expected to be present in the alkaline medium (I).

The strong bathochromic shift observed in alkaline solution indicates that the condensation product is the N^4 derivative and not the N^1, since the latter is expected to be insoluble in alkali and possesses identical spectra both in alkaline and neutral solutions.

Condensation of 2-chloro-5-nitropyridine with 1-aminohydantoin in dry pyridine, according to a method reported by Whitmore *et al.* (9), resulted in decomposition of the aminohydantoin with liberation of ammonia (confirmed qualitatively) which caused ammonolysis of the halonitropyridine yielding 2-amino-5-nitropyridine. The same happened when 1-aminohydantoin was replaced with 3-amino-2-oxazolidone. However, when the condensation of the aminohydantoin and the aminooxazolidone with 2-chloro-5-nitropyridine was conducted in aqueous hydrochloric acid in the presence of ethanol following Banks' directions (10), in 10% acid, the haloheterocycle was hydrolyzed to the corresponding hydroxy compound while with 1% acid the condensation was successful with the former amine and with the latter the starting materials were recovered unaffected. Furthermore, in the case of aminooxazolidone, no success attended Mangini's method (11–13), which consists of heating the reactants under reflux with absolute alcohol in presence of fused sodium acetate.

2-Chloro-5-nitropyridine was condensed with semicarbazide by refluxing together equimolecular quantities of the 2 reactants in the presence of 1% hydrochloric acid and ethanol. From the reaction mixture a high melting substance was isolated. It was obvious from the microanalytical data that 2 molecules of the halonitropyridine had condensed with 1 molecule of semicarbazide. Theoretically, 5 structures are possible for such a condensation product (II).

Structures II*a* and II*b* were excluded by the fact that no ammonia evolved when the condensation

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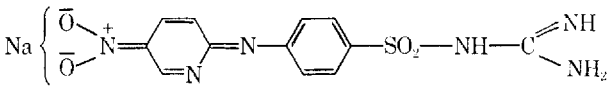
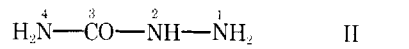
[1] The products at present are under preliminary screening for possible amebicidal action or any other useful pharmacological activity.

[2] The ultraviolet and infrared spectra were run on Spectracord model 4000 A and Infracord model 137 spectrophotometers, respectively.

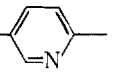


I

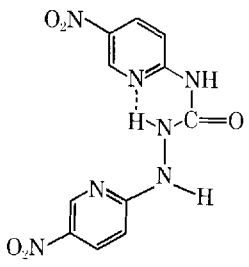


II

IIa = R in 1,1; IIb = R in 1,2; IIc = R in 4,4

IId = R in 2,4; IIe = R in 1,4; R = O_2N- 

product was heated with sodium hydroxide. Obviously, the reasons which prevented the formation of a compound having the structure IIa and IIb would also prevent the formation of IIc or IId. These reasons are probably steric effects. On the other hand, the infrared analysis[2] favored structure IIe and showed that it did exist in the chelated form (III).



III

The observed frequency of about 2985–3080 cm.$^{-1}$ (broad) may be ascribed to the chelated NH (*Reference 14*, p.195), while the sharp band at 3400 cm.$^{-1}$ may be assigned to the NH stretching frequency of the secondary amide (*Reference 14*, p. 176). Furthermore, the carbonyl stretching frequency of 5-nitro-2-acetylaminopyridine was found to occur at 1700 cm.$^{-1}$. Accordingly, the frequency of 1724 cm.$^{-1}$ may be attributed to the amide I band. Finally, the bands at 1997 and 1550 cm.$^{-1}$ may be assigned to the C=N (*Reference 14*, p. 226) and NO_2 (*Reference 14*, p. 250) stretch vibrations, respectively.

EXPERIMENTAL[3]

5-Nitro-2-acetylaminopyridine.—This compound was prepared from 5-nitro-2-aminopyridine by acetylation with acetic anhydride. The product was crystallized from benzene and it melted at 196° as reported (15).

p - (5 - Nitro - 2 - pyridylamino)benzenesulfonylguanidine.—*Method A.*—A mixture of 2-chloro-5-nitropyridine (3.16 Gm.) and sulfaguanidine (4.64 Gm.) was fused at 130–150° for 20 min. (Bobranski's conditions). The solid cake resulting was then extracted with boiling 2 *N* sodium hydroxide solution. The deep red extract was acidified with dilute hydrochloric acid and buffered by the addition of excess saturated solution of sodium acetate. The product which was obtained in 52% yield (3.3 Gm.) melted at 265–266° after 1 crystallization from ethanol.

Anal.[4]—Calcd. for $C_{12}H_{12}N_6O_4S$: C, 42.85; H, 3.57; N, 25.00; S, 9.52. Found: C, 42.90; H, 3.70; N, 24.30; S, 9.80.

Method B.—The above experiment was repeated but in the presence of anhydrous potassium carbonate (2.7 Gm.) and copper powder (0.2 Gm.) and the fusion conducted at 100–130° for 1 hr. (Ullmann's conditions). The solid cake thus obtained was then extracted with hot water, and the aqueous extract was acidified with 2 *N* acetic acid. The product after being crystallized from ethanol melted at 265–266°, and the melting point was not depressed on admixture with a pure specimen prepared by *Method A*.

5′ - Nitro - 2′ - pyridyl - 1 - aminohydantoin and N^1, N^4-Di(5′-nitro-2′-pyridyl) Semicarbazide.—These were prepared by the following general method.

A mixture of equimolecular amounts of 2-chloro-5-nitropyridine and the amino compound[5] (1-aminohydantoin in the former and semicarbazide in the latter) was refluxed with hydrochloric acid solution (1 ml. concentrated HCl, d 1.19, in 80 ml. of water and 20 ml. of ethanol) for 30–32 hr. The reaction mixture was then concentrated under diminished pressure to about 15–20 ml. when the condensation product separated out. The yield in both cases was 25% and the product was crystallized from ethanol.

The former compound melted at 247–249°.

Anal.—Calcd. for $C_8H_7N_5O_4$: C, 40.50; H, 2.95; N, 29.50. Found: C, 40.68; H, 3.05; N, 29.37.

The latter compound did not melt until 350°.

Anal.—Calcd. for $C_{11}H_9N_7O_5$: C, 41.00; H, 2.80; N, 30.70. Found: C, 41.13; H, 2.89; N, 31.02.

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[3] Melting points are uncorrected.

[4] Analyses were performed by Alfred Bernhardt, Germany.

[5] In the case of semicarbazide the 25% yield was obtained when 1 mole was condensed with 2 moles of the haloheterocycle, while with equimolecular amounts poorer yields were obtained.

Synthesis of Methylglyoxal-^{14}C

By VICTOR C. BRUM

Methylglyoxal-1,3-^{14}C and methylglyoxal-2-^{14}C of relatively high specific activity were prepared by the oxidation of acetone with selenium dioxide followed by distillation under a nitrogen atmosphere and concentration under reduced pressure. Analysis of the synthesized compounds by melting point determination and also by paper and gas-liquid chromatography indicated the samples were of high purity.

THE OCCURRENCE of methylglyoxal in the form of an aberrant metabolite in vitamin B_1 deficiency has held the interest of many investigators (1–8) in the field of nutrition and carbohydrate metabolism. Its existence in the aminoacetone cycle (9), its accumulation in dystrophic muscle (10), and its possible role as a carcinostatic agent (11) have resulted in a revived interest in this compound.

In order to study the metabolic pathway and fate of this compound in animals, radioactive methylglyoxal-1,3-^{14}C and methylglyoxal-2-^{14}C were prepared.

Three methods were considered for the preparation of the labeled compound. (*a*) The conversion of acetone to isonitroacetone and warming the latter with dilute sulfuric acid (12). (*b*) The distillation of dihydroxyacetone in a dilute aqueous solution from calcium carbonate or by *in vacuo* distillation from phosphorus pentoxide (13). (*c*) The oxidation of acetone with selenium dioxide followed by distillation under a nitrogen atmosphere and concentration under reduced pressure (14, 15).

Both methylglyoxal-2-^{14}C ($CH_3{}^{14}COCHO$) and methylglyoxal-1,3-^{14}C ($^{14}CH_3CO{}^{14}CHO$) may be obtained by method (*a*) utilizing ^{14}C-labeled acetone as a precursor. The preparation by method (*b*) requires ^{14}C-labeled dihydroxyacetone which can be prepared enzymatically utilizing ^{14}C-labeled glycerol as a precursor. Both methods (*a*) and (*b*) result in the formation of the following contaminants which are difficult to separate from methylglyoxal: glycols, formaldehyde, and formic acid.

These disadvantages do not exist for method (*c*) which was, therefore, selected for the synthesis of methylglyoxal-^{14}C.

Methylglyoxal-1,3-^{14}C was prepared from 0.5 mc. of acetone-1,3-^{14}C with a specific activity of 9.1 mc./mmole.[1] Methylglyoxal-2-^{14}C was prepared from 0.5 mc. of acetone-2-^{14}C with a specific activity of 5.4 mc./mmole.[1]

METHOD

Procedure for Refluxing.—To a 125-ml. conical flask containing 16.0 ml. (0.19 mole) of *p*-dioxane and 1.1 ml. of water was added 3.04 Gm. (0.027 mole) of purified selenium dioxide. The mixture was slowly brought up to 50° and maintained at this temperature while being constantly stirred by a hot plate magnetic stirrer. The selenium dioxide was completely dissolved in 20 min. The solution was cooled to 25° and transferred to a 50-ml. pear-shaped distillation flask. To this solution was added 2.0 ml. of the radioactive acetone and carrier. A water-cooled upright Leibig condenser was attached to the flask and the mixture was allowed to reflux gently for 4 hr. in an oil bath maintained at 100°. At the completion of the reflux period the solution was dark and tarry in appearance.

Procedure for Distillation.—On completion of the refluxing the distillation flask was removed and attached to a distillation assembly. A capillary was introduced into the distillation flask and served as an inlet tube for the admission of nitrogen gas at a pressure slightly above atmospheric. During the entire distillation process nitrogen gas was allowed to slowly bubble in to prevent the oxidation of methylglyoxal. The mixture was distilled over an oil bath by slowly raising the temperature to 130°. Three separate distillate fractions were collected in an ice bath.

Fraction I consisted of 5 ml. of a clear distillate collected at a vapor temperature of 80 to 90°. It was found to contain less than 5% methylglyoxal as determined by the *m*-nitrobenzhydrazide reaction.

Fraction II consisted of 8 ml. of a pale yellow distillate collected at a vapor temperature of 90 to 100° and contained a 15% solution of methylglyoxal in water and dioxane.

Fraction III consisted of 5 ml. of a distillate collected at a vapor temperature of 100 to 105° and contained a mixture of dioxane, water, oily droplets, and an unstable selenium methylglyoxal complex.

By collecting three fractions it was found that the highest yield came over in fraction II at a vapor temperature of 90 to 100°. Fractions I and III which distilled over at lower and higher temperatures, respectively, produced lower yields and indicated that the ideal temperature range for maximum yield was that of fraction II.

Procedure for Concentration.—The distillate fractions were transferred to a 50-ml. pear-shaped flask and attached to a vacuum system containing two 29 × 200 mm. culture tubes connected in series and immersed in Dewar flasks containing liquid nitrogen. These tubes served as traps for the lower boiling solvents.

At the end of 6 hr. of vacuum distillation at 50 mm. of mercury, water and dioxane were removed and 2 ml. of a solution pale yellow in color and syrupy in consistency remained.

Quantitative Determination of Methylglyoxal-^{14}C as the m-Nitrobenzoylosazone Derivative.—The methylglyoxal-1,3-^{14}C and methylglyoxal-2-^{14}C yields and specific activities were determined by diluting 25.0 μl. of the concentrate in 100 ml. of water and then adding 10 ml. of *m*-nitrobenzhydrazide reagent (16). The precipitate formed was quantitatively transferred to preweighed stainless steel planchets, dried to constant weight, and the radioactive samples counted using a proportional flow counter with a laboratory scaler. A 45.6% yield

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[1] Both ^{14}C labeled acetone precursors were obtained from Nuclear-Chicago Corp., Des Plaines, Ill.

of methylglyoxal-1,3-^{14}C with a specific activity of 9.0 mc./mmole and a 16.6% yield of methylglyoxal-2-^{14}C with a specific activity of 5.0 mc./mmole was obtained.

The purity of the radioactive compounds was assayed by melting point determinations, paper, and gas-liquid chromatography.

The melting point of the 2,4-dinitrophenylhydrazine derivatives of the ^{14}C-labeled methylglyoxal determined on a Fisher-Johns apparatus was found to be 297–298°, uncorrected, in agreement with the theoretical value (17).

Analysis by paper chromatography (18, 19) was carried out with the following modifications. To a solution containing 50.0 mcg. of methylglyoxal-^{14}C was added 0.2 ml. of a 2,4-dinitrophenylhydrazine reagent (0.1% in 2 *N* hydrochloride). After the mixture was allowed to stand for 1 hr., 1 ml. of chloroform was added. The hydrazone formed was extracted by vigorous shaking followed by a 5-min. period of centrifugation at 2000 r.p.m. to separate the layers.

A 100.0 μl. aliquot of the chloroform layer was spotted on Whatman No. 1 paper. The ascending technique was employed and the development solvent used was *n*-butanol–ethanol–aqueous 2 *N* ammonium hydroxide (7:1:2, by volume). After 16 hr., the paper was dried at room temperature in a fume hood and sprayed with alcoholic sodium hydroxide solution (2% sodium hydroxide in 90% ethanol).

The colored spots were identified by comparison with the color and R_f values of a known standard run simultaneously.

Two isomers of methylglyoxal were observed: a blue-violet spot with an R_f value of 0.00 and a pinkish-brown spot with an R_f value of 0.90. Values were in agreement with those reported in the literature (19).

The colored spots identified as methylglyoxal were cut from the paper, macerated 3 times with 0.5-ml. aliquots of 1 *N* ammonium hydroxide, and each quantity was centrifuged. The supernatants of the washings were pooled, transferred to a planchet, evaporated to dryness, and counted. All samples prepared in this manner and identified as methylglyoxal-^{14}C were found to be highly radioactive.

The samples of ^{14}C-labeled methylglyoxal and standards were successfully analyzed by gas-liquid chromatography. The purpose of analyzing the radiolabeled methylglyoxal-^{14}C by gas chromatography was to determine its purity and not its radioactivity since the radioactivity of the compound had previously been established by both paper chromatography and as the *m*-nitrobenzoylosazone of methylglyoxal-^{14}C. The instrument used was a Perkin-Elmer gas chromatograph model 801 with a 6 ft. × 0.085 in. i. d.

The stationary phase used was SE 30/EPON 1001 with a gas-chrom P as the support (ratio of 4/0.2%). Helium gas was used as the carrier gas and introduced at a flow rate of 40 ml./min. The instrument utilized a flame detector and the analysis was carried out using hydrogen gas at 18 psig and air at 46 psig. The temperature of the injection block was 280°, detector 220°, and the initial temperature of the column 200°. A Leeds and Northrop model G (5 MV range) was used and the data recorded at a chart speed of 0.5 in./min.

One major peak was recorded with another following 2.0 cm. behind which represented the other isomer. There was no decomposition, little tailing, and the purity of the methylglyoxal-^{14}C appeared satisfactory from a quantitative as well as qualitative point of view.

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Separation and Analysis of Degradation Products of Tetracycline by Gel Filtration on Sephadex G-25

By B. W. GRIFFITHS

The anhydrotetracycline and 4-epi-anhydrotetracycline degradation products of tetracycline have been found to separate from tetracycline on a column of Sephadex G-25. The tetracycline elutes as the "fast" component, whereas the degradation products which are not separated from each other elute as the "slow" component. The ease of column preparation, rapidity of analysis, and repetitive use of a single column make the technique a useful screening procedure for gross contamination of tetracycline preparations with anhydro- and 4-epi-anhydrotetracycline degradation products.

In view of recent findings that relate a reversible Fanconi-type syndrome to the ingestion of degraded tetracycline capsules (1–5), interest has developed in the analytical determination of products of degradation of tetracycline (TC). Particular interest has centered on the 4-epi-anhydrotetracycline derivative, since a similar syndrome to the above was induced in rats and dogs on the ingestion of large doses of this compound (6).

A column chromatographic method has been developed by Kelly (7) for the determination of both anhydrotetracycline (ATC) and 4-epi-anhydrotetracycline (EATC) in the presence of large quantities of TC. In the author's experience the method was found workable and reproducible but had the disadvantage of tedious column preparation and the limitation of a single analysis per column.

During a search for an alternative method, it was found that a partially degraded TC compound separated into 2 colored components on a column of Sephadex G-25. The "fast" component was found to have the U.V. characteristics of TC while the "slow" one had those of ATC or EATC. The potential of this observation for use in analysis was studied and a test was developed for the gross analysis of ATC and EATC in pharmaceutical preparations of TC. Although the method is not expected to be a substitute for one providing a separate analysis for ATC and the epimer, the features of ease of column preparation, rapidity of analysis, and repetitive use of the same column make it a technique highly useful for the routine screening of TC preparations for gross ATC and EATC content.

EXPERIMENTAL

Reagents and Materials

Compounds of Tetracycline and Derivatives.—Commercial tetracycline HCl (TC) of a high degree of purity by microbiological assay was used. Anhydrotetracycline (ATC) and 4-epi-anhydrotetracycline (EATC) were obtained from Lederle Laboratories.

Sephadex Columns.—*Acid Column.*—A pledget of glass wool was placed in the outlet of a glass column (1.8 × 35 cm.) over which were placed small glass beads to a depth of 1 cm. The Sephadex G-25 (medium) previously swollen in 0.03 *N* HCl was applied as a slurry to the closed column. At this normality the epimerization rate of tetracycline is small and acid-catalyzed dehydration to anhydrotetracycline does not occur (8). After about 5 min. the outlet was opened and more slurry added until a bed length of 23 cm. was attained. Flow rates of 180–200 ml./hr. were obtained. An overhead volume of about 400 ml. provided continuous delivery of solvent. Fractions were collected usually in 5-ml. portions with the aid of an LKB Radi-Rac fraction collector.

Alkaline Column.—The Sephadex was equilibrated with 0.04 *M* phosphate buffer, pH 7.7–7.8. The column preparation followed the above outline with the exception that the length of the bed was 30.5 cm.

Method for Tetracyline Oral Preparations

The powdered tablet or capsule material is accurately weighed and dissolved in 0.03 *N* HCl to give 20 mg. of TC/ml. The insoluble residue is centrifuged and 0.5 ml. of the supernatant is applied to the acid column and washed in gently with the HCl solvent. The TC passes rapidly through the column while the ATC and EATC elute as a slower single component which is visible as a diffuse, bright yellow band. This band, which is eluted after approximately 115 ml. of solvent has passed over the column, is detected by U.V. analysis at 273 mμ. The routine collection of samples is implemented at a slightly earlier stage in the elution, and the occasional check of samples at 273 mμ indicates the complete elution of the band. The contents of the tubes are transferred to a volumetric flask (100 or 250-ml.) and corrected to volume with the dilute HCl. The absorption is read at 273 mμ, and the content of degradation product is expressed as ATC. The absorptivity (defined as absorbance/mcg. ATC/ml., 1-cm. light path) of ATC at wavelength maximum of 273 mμ was found to be 1.01×10^{-1}. The ATC obeyed Beer's law over the range of 1.0–10 mcg./ml.

The oral suspensions and syrups are diluted with a volume of solvent to give an estimated tetracycline concentration of 10 mg./ml. A volume of 0.5 ml. of the solution (centrifugation is necessary for clarification of the suspension) is transferred to the column and eluted with either the acid or phosphate buffer solvent. When the analysis is carried out with the latter solvent the absorption of the pooled tube contents from the second U.V. peak is read at wavelength maximum of 269.5 mμ. The ATC absorptivity at this wavelength was found to be 8.2×10^{-2}. The absorptivities for EATC at the wavelength maxima of 273 and 269.5 mμ were slightly lower than those for ATC 9.6×10^{-2} and 7.1×10^{-2}, respectively.

RESULTS AND DISCUSSION

In an initial experiment it was observed that a partly degraded commercial tetracycline preparation separated into 2 colored components on Sephadex

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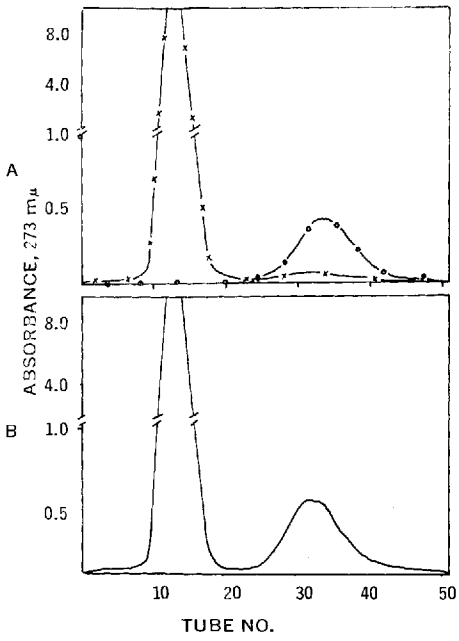

Fig. 1.—*A*. The elution diagram of tetracycline HCl and anhydrotetracycline on Sephadex G-25. Key: ×, tetracycline HCl, applied 1.0 ml. of 5 mg./ml.; O, anhydrotetracycline, applied 1.0 ml. of 200 mcg./ml. Sephadex column, 1.8 × 23 cm. Elution solvent, 0.03 *N* HCl. *B*. The elution diagram of 5 mg./ml. tetracycline HCl containing 200 mcg./ml. anhydrotetracycline: 1.0 ml. solution added to column; solvent and column conditions as above; collected 5-ml. samples.

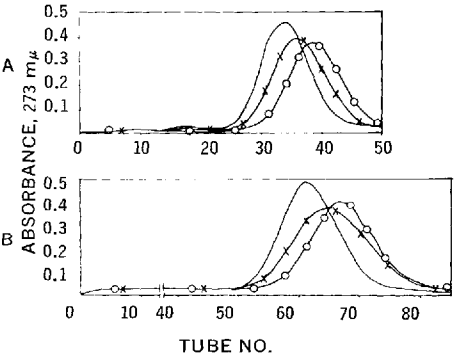

Fig. 2.—Key: ——, the elution diagrams of anhydrotetracycline; O, 4-epi-anhydrotetracycline; ×, an equal mixture of the 2 compounds. All samples were added in 1.0-ml. vol.; ATC = 200 mcg., EATC + ATC = 100 mcg. each and, EATC = 200 mcg., the elution solvent was 0.03 *N* HCl; the Sephadex column in *A* was 1.8 × 23 cm.; the Sephadex column in *B* was 1.8 × 45 cm.; collected 5-ml. samples.

G-25. The first elution band had the U.V. characteristics of tetracycline while the second had those of anhydrotetracycline and/or 4-epi-anhydrotetracycline.

Confirmation of this observation was made by the application of pure products of TC, ATC, and EATC to Sephadex G-25 (Figs. 1 and 2). In Fig. 1, *A*, TC and ATC, when applied separately to the same acid column, were found to elute as 2 distant peaks. It may be seen that the TC contained a small percentage of ATC as evidenced by the significant absorption in the region of the ATC elution. The TC showed full recovery of biological activity by microbiological plate assay. Figure 1, *B*, shows the identical elution pattern for a mixture of TC and ATC.

Figure 2, *A*, outlines the elution diagrams of ATC, EATC, and an equal mixture of the latter from the same acid column of Sephadex. It is of interest that the ATC and EATC exhibited slightly different elution rates when they were applied separately to the column, whereas an admixture eluted as a single band with an elution rate which was an apparent mean of that for the separate components. Figure 2, *B*, shows a similar effect on a longer column of Sephadex.

The time required for the elution of the TC degradation products from either the acid or alkaline columns was about 1 hr., and continuous sampling allowed for the analysis of several TC preparations in 1 day on a single column. In 2 experiments in which 200 mcg. of ATC was applied to a column, the recoveries were 92.5 and 97%. The recovery of EATC under the same conditions was 97.1%. No significant changes in the absorptivities were found for ATC and EATC in either 0.03 *N* HCl or 0.04 *M* phosphate buffer for periods of up to 4 hr.

During the analysis of certain syrups of TC containing amaranth dye on acid columns, it was found that the dye contaminated the ATC band to produce false high results. An examination of several solvents of varied pH revealed that the amaranth was retarded at pH 7.7 so as to completely separate from the "faster" ATC band. The slight overlap of the

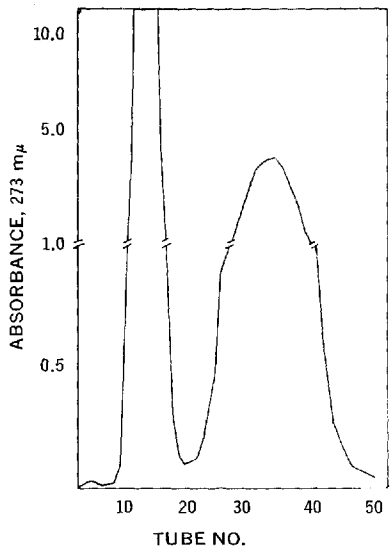

Fig. 3.—The gel filtration of tetracycline syrup on Sephadex G-25. Sephadex column, 1.8 × 23 cm.; elution solvent, 0.03 *N* HCl; 0.5 ml. of syrup (25 mg./ml. tetracycline HCl) added to column; collected 5-ml. samples.

TC band with the ATC band under these conditions required that the column be lengthened for their adequate separation.

Although the main effect of Sephadex is to separate molecules with respect to size, some secondary effects have been noted. Porath (9) and Gelotte (10) have found that heterocyclic and aromatic compounds interact with the Sephadex bed material which results in their delayed elution. The differences in elution rates between TC and ATC (as well as ATC and EATC) are presumed to be due to a similar adsorption phenomenon.

Of a group of about 50 tetracycline preparations analyzed on Sephadex, only about 7 of these contained degradation products in excess of 1% of the labeled amount of tetracycline. This figure of 1% represents the arbitrary amount (tentative) above which the samples were analyzed for both ATC and EATC by the partition chromatography method (7). Figure 3 shows the elution diagram of a tetracycline syrup containing an unusually high quantity of degradation products. The amount expressed as ATC was found to be 4.35 mg./ml. of syrup. The analysis of the same product by the partition chromatography method gave a gross value of 4.10 mg./ml. syrup, most of which was in the form of EATC. In general, the analysis of the TC degradation products by Sephadex analysis has been in close agreement with that by partition chromatography when appreciable quantities were present.

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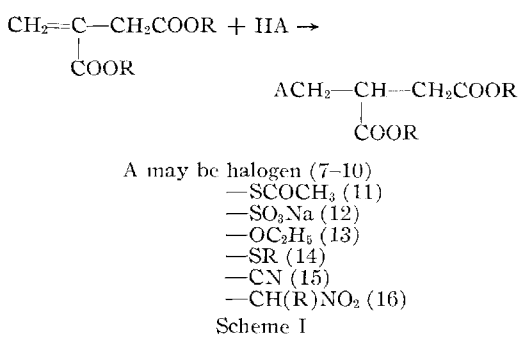
Synthesis of 1,2-Diethyl-4-(2-hydroxyethyl)pyrazolidine

By MILTON J. KORNET

The reaction of 1,2-diethylhydrazine with monomethyl itaconate has been investigated and found to give a mixture of 1,2-diethyl-4-carbomethoxymethyl-3-pyrazolidinone and its hydrolysis product, the corresponding acid. Reduction of either the ester or the acid affords 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine.

THE AUTHOR'S interest in compounds which contain both an alcoholic hydroxyl group and an alkylated hydrazine group as necessary intermediates in the synthesis of new medicinals prompted the preparation of the title compound. Recently the synthesis of 1,2-diethyl-3-hydroxymethylpyrazolidine, a molecule which embodies the above structural features, was accomplished. Esterification of the latter alcohol with several aromatic acids afforded esters whose hydrochloride salts exhibited local anesthetic activity (1). As the first step in the synthesis of 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine, it was decided to investigate the reaction of 1,2-diethylhydrazine with an itaconic acid derivative.

A large number of reagents have been added to itaconic acid and its esters (2–16). Unsymmetrical reagents add contrary to Markovnikoff's rule and the addition may be represented by Scheme I.



Scheme I

Itaconic acid reacts with primary amines to give 1-substituted-4-carboxy-2-pyrrolidinones (17). The latter are formed by the addition of the amine to the β-carbon of the double bond followed by ring closure to the 5-membered ring with the elimination of a molecule of water.

In the reaction of 1,2-diethylhydrazine, with an itaconic acid derivative, ring closure to either a 5- or 6-membered ring is possible. Because of this possibility monomethyl itaconate was utilized. Separation of products could be more easily achieved since a mixture would consist of a 5-membered ring methyl ester and a 6-membered ring carboxylic acid. Treatment of 1,2-diethylhydrazine (I) with monomethyl itaconate (II) afforded a mixture of 43.9% of 1,2-diethyl-4-carbomethoxymethyl-3-pyrazolidinone (IV) and 44.6% of its hydrolysis product, 1,2-diethyl-4-carboxymethyl-3-pyrazolidinone (IV*a*). Both compounds gave crystalline picrate derivatives. Compound IV can be visualized as arising from an addition of I to the β-carbon of the double bond of II followed by ring closure *via* the carboxyl group with the elimination of water. The acid (IV*a*) undoubtedly arises from the hydrolysis of IV during the course of the reaction. Elemental analyses and infrared spectra are in agreement with the proposed structures for IV and IV*a*. That the isomeric acid, (III) which would arise from the addition of I to the β-carbon of the double bond of II followed by ring closure *via* the ester carbonyl with simultaneous

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TC band with the ATC band under these conditions required that the column be lengthened for their adequate separation.

Although the main effect of Sephadex is to separate molecules with respect to size, some secondary effects have been noted. Porath (9) and Gelotte (10) have found that heterocyclic and aromatic compounds interact with the Sephadex bed material which results in their delayed elution. The differences in elution rates between TC and ATC (as well as ATC and EATC) are presumed to be due to a similar adsorption phenomenon.

Of a group of about 50 tetracycline preparations analyzed on Sephadex, only about 7 of these contained degradation products in excess of 1% of the labeled amount of tetracycline. This figure of 1% represents the arbitrary amount (tentative) above which the samples were analyzed for both ATC and EATC by the partition chromatography method (7). Figure 3 shows the elution diagram of a tetracycline syrup containing an unusually high quantity of degradation products. The amount expressed as ATC was found to be 4.35 mg./ml. of syrup. The analysis of the same product by the partition chromatography method gave a gross value of 4.10 mg./ml. syrup, most of which was in the form of EATC. In general, the analysis of the TC degradation products by Sephadex analysis has been in close agreement with that by partition chromatography when appreciable quantities were present.

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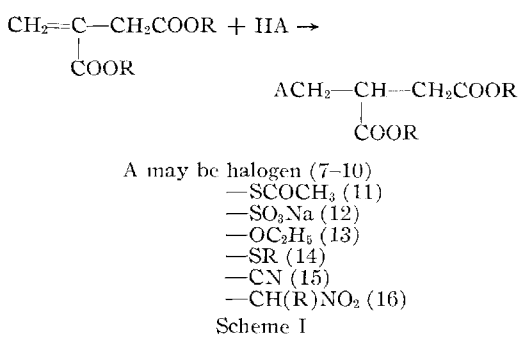
Synthesis of 1,2-Diethyl-4-(2-hydroxyethyl)pyrazolidine

By MILTON J. KORNET

The reaction of 1,2-diethylhydrazine with monomethyl itaconate has been investigated and found to give a mixture of 1,2-diethyl-4-carbomethoxymethyl-3-pyrazolidinone and its hydrolysis product, the corresponding acid. Reduction of either the ester or the acid affords 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine.

THE AUTHOR'S interest in compounds which contain both an alcoholic hydroxyl group and an alkylated hydrazine group as necessary intermediates in the synthesis of new medicinals prompted the preparation of the title compound. Recently the synthesis of 1,2-diethyl-3-hydroxymethylpyrazolidine, a molecule which embodies the above structural features, was accomplished. Esterification of the latter alcohol with several aromatic acids afforded esters whose hydrochloride salts exhibited local anesthetic activity (1). As the first step in the synthesis of 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine, it was decided to investigate the reaction of 1,2-diethylhydrazine with an itaconic acid derivative.

A large number of reagents have been added to itaconic acid and its esters (2–16). Unsymmetrical reagents add contrary to Markovnikoff's rule and the addition may be represented by Scheme I.



Scheme I

Itaconic acid reacts with primary amines to give 1-substituted-4-carboxy-2-pyrrolidinones (17). The latter are formed by the addition of the amine to the β-carbon of the double bond followed by ring closure to the 5-membered ring with the elimination of a molecule of water.

In the reaction of 1,2-diethylhydrazine, with an itaconic acid derivative, ring closure to either a 5- or 6-membered ring is possible. Because of this possibility monomethyl itaconate was utilized. Separation of products could be more easily achieved since a mixture would consist of a 5-membered ring methyl ester and a 6-membered ring carboxylic acid. Treatment of 1,2-diethylhydrazine (I) with monomethyl itaconate (II) afforded a mixture of 43.9% of 1,2-diethyl-4-carbomethoxymethyl-3-pyrazolidinone (IV) and 44.6% of its hydrolysis product, 1,2-diethyl-4-carboxymethyl-3-pyrazolidinone (IV*a*). Both compounds gave crystalline picrate derivatives. Compound IV can be visualized as arising from an addition of I to the β-carbon of the double bond of II followed by ring closure *via* the carboxyl group with the elimination of water. The acid (IV*a*) undoubtedly arises from the hydrolysis of IV during the course of the reaction. Elemental analyses and infrared spectra are in agreement with the proposed structures for IV and IV*a*. That the isomeric acid, (III) which would arise from the addition of I to the β-carbon of the double bond of II followed by ring closure *via* the ester carbonyl with simultaneous

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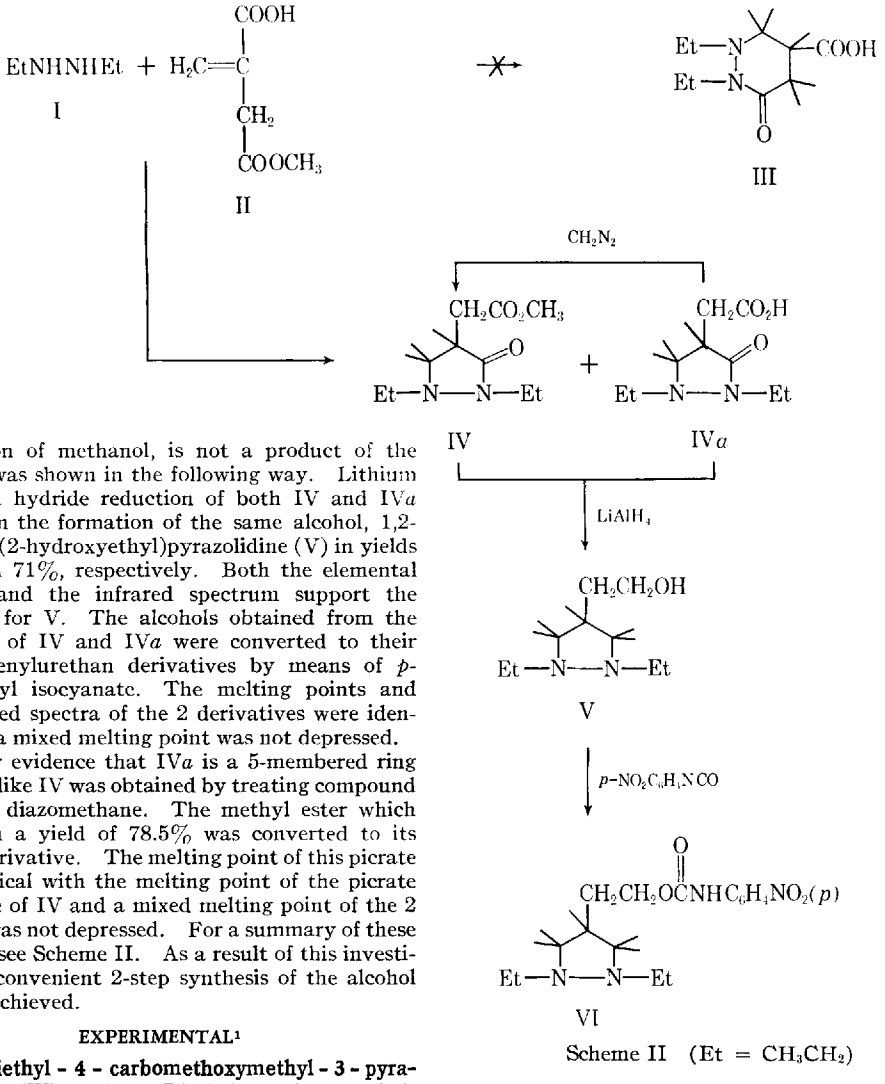

Scheme II (Et = CH_3CH_2)

elimination of methanol, is not a product of the reaction was shown in the following way. Lithium aluminum hydride reduction of both IV and IV*a* resulted in the formation of the same alcohol, 1,2-diethyl-4-(2-hydroxyethyl)pyrazolidine (V) in yields of 70 and 71%, respectively. Both the elemental analysis and the infrared spectrum support the structure for V. The alcohols obtained from the reduction of IV and IV*a* were converted to their *p*-nitrophenylurethan derivatives by means of *p*-nitrophenyl isocyanate. The melting points and the infrared spectra of the 2 derivatives were identical and a mixed melting point was not depressed.

Further evidence that IV*a* is a 5-membered ring structure like IV was obtained by treating compound IV*a* with diazomethane. The methyl ester which formed in a yield of 78.5% was converted to its picrate derivative. The melting point of this picrate was identical with the melting point of the picrate derivative of IV and a mixed melting point of the 2 picrates was not depressed. For a summary of these reactions see Scheme II. As a result of this investigation a convenient 2-step synthesis of the alcohol (V) was achieved.

EXPERIMENTAL[1]

1,2 - Diethyl - 4 - carbomethoxymethyl - 3 - pyrazolidinone (IV) and 1,2-Diethyl-4-carboxymethyl-3-pyrazolidinone (IVa).—To a solution of 9.25 Gm. (0.105 mole) of 1,2-diethylhydrazine (I) (18) in 5 ml. of anhydrous methanol was added dropwise a solution of 14.4 Gm. (0.100 mole) of monomethyl itaconate (II) in 13 ml. of absolute methanol with stirring (magnetic) and ice-bath cooling. The reaction mixture was allowed to stir and come to room temperature overnight and then refluxed for 4.5 hr. The methanol was distilled *in vacuo* on a water bath and the residue was distilled under reduced pressure and gave 9.39 Gm. (43.9%) of the ester (IV), b.p. 90° (0.1 mm.), n_D^{20} 1.4724, $\lambda_{max.}^{CHCl_3}$ 5.8 μ (ester C=O), 6.0 μ (amide C=O), and 8.93 Gm. (44.6%) of the corresponding acid (IV*a*), b.p. 169° (0.18 mm.), n_D^{20} 1.4943, $\lambda_{max.}^{CHCl_3}$ 5.8–6.2 μ (carbonyl of acid and amide), 2.8–4.2 μ (associated OH of acid).

Anal.—Calcd. for $C_{10}H_{18}N_2O_3$ (ester): C, 56.05; H, 8.46; N, 13.08. Found: C, 55.74; H, 8.52; N, 12.90.

Anal.—Calcd. for $C_9H_{16}N_2O_3$ (acid): C, 53.98; H, 8.05; N, 13.99. Found: C, 53.59; H, 8.32; N, 13.90.

The ester was converted into its picrate derivative and recrystallized from absolute ethanol, m.p. 119–120.5°.

Anal.—Calcd. for $C_{16}H_{21}N_5O_{10}$: C, 43.34; H, 4.77; N, 15.80. Found: C, 43.47; H, 4.99; N, 15.91.

The acid was converted into its picrate derivative and recrystallized from absolute ethanol, m.p. 131–132°.

Anal.—Calcd. for $C_{15}H_{19}N_5O_{10}$: C, 41.96; H, 4.46; N, 16.31. Found: C, 41.73; H, 4.86; N, 16.24.

1,2-Diethyl-4-(2-hydroxyethyl)pyrazolidine (V) by Reduction of the Ester (IV).—A solution of 7.57 Gm. (0.0354 mole) of IV in 10 ml. of anhydrous ether was added dropwise to a suspension of 1.97 Gm. (0.052 mole) of lithium aluminum hydride in

[1] Melting points were determined with the Fisher-Johns melting point apparatus and are corrected. Infrared spectra were recorded on a Beckman IR 8 spectrophotometer using sodium chloride optics. Microanalyses were performed by Dr. Kurt Eder, Geneva, Switzerland.

30 ml. of ether with stirring (magnetic). After refluxing overnight the complex was decomposed with 40% KOH, and the salts were extracted with ether. The combined ether layers were dried over $MgSO_4$, and the ether was distilled on a water bath. The residue remaining was distilled and gave 4.28 Gm. (70.3%) of a colorless oil, b.p. 86° (0.33 mm.), n_D^{20} 1.4763, $\lambda_{max.}^{CHCl_3}$ 2.8–3.3 μ (OH).

Anal.—Calcd. for $C_9H_{20}N_2O$: C, 62.75; H, 11.70; N, 16.26. Found: C, 62.75; H, 11.73; N, 16.21.

A *p*-nitrophenylurethan derivative (19) was prepared and recrystallized from carbon tetrachloride, m.p. 115.5–116.5°, $\lambda_{max.}^{KBr}$ 2.94 μ (NH), 5.76 μ (C=O).

Anal.—Calcd. for $C_{16}H_{24}N_4O_4$: C, 57.13; H, 7.19; N, 16.66. Found: C, 57.20; H, 7.00; N, 16.76.

1,2 - Diethyl - 4 - (2 - hydroxyethyl)pyrazolidine (V) by Reduction of the Acid (IVa).—The procedure for the reduction of the acid (IV*a*) was analogous to that used for reduction of the ester (IV). The alcohol (V), b.p. 80° (0.22 mm.), n_D^{20} 1.4780, was obtained in 71.5% yield. It was converted to its *p*-nitrophenylurethan derivative and recrystallized from carbon tetrachloride, m.p. 115.5–116.5°. The identity of this derivative was confirmed by a mixed melting point and comparison of infrared spectra.

Methylation of 1,2-Diethyl-4-carboxymethyl-3-pyrazolidinone.—To a solution of 7.90 Gm. (0.0395 mole) of IV*a* in 25 ml. of ether was added sufficient ethereal diazomethane to give a yellow color which persisted. The solution was dried with $MgSO_4$ and the ether was evaporated on a steam bath. The remaining residue was distilled, b.p. 90° (0.1 mm.), n_D^{20} 1.4728, to give 6.64 Gm. (78.5%) of the ester. A picrate was prepared and recrystallized from absolute ethanol, m.p. 119–120.5°. The identity of this picrate was confirmed by a mixed melting point.

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Books

REVIEWS

Dictionary of Organic Compounds. Fourth revised edition in five volumes, and *First Supplement, 1965.* Oxford University Press, New York, N. Y., 1965. xxx + 3282 pp. 20 × 26.5 cm. Price $280.00.

The Fourth Edition of the "Dictionary of Organic Compounds" has undergone extensive revision from the preceding 3rd edition published in November 1953. The entries, which total 12,000 and represent 7,000 new compounds, are contained in five volumes instead of four.

A major change is the general policy of adopting chemical synonyms as principal entries with appropriate cross-references to popular or trivial names used predominantly in previous editions of the "Dictionary." A second important change is the inclusion of three double bonds for benzenoid rings in place of the simple hexagon to denote aromatic compounds. The implications of this revision are twofold. First, the aromatic compounds are more easily distinguished at first glance, and second a simplification of the structural formulas of saturated ring compounds has been made possible. The latter are now represented by a simple polygon and the compact structure saves considerable space alloted to the structural formula.

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The Uniqueness of Biological Materials. By A. E. NEEDHAM. Pergamon Press, Inc., 122 East 55th Street, New York, N. Y. 10022, 1965. xi + 593 pp. 16 × 23 cm. Price $15.00.

"Is the uniqueness of life inherent in the material of living organisms?" By posing this question the author states one of the reasons for writing the book. The other reason is a practical one. It was prompted by the need to persuade students that the study of the properties of biological materials such as lipids, carbohydrates, proteins, nucleic acids, and other cell components is not only essential but also very interesting.

The evidence as surveyed in the various chapters of the book seems to show rather consistently that the compounds used by living organisms are outstanding if not absolutely unique among the materials available on earth. It is further pointed out that these substances are unique in respects which make them ideal for some particular biological function.

All of the relevant elements and compounds are surveyed systematically. The author concentrates on the significance and interpretation of the properties studied rather than on the properties themselves. Biological rather than any other applications of the properties are stressed. Illustrations by use of figures, tables, and formulas are numerous.

The coverage of the subject matter is comprehensive; the bibliography, while not too extensive, is up-to-date and ample.

The concluding chapter entitled, "The Origin and Evolution of Biological Uniqueness," is very stimulating.

The book is very valuable to not only students of biochemistry, biology, and zoology, but also to students of chemistry. Students of pharmacy will find the text very informative and interesting.

Reviewed by Ernst R. Kirch
College of Pharmacy
University of Illinois
Chicago

The Structure of Lipids by Spectroscopic and X-Ray Techniques. By D. CHAPMAN. John Wiley & Sons, Inc., New York, N. Y., 1965. xii + 323 pp. 16 × 24 cm. Price $10.50.

In the past ten to fifteen years there has been a considerable increase in research in the field of lipids. This intensified interest in this class of compounds has stemmed largely from the growing interest in the causes of lipid diseases such as atherosclerosis.

In this book the author has described a number of modern physicochemical experimental methods that have been used extensively in recent years to study lipid molecules in the solid and liquid states. In each instance a great deal of data are presented and their relationships to intra- and intermolecular structure and interactions are discussed. More than one-half of the book is devoted to X-ray diffraction and infrared spectroscopic studies with particular emphasis on crystal polymorphism. Separation techniques, ultraviolet, nuclear magnetic resonance, electron spin resonance, and mass spectroscopy and their application to lipids are discussed in the remainder of the book. It is shown that the combined applications of these techniques, particularly X-ray, infrared, and NMR, have unraveled a number of difficult problems such as the existence of the multiple melting points of triglycerides and their relationship to X-ray data.

The author has chosen to organize the subject matter from the standpoint of experimental techniques rather than from that of lipid chemistry. As a result, the book is somewhat difficult to read; but, on the other hand, this arrangement has permitted the presentation of a large amount of data.

It is the reviewer's opinion that the book is an outstanding contribution to the field and very useful for researchers in the field.

Reviewed by William I. Higuchi
College of Pharmacy
University of Michigan
Ann Arbor

NOTICES

La Relation Medecin-Malade Au Cours Des Chimiotherapies Psychiatriques. Preface du PROFESSEUR J. DECHAUME. Textes Publies par P. A. LAMBERT. Masson et Cie, Editeurs, Libraires De L'Academie De Medecine, 120 Boulevard Saint-Germain, Paris VIe, France, 1965. 222 pp. 16 × 24 cm. Paperbound.

Antibiotika-Fibel: Antibiotika und Chemotherapie. 2nd rev. ed. By A. M. WALTER and L. HEILMEYER. Georg Thieme Verlag, Postfach 732, Herdweg 63, 7000 Stuttgart 1, Germany, 1965. U. S. and Canadian agent: Intercontinental Medical Book Corp., New York 16, N. Y. 897 pp. 14.8 × 21 cm. Price DM 88.

Plantas Medicinales De Puerto Rico. By E. NÚÑEZ MELÉNDEZ. Universidad de Puerto Rico, Estacion Experimental Agricola, Río Piedras, Puerto Rico, 1964. 245 pp. 15 × 23 cm. Paperbound.

Functions of the Corpus Callosum. Ciba Foundation Study Group. Edited by E. G. ETTLINGER. Little, Brown and Co., Boston, Mass., 1965. xii + 156 pp. 12.5 × 19 cm. Price $3.75.

Advanced Practical Inorganic Chemistry. By D. M. ADAMS and J. B. RAYNOR. John Wiley & Sons, Inc., 605 Third Ave., New York 16, N. Y., 1965. xiv + 182 pp. 15 × 23.5 cm. Price $6.00.

Hashish: Its Chemistry and Pharmacology. Ciba Foundation Study Group. Edited by G. E. W. WOLSTENHOLME, F. I. BIOL, and J. KNIGHT. Little, Brown and Co., Boston, Mass., 1965. 96 pp. 12.5 × 19 cm. Price $2.95.

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The author has chosen to organize the subject matter from the standpoint of experimental techniques rather than from that of lipid chemistry. As a result, the book is somewhat difficult to read; but, on the other hand, this arrangement has permitted the presentation of a large amount of data.

It is the reviewer's opinion that the book is an outstanding contribution to the field and very useful for researchers in the field.

Reviewed by William I. Higuchi
College of Pharmacy
University of Michigan
Ann Arbor

NOTICES

La Relation Medecin-Malade Au Cours Des Chimiotherapies Psychiatriques. Preface du PROFESSEUR J. DECHAUME. Textes Publies par P. A. LAMBERT. Masson et Cie, Editeurs, Libraires De L'Academie De Medecine, 120 Boulevard Saint-Germain, Paris VIe, France, 1965. 222 pp. 16 × 24 cm. Paperbound.

Antibiotika-Fibel: Antibiotika und Chemotherapie. 2nd rev. ed. By A. M. WALTER and L. HEILMEYER. Georg Thieme Verlag, Postfach 732, Herdweg 63, 7000 Stuttgart 1, Germany, 1965. U. S. and Canadian agent: Intercontinental Medical Book Corp., New York 16, N. Y. 897 pp. 14.8 × 21 cm. Price DM 88.

Plantas Medicinales De Puerto Rico. By E. NÚÑEZ MELÉNDEZ. Universidad de Puerto Rico, Estacion Experimental Agricola, Río Piedras, Puerto Rico, 1964. 245 pp. 15 × 23 cm. Paperbound.

Functions of the Corpus Callosum. Ciba Foundation Study Group. Edited by E. G. ETTLINGER. Little, Brown and Co., Boston, Mass., 1965. xii + 156 pp. 12.5 × 19 cm. Price $3.75.

Advanced Practical Inorganic Chemistry. By D. M. ADAMS and J. B. RAYNOR. John Wiley & Sons, Inc., 605 Third Ave., New York 16, N. Y., 1965. xiv + 182 pp. 15 × 23.5 cm. Price $6.00.

Hashish: Its Chemistry and Pharmacology. Ciba Foundation Study Group. Edited by G. E. W. WOLSTENHOLME, F. I. BIOL, and J. KNIGHT. Little, Brown and Co., Boston, Mass., 1965. 96 pp. 12.5 × 19 cm. Price $2.95.

edition do not coincide with current literature values. Admittedly, it would constitute a major undertaking to research and revise the melting points of the principal entries, but such effort would be cognizant of the high quality of standards associated with this "Dictionary."

The Uniqueness of Biological Materials. By A. E. NEEDHAM. Pergamon Press, Inc., 122 East 55th Street, New York, N. Y. 10022, 1965. xi + 593 pp. 16 × 23 cm. Price $15.00.

"Is the uniqueness of life inherent in the material of living organisms?" By posing this question the author states one of the reasons for writing the book. The other reason is a practical one. It was prompted by the need to persuade students that the study of the properties of biological materials such as lipids, carbohydrates, proteins, nucleic acids, and other cell components is not only essential but also very interesting.

The evidence as surveyed in the various chapters of the book seems to show rather consistently that the compounds used by living organisms are outstanding if not absolutely unique among the materials available on earth. It is further pointed out that these substances are unique in respects which make them ideal for some particular biological function.

All of the relevant elements and compounds are surveyed systematically. The author concentrates on the significance and interpretation of the properties studied rather than on the properties themselves. Biological rather than any other applications of the properties are stressed. Illustrations by use of figures, tables, and formulas are numerous.

The coverage of the subject matter is comprehensive; the bibliography, while not too extensive, is up-to-date and ample.

The concluding chapter entitled, "The Origin and Evolution of Biological Uniqueness," is very stimulating.

The book is very valuable to not only students of biochemistry, biology, and zoology, but also to students of chemistry. Students of pharmacy will find the text very informative and interesting.

Reviewed by Ernst R. Kirch
College of Pharmacy
University of Illinois
Chicago

The Structure of Lipids by Spectroscopic and X-Ray Techniques. By D. CHAPMAN. John Wiley & Sons, Inc., New York, N. Y., 1965. xii + 323 pp. 16 × 24 cm. Price $10.50.

In the past ten to fifteen years there has been a considerable increase in research in the field of lipids. This intensified interest in this class of compounds has stemmed largely from the growing interest in the causes of lipid diseases such as atherosclerosis.

In this book the author has described a number of modern physicochemical experimental methods that have been used extensively in recent years to study lipid molecules in the solid and liquid states. In each instance a great deal of data are presented and their relationships to intra- and intermolecular structure and interactions are discussed. More than one-half of the book is devoted to X-ray diffraction and infrared spectroscopic studies with particular emphasis on crystal polymorphism. Separation techniques, ultraviolet, nuclear magnetic resonance, electron spin resonance, and mass spectroscopy and their application to lipids are discussed in the remainder of the book. It is shown that the combined applications of these techniques, particularly X-ray, infrared, and NMR, have unraveled a number of difficult problems such as the existence of the multiple melting points of triglycerides and their relationship to X-ray data.

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Review Article

The Adrenergic Receptor

By RAYMOND P. AHLQUIST

At this moment in time it is appropriate to review briefly the status of the adrenergic receptor.

The nomenclature of α and β receptors introduced by this laboratory in 1948 (1) is now used internationally (27, 37, 48, 70, 77, 105).

A new class of drugs having potential therapeutic value, the β adrenergic blocking agents, has appeared (23, 24, 26, 44, 70, 77, 115, 117, 118, 125).

In the study of receptors the biological approach based on observations of tissue and organ response is yielding to a biochemical approach based on studies of binding, membranes, and enzyme kinetics. Therefore, this review will attempt only to summarize the biological studies of the adrenergic receptor. Some biochemical views will be found in papers by Ariens (12, 13), Belleau (19), Burn (34), Furchgott (64–67), Volle (139), and Bloom (26).

For purposes of this review the adrenergic receptor is defined as the specific molecular site or structure in or on effector cells with which molecules of adrenergic agonists (epinephrine, etc.) react in order to elicit the characteristic response of the cell (67). There is some tendency to call any and all sites of uptake or binding receptors (85). However, the receptor is usually considered to be the site of drug-effector interaction that produces an observable response.

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It is the opinion of this reviewer that the adrenergic receptor is the most important link in the adrenergic neuroeffector transmission chain. Effector cells without adrenergic receptors, the ciliary muscle of the eye, for example, cannot respond to adrenergic agonists. This is true whether the agonist is administered by a pharmacologist, a nerve end, or the adrenal medulla. Although this is obviously circular reasoning (the response depends on a receptor and the receptor is defined by the response) it is, however, the way in which all receptors have originated. Regardless of the exact chemical structure of the adrenergic transmitter the effector response is controlled by the receptor and by the natural function of the effector cell.

There are others, however, that do not place much importance on the receptor. For example, Euler puts most emphasis on structure, synthesis, storage release, and uptake of the transmitter (60).

There are two classic ways to characterize biologically the adrenergic receptors; Sir Henry Dale pioneered both methods. One is to compare responses to structurally different but chemically related agonists (16). The other is to compare responses to specific receptor blocking agents (47). This review will consider both views of the adrenergic receptor.

As previously pointed out by this reviewer (4), experimental design, deliberate or unconscious, favors results that best support the experimenter's currently held notions. Reviewers, including the

present one, are not immune to this phenomenon. Therefore, comparative potencies assigned to various agonists are not necessarily the same as those assigned by the authors of the quoted papers. In some cases the authors made no estimates of comparative potency although their published results give sufficient information to allow these estimates to be made. In other cases, older papers have been reinterpreted in the light of newer ideas.

HISTORICAL VIEW

Ehrlich proposed the basic ideas of receptor theory (52). He considered chemicals as having two functional parts: a selective group that governs distribution in the body, tissues, and cells, and a pharmacophore group that evokes the specific effect produced by the chemical. In modern terms, affinity and intrinsic activity (13) could be substituted for Ehrlich's terms.

Dale was the first to make significant use of the receptor concept in connection with the sympathetic nervous system (47). He recognized that the sympathetic neuromyal junction could be viewed as "the receptive mechanism for adrenaline," and he used this concept to explain the differential blocking effect of the ergot alkaloids.

Initiated by the work and ideas of Cannon (38), theories of differential activity based on the chemical structure of the transmitter started to develop. Cannon's sympathins E and I were followed by sympathins A (from adrenal) and N (from adrenergic nerves) (14, 58, 59); sympathin A was thought to be epinephrine and N norepinephrine.

In the course of a search for a compound to prevent the myometrial stimulation induced by vasopressin some unexpected (to this experimenter) observations were made. These were: phenylephrine, a potent vasoconstrictor, *relaxed* the smooth muscle of the gut while a methyl derivative of epinephrine, a potent depressor agent, did not as readily relax the gut; isoproterenol in high dosage *contracted* rabbit uterus; and arterenol was *less* potent than epinephrine as a vasoconstrictor. These findings suggested a more thorough comparison of closely related catecholamines. The relative potencies of five amines, including epinephrine, were studied on a variety of effectors. The conclusions drawn from the results were as follows (1, 4, 5, 7).

1. There are two distinct types of adrenotropic (adrenergic) receptors as determined by their relative responsiveness to closely related sympathomimetic amines.

(*a*) The α receptor is associated with most of the excitatory functions (vasoconstriction, and contraction of the smooth muscle of the uterus, nictitating membrane, ureter, and pupillary dilator) and one important inhibitory function (intestinal relaxation).

(*b*) The β receptor is associated with most of the inhibitory functions (vasodilation, and inhibition of the uterine and bronchial smooth muscle) and one excitatory function (myocardial stimulation).

2. Epinephrine is the one amine that is most active on both α and β receptors; the adrenergic receptor seems to be designed to fit best with the molecular shape of epinephrine (5).

Two of the catecholamines used in this study, the l-methyl derivatives of arterenol and epinephrine, were dropped from the experimental procedures because of questionable purity and optical activity. Furthermore, the *levo*-rotatory isomer of arterenol (levarterenol) became commonly available. Therefore, for testing adrenergic receptors by the comparative potency method only epinephrine,[1] levarterenol,[1] and isoproterenol[1] are usually considered.

Two other adrenergic receptor theories have been proposed. Lands (86), on the basis of responses to a large variety of sympathomimetic amines, suggested receptors A c (excitatory), A r (inhibitory), and A cr (undifferentiated). The undifferentiated receptor was to be found in the heart and presumably the intestine, and responded equally to almost all sympathomimetic amines. Furchgott (65) added two receptors. The γ receptor for glycogenolysis and the δ receptor for intestinal inhibition. As will be described below the receptor blocking agents seem to have clarified partly the cardiac and intestinal receptor. However, catecholamine metabolic effects, including glycogenolysis, may require a different receptive mechanism.

ALPHA ADRENERGIC RECEPTORS

The α receptor is characterized by being most responsive to epinephrine and least responsive to isoproterenol. In terms of comparative potency the order of activity is, epinephrine is more potent than levarterenol which is more potent than isoproterenol.

Eye.—There is no doubt that epinephrine is the most potent catecholamine on the adrenergically controlled smooth muscle of the eye. This is true whether the racemic or *levo* forms of epinephrine and norepinephrine are com-

[1] In discussing comparative potencies of the catecholamines it should be understood that epinephrine and levarterenol are *levo* rotatory compounds and that isoproterenol is a racemic mixture. These are the forms compared unless otherwise specified. In some of the older studies, prior to 1948, racemic epinephrine was compared to racemic norepinephrine (arterenol). In some more recent studies *levo* isoproterenol has been used.

pared. This is shown by mydriasis produced by intra-arterial injection in the intact cat (1, 58, 78), by intraocular injection in the dog or rabbit (21), or in the isolated eye of the rat (18).

The smooth muscle controlling the nictitating membrane, usually studied in the cat, has long been used to show the difference in potency between epinephrine and levarterenol (1, 33, 134, 142). On the isolated membrane of the cat, epinephrine is five times more potent than levarterenol (133). The chronically denervated or cocaine pretreated nictitating membrane becomes supersensitive to both epinephrine and levarterenol (33, 134). The increase in sensitivity to the latter exceeds that to the former so that the potency difference between these amines becomes smaller. However, epinephrine remains the more potent.

The smooth muscle of the orbit of the eye seems to be more sensitive to epinephrine than to levarterenol (personal observation). However, a quantitative study has apparently been done only in the rat; epinephrine was found to be about twice as potent as levarterenol (68).

What is the effect of isoproterenol on the iris dilator and the nictitating membrane? On intraocular administration, this amine produces mydriasis by causing contraction of the radial muscle (62); this is an α receptor response as shown below by tests with specific blocking agents. A similar result is obtained in the isolated eye of the rat (18). In the intact eye of the cat mydriasis is obtained only with very large doses of isoproterenol (1). On the isolated nictitating membrane of the cat isoproterenol in a concentration of 1 mcg./ml. produces relaxation if the muscle is in spasm (123); a concentration of 10 mcg./ml. produces only contraction (133). In summary, isoproterenol activates the α receptors associated with the eye, and its potency is a tenth to a hundredth that of epinephrine, depending on the test method used to compare the drugs.

Spleen.—The smooth muscle of this organ is contracted by the catecholamines. Epinephrine is more potent than levarterenol. This has been determined using measurements of whole spleen size in anesthetized dogs (9, 41), contraction of isolated strips of cat spleen (22), and by hematocrit increases in sheep (135). Isoproterenol in relatively high dosage also produces splenic contraction in the dog or cat (22, 102).

Seminal Vesicles.—When tested on isolated preparations from the rat, epinephrine is more potent than levarterenol in causing contraction (42, 126).

Retractor Penis.—The smooth muscle of this canine structure *in situ* is contracted by catecholamines. Epinephrine is more potent than levarterenol (91, 95). Occasionally, high doses of isoproterenol will produce contraction.

Myometrium.—It has long been known that the myometrial response to epinephrine varies from species to species and depends on the hormonal status at the time of experiment (72). Rabbit or dog uterus, *in situ* or isolated, contracts in response to epinephrine or levarterenol; the former is the more potent (1, 3, 58, 142). Results similar to those obtained in the rabbit have recently been found in the sloth (*Choloepus hoffman* Peters) (114). Isoproterenol produces both relaxation and contraction, the latter occurring only with high concentrations (1). The uterus of the pregnant cat also responds to epinephrine and levarterenol with contraction, but the latter is now the more potent (142). This is due to the fact that the inhibitory receptor is dominant over the excitatory receptor. The dominance between receptors varies from the rabbit, in which the excitatory is predominant, to the rat, in which the inhibitory is dominant. In the human female both receptors are apparently present since epinephrine can produce either relaxation or contraction depending on dosage, and levarterenol produces contraction (43, 81, 116, 145).

Arterial Pressure (Pressor Response).—The acute transient rise in mean arterial pressure in the anesthetized animal is the classic hallmark of sympathomimetic activity. Levarterenol under ordinary circumstances, administered intravenously, is a more potent pressor agent than epinephrine (1, 16, 42, 58, 78, 94, 95, 142). However, epinephrine is the more potent agent in eviscerated dogs (42), in dogs anesthetized with ether (131), and in rabbits (1).

Isoproterenol produces a depressor response in most species of animals. In the rabbit a pressor response may occur (1).

While it is true that arterial blood pressure responses give clues as to how drugs effect the peripheral resistance and cardiac action, other more direct measurements are needed. Arterial pressure can be elevated by either vasoconstriction or cardiac stimulation. Reflex effects initiated by pressure changes can conceal or even reverse the responses due to direct drug action.

Vasoconstriction.—Epinephrine is the most potent adrenergic vasoconstrictor. This has been demonstrated in dogs in the renal circulation (1, 9, 124), in the skin (75), in the mesenteric circulation (1, 73), and in the femoral

circulation (9). Epinephrine is more potent than levarterenol as an intracutaneous vasoconstrictor (42, 78, 95). It is also more potent in the perfused rabbit ear (95) and perfused frog (142).

Levarterenol is more potent than epinephrine as a vasoconstrictor in the canine skeletal muscle vascular bed (74). This is due to the fact that epinephrine is a more potent vasodilator than is levarterenol (see below).

Coronary blood flow is increased by the catecholamines. There is, however, little conclusive evidence that this is a direct relaxing effect on coronary smooth muscle. Changes in heart rate, ventricular contractile force, and diastolic pressure can markedly change coronary flow by mechanical or metabolic means. These effects complicate and obscure attempts to measure the direct coronary effects of the catecholamines. It is possible that epinephrine and levarterenol are direct coronary vasoconstrictors.

Aortic Muscle.—The smooth muscle in rabbit aorta is contracted by the catecholamines. Epinephrine is equipotent with levarterenol, and isoproterenol is the least potent (63, 64, 144). These studies include blocking agents, and the results are consistent with existence of a single receptor (α).

Intestinal Smooth Muscle.—On the two standard experimental preparations for testing drugs on the gut, isolated rabbit ileum and intact canine intestine, *all catecholamines and all sympathomimetics*, produce an inhibitory effect. In comparing epinephrine and levarterenol the majority of studies show epinephrine to be the more potent (1, 9, 33, 37, 58, 142). Isoproterenol is sometimes the least potent of the three catecholamines, and sometimes the most potent. And in intact animals, isoproterenol often produces stimulation of the ileum instead of inhibition. Although an α receptor could be assigned to intestinal smooth muscle, it will be shown below that this would not be a complete explanation.

BETA ADRENERGIC RECEPTOR

The β receptor is most responsive to isoproterenol and epinephrine and in general is least responsive to levarterenol. In terms of potency: isoproterenol > epinephrine > levarterenol. Only two smooth muscle inhibitory responses seem unequivocally to be controlled by a β receptor. These are: the bronchial smooth muscle and the rat myometrium. All other smooth muscle adrenergic responses are best described as being controlled by a balance between α and β activity.

Bronchial Smooth Muscle.—The relative potencies of the catecholamines on this muscle are, isoproterenol > epinephrine > levarterenol (42, 78, 95). This relationship is the same whether the test method used is protection against histamine asthma in guinea pigs, perfusion of the isolated lung, or mechanical response of tracheal ring chains.

Myometrium.—The isolated uterus of the rat is relaxed by all of the catecholamines and, indeed, by all sympathomimetic compounds tested. The relative potencies of the catecholamines are: isoproterenol > epinephrine > levarterenol (1, 95, 142). As stated above, the myometrium of other species appears to have both α and β receptors. As will be described below the myometrium can be used to detect the specific blocking agents of these receptors.

Arterial Pressure (Depressor Response).—Isoproterenol injected intravenously produces a transient but well marked fall in pressure in most species (1, 87, 114). There is no unequivocal evidence that epinephrine or levarterenol can produce a similar depressor response. However, after an α adrenergic blocking agent (see below) epinephrine evokes a depressor response. This change from a pressor response to a depressor response is termed epinephrine reversal. This is considered to be due to an unmasking of a vasodilator action that is normally concealed by a predominant vasoconstricting effect. In addition, epinephrine and levarterenol evoke special depressor reflexes (2, 76).

Vasodilation.—Intra-arterial injections of epinephrine produce vasodilation in the vascular bed of skeletal muscle of dog and man (15, 74, 121). Epinephrine is said to increase hepatic blood flow in man (17).

Isoproterenol produces vasodilation when injected intra-arterially in the femoral and mesenteric vascular beds (1). In the renal vascular bed isoproterenol has either no significant effect (1) or produces some vasoconstriction (124).

Although there have been few detailed comparative studies of the potency of the three principal catecholamines the information available shows isoproterenol > epinephrine > levarterenol as direct vasodilators.

Heart.—Isoproterenol is the most potent of the three catecholamines in producing a positive chronotropic effect on the heart (1, 80, 87). Epinephrine is probably more potent than norepinephrine (11), but reflexes due to pressure changes can obscure the positive chronotropic effect. For example, in animals with intact buffer reflexes, vagal bradycardia may

completely overshadow any tachycardia. Levarterenol in this case produces more bradycardia (less positive chronotropic effect) than epinephrine (9).

In man, levarterenol produces reflex bradycardia while epinephrine produces sinus tachycardia (71, 145).

The relative potency for producing a positive inotropic cardiac effect is isoproterenol > epinephrine = levarterenol (46, 61, 78, 87, 120). In certain amphibia epinephrine is definitely more potent than levarterenol (142) and the same is true in isolated rabbit hearts under some experimental conditions (1).

Intestinal Smooth Muscle.—The potency of isoproterenol as compared to epinephrine and levarterenol is difficult to determine. On isolated strips of rabbit ileum the response seems to depend on the order of administration. Using equimolar doses, if isoproterenol is applied first, and applied only once to each strip, it is the least potent catecholamine in producing cessation of movement. On the other hand, if isoproterenol is applied repeatedly or after the other two amines, it appears to be the most potent. If the strip is pretreated with atropine its response to isoproterenol becomes more uniform, but the relative potency is still variable. The evidence makes it difficult to assign either an α or a β receptor on the basis of relative response to the catecholamines. Furchgott (64) assigned a δ receptor. However, an alternative explanation will be presented below under β adrenergic blocking agents.

ALPHA RECEPTOR BLOCKADE

The classical adrenergic blocking agents such as dibenamine, phenoxybenzine, and phentolamine have long been known to block most of the excitatory responses to epinephrine and other catecholamines. The excitatory responses that are not blocked are the positive inotropic and chronotropic effects on the heart. This class of drugs has been reviewed extensively (110, 111). Green and co-workers have published extensively on adrenergic block in skeletal muscle vascular bed (74), mesenteric bed (73), and skin (75). The iris has been studied by Bennet *et al.* (21), the spleen by Bickerton (22), and the isolated seminal vesicles by Stone and Loew (126).

Levy and Ahlquist (91) have described a general method for examining adrenergic blocking agents. This consists of recording arterial pressure, heart rate, intestinal contraction, and contractions of the retractor penis in the anesthetized dog. Four test amines are administered before and after the unknown blocking agents. These are: epinephrine and ethylnorepinephrine (α and β activators), phenylephrine (a relatively pure α activator), and isoproterenol (the most potent β activator). An α blocking agent diminishes or prevents the effect of epinephrine and phenylephrine on the retractor penis, blocks the pressor action of phenylephrine, reverses the pressor action of epinephrine, and does not essentially alter the responses to isoproterenol.

It was found that these blocking agents also blocked the inhibitory effect of phenylephrine on the intestine (8).

It is fair to say that all responses described above as being controlled by α receptors are blocked by the agents known as classic adrenergic blocking agents. This includes vasoconstriction and contraction of iris dilator, seminal vesicle, spleen, and retractor penis.

BETA ADRENERGIC BLOCKADE

In 1958 Powell and Slater described the actions of the dichloro analog of isoproterenol, DCI (114). This compound had effects that could only be described as due to blockade of the β adrenergic receptors (122). Moran (106) suggested that the term "*beta* adrenergic blocking agent" was most appropriate. This was the start of a continuing search for new β adrenergic blocking agents for possible therapeutic use in cardiac arrhythmias.

It is of historical interest that at least two compounds preceded dichloroisoproterenol as β blocking agents. Ethylnorepinephrine, to be described in greater detail below, in large doses had β adrenergic blocking activity. The first dose of this compound administered intravenously produced a transient pressor response followed by a more prolonged depressor response. If the dose was immediately repeated the pressor response increased and the depressor response decreased. After three or four doses of about 0.5 mg./Kg., ethylnorepinephrine produced only a pressor response (132). The reason for this "reversal" was not found until 25 years later (90).

Butylsympatol blocked the depressor response to isoproterenol and increased the pressor response to epinephrine (41, 113).

Many substances have been found to have β adrenergic receptor blocking properties. At the present time the principal compounds are as follows.

Dichloroisoproterenol, DCI (88, 90, 115).

Naphthylisoproterenol, 1-(2-naphthyl)-2-isopropylaminoethanol, nethalide, pronethalol (24).[2]

[2] Trademarked as Alderlin.

1 - Isopropylamine - 3 - (1 - naphthyloxy) - 2-propanol, propranolol (23).[3]

4 - (2 - Isopropylamino - 1 - hydroxyethyl) methanesulfanilide, MJ1999 (51, 125).

4 - (2 - Methylamino - 1 - hydroxypropyl) methanesulfonanilide, MJ1998 (51, 125).

Arterial Pressure.—The β adrenergic blocking agents diminish or block the depressor response to isoproterenol (91). This effect can serve as a basic indicator for these compounds. A more sensitive screening test has been described; this is known as the "ethylnorepinephrine reversal" test (90). This catecholamine in a dose of 50 mcg./Kg. in anesthetized dogs treated with atropine consistently produces a small pressor response followed by a more prolonged depressor response. Blood flow studies show this to be due to vasoconstriction followed by vasodilation. Following an effective dose of a β blocking agent, ethylnorepinephrine produces only a pressor response due to peripheral vasoconstriction. Many vasoconstrictors of prolonged action also produce ethylnorepinephrine reversal by obscuring the dilator effect of this catecholamine. Therefore, to be certain only β receptor blockade is involved, blood flow studies should be done. Isoproterenol block *but not reversal* must also be present.

The β blocking agents also potentiate the pressor action of epinephrine (6). This is consistent with the idea that the pressor response to epinephrine is reduced by the vasodilating action of this catecholamine.

Bronchial Smooth Muscle.—The bronchodilation produced by epinephrine, norepinephrine, or isoproterenol is blocked by β adrenergic blocking agents (100). Epinephrine and levarterenol now produce a contraction that is blocked by α adrenergic blocking agents.

Myometrium.—Inhibition of the feline myometrium *in situ* produced by epinephrine is blocked by β adrenergic blocking agents (122). Inhibition of isolated uteri by epinephrine is also blocked by these agents (65, 92, 115). All of the β blocking agents have some intrinsic activating effect on the β receptors. This renders the assessment of blockade of inhibitory adrenergic effects difficult (137). For example, isolated strips of rat myometrium, are persistently relaxed by the β blocking agents.

Intestinal Smooth Muscle.—The inhibitory effect of isoproterenol on the canine intestine *in situ* is blocked by a β blocking agent (4, 87). In the same experiments the inhibitory effect of phenylephrine was blocked by an α blocking agent. When it became apparent that the inhibitory effect of epinephrine was blocked only by a combination of an α and a β blocker the conclusion was drawn that the intestine has both types of receptors and that both control inhibition. Confirmatory results have been obtained using isolated intestine (96, 143).

Myocardium.—Cotton *et al.* (45) found evidence that led them to believe that the α blocking agents, phenoxybenzamine and phentolamine, blocked the positive inotropic effect of epinephrine in the open-chest dog. Following the description of the blockade of the myocardial actions of epinephrine by dichloroisoproterenol (49, 50, 106) the effect of blocking agents was re-examined. It was found that the relative increase in force of contraction produced by epinephrine was reduced by phenoxybenzamine. However, the α blocking agent had by itself markedly increased the force of contraction. This increase in control level resulted in the decrease in relative response to epinephrine. The absolute increase, however, was not reduced. Therefore, the suggestion that α blocking agents prevent the cardiac effects of the catecholamines was withdawn (107, 112).

DCI blocks the effects of catecholamines in dog heart-lung preparations (62) and blocks action of epinephrine to increase automaticity in isolated hearts (50).

Pronethalol blocks positive inotropic and chronotropic effects of catecholamines (24, 83, 84). This substance also prevents hydrocarbon-epinephrine fibrillation (108), fibrillation due to cardiac glycosides (138), and blocks catecholamine induced heart rate increases in man (40). It also blocks the effects of catecholamines on the heart-lung preparation (48).

Propranolol is somewhat more potent in blocking action than pronethalol and is said to have practically no intrinsic β activating effect (23). It also acts as an antifibrillatory substance (20). In man propranolol decreases heart rate, cardiac output, arterial pressure, and cardiac work (57).

Any agent that blocks the positive chronotropic effects of catecholamines should be a potential antiarrhythmic drug. Propranolol is now undergoing extensive clinical testing (70, 77, 117, 118). There is some question, however, whether the demonstrable antiarrhythmic effect is due to β blockade or to some other effect (69, 93).

A modification of the ethylnorepinephrine reversal test (see above) based on the blockade of the positive chronotropic effect has been suggested (130). In anesthetized dogs with intact buffer reflexes, slight, transient direct, and reflex tachycardia is produced by ethylnorepinephrine.

[3] Trademarked as Inderal.

Following β blocking agents this response is converted to one of reflex bradycardia. This test is sensitive enough to detect the β blocking activity of 10–50 mcg./Kg. of pronethalol.

METABOLIC EFFECTS OF CATECHOLAMINES

In addition to the effects on smooth and cardiac muscle, epinephrine and related compounds produce a variety of metabolic effects. This includes hepatic glycogenolysis, lipolysis in adipose tissue, and an increase in blood lactic acid. It is tempting to assign an α or β receptor for each of these actions. However, as will be pointed out, this is not possible at the present time.

Comparative Potencies of Catecholamines.—As far as these metabolic effects are concerned the only consistent finding reported is that epinephrine is the most potent catecholamine when all effects are considered. This has been found for hyperglycemia in rabbits (42), hyperglycemia in rats (136), hepatic glycogenolysis (54, 140), increase in hepatic active phosphorylase in rabbit liver slices (127), lipolysis from adipose tissue (141, 146), and increased blood lactic acid (54, 99).

It is the lack of uniformity of the comparative potency of isoproterenol to epinephrine that prevents a clear assignment of a receptor. In some cases isoproterenol is the least potent. In some cases it is even inactive, for example, in producing hepatic glycogenolysis (82). Only in the case of increasing active phosphorylase in the heart (101) and the increase in cyclic 3-5-AMP (128) is isoproterenol the more potent.

On the basis of comparative potencies it is not possible to assign a single receptor, although it seems that a β type receptor would be appropriate.

Blocking Agents.—The β adrenergic receptor blocking agents have been found to block epinephrine induced hyperglycemia in cats (53, 55), myocardial and skeletal muscle glycogenolysis (82), increase in active phosphorylase in the dog heart (101), rat diaphragm and liver (10), increase in plasma free fatty acid (35), and free fatty acid release from adipose tissue (28).

Many of the same effects are also blocked by α blocking agents (10, 28, 29, 39, 53). In addition, the compound isopropylmethoxamine which is not a β blocking agent (89, 129) blocks these effects too. And most recently, the compound *N*-tertiary butyl methoxamine, another substance with no β blocking properties has been shown to block these metabolic effects (36). However, there is some evidence that methoxamine itself may have some β blocking properties (25).

In the opinion of the reviewer the adrenergic metabolic actions do not seem to be controlled by a receptor that fits with the smooth or cardiac muscle effects. Celander's (39) idea that the sympathetic adrenergic nerves and the adrenal medulla have two different general controlling effects seems most attractive. In his view epinephrine from the adrenal should be regarded solely as a metabolic hormone. The theory set forth by the Lundholms (97, 98) that the vasodilator and inhibitory actions of the catecholamines are secondary to metabolic changes deserves further study. Furchgott's original suggestion of a γ adrenergic receptor (65) for metabolic effects appears to be useful.

CONCLUSIONS

1. How valid is the adrenergic receptor concept? It is as valid as any other receptor mechanism. It would be better to define a site of drug or hormone action by the actual enzyme or enzymes involved. However, if these are not known, some other way to characterize sites of action is needed.

The receptor concept correctly describes the site of drug-effector interaction as belonging to the effector cell. The effector responds to the drug (or hormone). Drugs do not act on just any cell.

2. What is the usefulness of the adrenergic receptor concept? In the first place it allows prediction of drug action. For example, the β receptor blocking agents were characterized before any such compounds were recognized. Dale (47) and Rothlin *et al.* (119) suggested that ergot alkaloids could block the effects of epinephrine on the heart and on the gut. However, the predominant α adrenergic blocking actions of these alkaloids strongly interfered with early definitive studies of β blockade. Holzbauer and Vogt (79) tested 27 different substances, including classical adrenergic blocking agents, on the rat uterus without finding a single β blocker.

It also follows that different kinds of chemicals than previously thought to be adrenergic blocking agents should be sought to block the metabolic effects of catecholamines.

Prediction of drug response is also necessary in determining what are the active parts of a given chemical structure. Biochemical studies of drug-enzyme interactions have usually been based on structure-response studies.

3. Can adrenergic receptor studies determine the nature of the adrenergic neuro-transmitter? Considering all adrenergic responses, epinephrine is the most potent catecholamine. Therefore, if potency is a measure of drug-receptor interac-

tion, the adrenergic receptor seems to be designed to "fit" epinephrine. However, potency can also reflect changes in metabolism, binding to inactive sites, membrane penetration, and other things beside drug-receptor interaction. However, epinephrine is the most potent, *in vivo* or *in vitro*, and under many different circumstances. From this we must assume that the superior potency of epinephrine is a true drug-receptor property.

Until definitive evidence to the contrary is obtained there is no reason for this reviewer to discard his assumption that epinephrine is the ultimate adrenergic neuro-hormone. Epinephrine is the end product of the catecholamine biosynthetic pathway (30). It is the receptor that determines the effector response. The reviewer has no information on the exact mechanism of the transmitter-receptor interaction. Therefore, it can be assumed that although adrenergic transmission is based on epinephrine as the transmitter the receptor would allow precursors such as levarterenol or even dopamine to affect transmission if necessary.

4. On the basis of relative responsiveness to sympathomimetic amines, and on the basis of specific blockade, the α receptor is associated with all adrenergic excitatory smooth muscle responses and with intestinal relaxation.

On the same basis the β receptor is associated with all adrenergic inhibitory effects on smooth muscle and with the adrenergic positive inotropic and chronotropic cardiac effects.

On the basis that epinephrine is usually the most potent catecholamine metabolically, and that a specific class of blocking agents for these effects has not yet been found, a γ receptor could be assigned to the adrenergic metabolic effects.

5. The adrenergic receptor concept should not interfere with or negate other studies or findings in regard to the cellular responses to catecholamines. The biophysical changes as described by Bulbring (31), for example, are a step beyond the drug-receptor interaction.

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Simplified Computation of Confidence Intervals for Relative Potencies Using Fieller's Theorem

By C. PHILIP COX and DONNA J. RUHL

Confidence intervals for relative potencies in bioassays are usually calculated by using Fieller's theorem, but the procedures presented in standard texts are computationally cumbersome. It is shown that Fieller's formula can be expressed in an alternative form which takes advantage of calculated quantities from the analysis of variance (ANOV) and thus simplifies computations. Slope ratio assays and parallel line assays are discussed, and 2 examples illustrate the use of the proposed alternatives.

CONFIDENCE intervals for relative potencies in bioassays based on normally distributed responses are calculated from the formula derived by Fieller (1) which, for present purposes, is restated as follows. Suppose that the ratio estimate of $\rho = \mu/\gamma$ is

$$R = u/v \qquad \text{(Eq. 1)}$$

where u, the unbiased estimate of μ, and v, the unbiased estimate of γ, are linear combinations of variates which are normally distributed with variance σ^2. Suppose also that the variance and covariance estimates are

$$V(u) = a_u s^2, \quad V(v) = a_v s^2, \quad CV(u, v) = a_{uv} s^2 \qquad \text{(Eq. 2)}$$

where s^2, with f degrees of freedom, is the unbiased estimate of σ^2, and a_u, a_v, and a_{uv} are known constants depending on the construction of u and v. The usual derivation of Fieller's theorem leads to R_L and R_U, the lower and upper 100 $(1 - \alpha)\%$ confidence limits on ρ, as

$$R_L, R_U = \frac{Rv^2 - s^2F_c a_{uv}}{v^2 - s^2F_c a_v} \mp \frac{\sqrt{s^2F_c\{a_u v^2 - 2a_{uv}uv + a_v u^2 - s^2F_c(a_u a_v - a_{uv}^2)\}}}{v^2 - s^2F_c a_v} \qquad \text{(Eq. 3)}$$

where F_c, the $100(1 - \alpha)\%$ tabulated critical value from the F-distribution, with 1 and f degrees of freedom, has replaced t^2 in the usual formulation.

Users (2) know that, as usually presented, the formula is computationally cumbersome, and it will be shown that the formula can be thrown into a simpler alternative form. The beneficial results for slope ratio and, particularly, parallel line assays are exemplified.

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SLOPE RATIO ASSAYS WITH ONE TEST PREPARATION

For slope ratio assays with responses at the zero-dose level and at doses x_{Si} and x_{Tj}, $i = 1, 2, \ldots, k_1$ and $j = 1, 2, \ldots, k_2$, for the standard and test preparations, respectively, the relative potency estimate, R, is obtained as

$$R = b_T/b_S \qquad \text{(Eq. 4)}$$

where b_S and b_T are the estimated slopes of the (x, y) dose-response lines for the standard and test preparations, respectively. The 2 slopes are given by

$$b_S = \frac{1}{\Delta}\{(\Sigma' x_T^2)(\Sigma' x_S y) - (\Sigma' x_S x_T)(\Sigma' x_T y)\}$$

$$b_T = \frac{1}{\Delta}\{-(\Sigma' x_S x_T)(\Sigma' x_S y) + (\Sigma' x_S^2)(\Sigma' x_T y)\} \qquad \text{(Eq. 5)}$$

where, if Σ denotes summation over all the observations for each preparation, Σ' denotes "corrected" summation so that the quantities in Eq. 5 are obtained as follows:

$$\Sigma' x_S^2 = \Sigma x_{Si}^2 - \frac{(\Sigma x_{Si})^2}{N},$$

$$\Sigma' x_T^2 = \Sigma x_{Tj}^2 - \frac{(\Sigma x_{Tj})^2}{N},$$

$$\Sigma' x_S x_T = \frac{-(\Sigma x_{Si})(\Sigma x_{Tj})}{N} \qquad \text{(Eq. 6)}$$

$$\Sigma' x_S y = \Sigma x_{Si} y_{Si} - \frac{G(\Sigma x_{Si})}{N},$$

$$\Sigma' x_T y = \Sigma x_{Tj} y_{Tj} - \frac{G}{N}(\Sigma x_{Tj}) \qquad \text{(Eq. 7)}$$

in which N is the total number of observations, G is the grand total of all the responses, and

$$\Delta = (\Sigma' x_S^2)(\Sigma' x_T^2) - (\Sigma' x_S x_T)^2 \qquad \text{(Eq. 8)}$$

Hence, by comparison with Eqs. 1 and 2

$$u = b_T, \quad v = b_S \qquad \text{(Eq. 9)}$$

and Finney (3) shows that

$$a_u = \Sigma' x_S^2/\Delta, \quad a_v = \Sigma' x_T^2/\Delta, \quad a_{uv} = -\Sigma' x_S x_T/\Delta \qquad \text{(Eq. 10)}$$

If now quantities F_r and F_S are defined as

$$2F_r = (\text{regressions sum of squares})/s^2 \qquad \text{(Eq. 11)}$$

$$F_S = b_S^2/a_v s^2 \qquad \text{(Eq. 12)}$$

then it is shown in the *Appendix* that the confidence interval on the ratio, ρ, can be expressed as

$$R_L, R_U = \frac{RF_S - F_c(a_{uv}/a_v) \mp \sqrt{F_c(2F_r - F_c)/\Delta a_v^2}}{(F_S - F_c)} \qquad \text{(Eq. 13)}$$

Using Eq. 10, Eq. 13 can be put into the alternative form

$$R_L, R_U = \frac{R(\Delta b_S^2/s^2) + F_c(\Sigma' x_S x_T) \mp \sqrt{F_c(2F_r - F_c)\Delta}}{(\Delta b_S^2/s^2) - F_c(\Sigma' x_T^2)} \qquad \text{(Eq. 14)}$$

With Δ from Eq. 8, the alternatives given in Eqs. 13 and 14 are already more convenient than the usual form in the general case described. Further advantages occur in applications to the common symmetrical assays for which explicit values of a_u, a_v, and a_{uv} are available.

Thus, for the symmetrical 5-point assay with a zero dose and coded doses of 0.5 and 1 for each of the standard and test preparations,

$$\Sigma' x_S^2 = \Sigma' x_T^2 = \frac{16N}{100} \text{ and } \Sigma' x_S x_T = \frac{-9N}{100} \qquad \text{(Eq. 15)}$$

so that, from Eqs. 8 and 10,

$$\Delta = 175N^2/10^4, \; a_u = a_v = 64/7N, \; a_{uv} = 36/7N \qquad \text{(Eq. 16)}$$

Substitution in Eq. 13 leads to the very simple expression of the confidence limits as

$$R_L, R_U = \frac{16F_S R - 9F_c \mp \sqrt{175F_c(2F_r - F_c)}}{16(F_S - F_c)} \qquad \text{(Eq. 17)}$$

where, from Eq. 12,

$$F_S = 7Nb_S^2/64s^2 \qquad \text{(Eq. 18)}$$

Taking $175/256 \doteq 0.6836$, Eq. 17 becomes

$$R_L, R_U = \frac{F_S R - (0.5625)F_c \mp \sqrt{(0.6836)F_c(2F_r - F_c)}}{(F_S - F_c)} \qquad \text{(Eq. 19)}$$

In microbiological assays the quantity F_c/F_S, which is g as used by Finney (3), is often small enough to be neglected. In this case Eqs. 17 and 19 reduce to the very convenient approximate form

$$R_L, R_U = R \mp \frac{20}{Nb_S^2}\sqrt{F_c(\text{regressions sum of squares})s^2/7} \qquad \text{(Eq. 20)}$$

Numerical Example.—Values from the assay by Wood (4), analyzed in Finney (3), will be used to illustrate the computational procedures. The numerical quantities required are

$$N = 20, \quad b_S = 118.629, \quad R = 0.6847, \quad s^2 = 14.43 \text{ (15 d.f.)}$$

From the ANOV, the regressions sum of squares is 31456.9, while, for 95% confidence limits, $F_c = F_{1,15}(0.05) = 4.54$ from standard tables. Since F_S from Eq. 18 will plainly be large relative to F_c, the approximate formula can safely be used in this case. Hence, from Eq. 20

$$R_L, R_U = 0.6847 \mp \frac{1}{(118.629)^2}\sqrt{(4.54)(31456.9)(2.06)} = 0.6462, 0.7232$$

which compare well with the accurate results that are obtained below and given in Finney (3). To obtain the accurate values, $2F_r$ and F_S are first computed as

$$2F_r = 31456.9/14.43 = 2179.9653$$

and, from Eq. 18,

$$F_S = (7)(20)(118.629)^2/(64)(14.43) = 2133.3566$$

so that, from Eq. 19,

$$R_L, R_U = \frac{1}{2128.8166}[(2133.3566)(0.6847) - (0.5625)(4.54) \mp \sqrt{(0.6836)(4.54)(2175.4253)}] = 0.6464, 0.7236$$

PARALLEL LINE ASSAYS

The logarithm of the relative potency estimate in parallel line assays is obtained by Finney (3) as M where

$$M = \bar{x}_S - \bar{x}_T - \frac{(\bar{y}_S - \bar{y}_T)}{b} \qquad \text{(Eq. 21)}$$

in which $\bar{x}_S$ and $\bar{x}_T$ are the mean log-doses, $\bar{y}_S$ and $\bar{y}_T$ are the mean responses for the standard and test preparations, respectively, and b is the estimated common slope of the log-dose, response lines. Since $\bar{x}_S$ and $\bar{x}_T$ are taken as fixed, the confidence interval is therefore obtained for the ratio quantity

$$M - \bar{x}_S + \bar{x}_T = -(\bar{y}_S - \bar{y}_T)/b \qquad \text{(Eq. 22)}$$

Here the numerator and denominator on the right hand side are statistically independent so that, from Eq. 2, $a_{uv} = 0$. It is shown in the *Appendix* that the confidence limits can be represented as

$$R_L, R_U = \frac{1}{(F_r - F_c)}\left\{RF_r \mp \sqrt{\frac{a_p}{a_r}F_c(F_p + F_r - F_c)}\right\} \qquad \text{(Eq. 23)}$$

where

$$a_p = \left(\frac{1}{n_S} + \frac{1}{n_T}\right), \; a_r = \left\{\sum_s (x - \bar{x})^2 + \sum_T (x - \bar{x})^2\right\}^{-1} \qquad \text{(Eq. 24)}$$

and

$$\left.\begin{aligned} F_p &= \text{the F-ratio for preparations in the ANOV} \\ F_r &= \text{the F-ratio for regression in the ANOV} \end{aligned}\right\} \qquad \text{(Eq. 25)}$$

and n_S and n_T are the total number of responses to the standard and test preparations, respectively.

Some simplification occurs if F_c/F_r is small enough to be neglected but, in practice, the gain is so slight that the use of Eq. 23 may be generally recommended. Simplified forms can, however, be presented for the common, balanced 4- and 6-point parallel assays using explicit values for a_p and a_r as follows.

Four-Point Parallel Line Assay.—If S_1, S_2, are the total responses to the lower and upper doses of the standard preparation, respectively, and T_1 and T_2 are the corresponding response totals for the test preparation, and linear contrasts L_p and L_r are defined as

$$\begin{aligned} L_p &= -(S_1 + S_2) + (T_1 + T_2) \\ L_r &= -(S_1 + T_1) + (S_2 + T_2) \end{aligned} \quad \text{(Eq. 26)}$$

the logarithm of the relative potency is calculated from the ratio of $R = dL_p/L_r$, where d, as used by Finney (3), is the logarithm of the ratio between successive doses, this ratio being the same for both preparations. Since $V(L_p) = V(L_r)$ from Eq. 26, the confidence limits for dL_p/L_r are found by using Eq. 23 with $a_p = a_r$.

$$\left(\frac{dL_p}{L_r}\right)_L, \left(\frac{dL_p}{L_r}\right)_U = \frac{d}{(F_r - F_c)}\left\{\frac{L_p}{L_r} F_r \mp \sqrt{F_c(F_p + F_r - F_c)}\right\} \quad \text{(Eq. 27)}$$

where, as before, F_c is the $100(1 - \alpha)\%$ tabulated F-value with 1 and f degrees of freedom, f being the number of degrees of freedom for s^2 in the ANOV, F_p and F_r are as defined in Eq. 25.

If g is computed as

$$g = F_c s^2/(\text{regression mean square}) \quad \text{(Eq. 28)}$$

an alternative computational form of Eq. 27 is then

$$\left(\frac{dL_p}{L_r}\right)_L, \left(\frac{dL_p}{L_r}\right)_U = \frac{d}{(1 - g)} \times \left\{\frac{L_p}{L_r} \mp \sqrt{g\left(1 - g + \frac{\text{preparations mean square}}{\text{regression mean square}}\right)}\right\} \quad \text{(Eq. 29)}$$

which is perhaps the most expedient formulation.

Six-Point Parallel Line Assay.—If L_p and L_r are the usual contrasts for the 6-point assay, corresponding to those in Eq. 26, the confidence limits for $\bar{y}_T - \bar{y}_S/b = 4dL_p/3L_r$ are obtained from Eq. 23 as

$$\left(\frac{4dL_p}{3L_r}\right)_L, \left(\frac{4dL_p}{3L_r}\right)_U = \frac{4d}{3(F_r - F_c)} \times \left\{\frac{L_p}{L_r} F_r \mp \sqrt{\frac{3}{2} F_c(F_p + F_r - F_c)}\right\} \quad \text{(Eq. 30)}$$

$$= \frac{4d}{3(1 - g)} \times \left\{\frac{L_p}{L_r} \mp \sqrt{\frac{3g}{2}\left(1 - g + \frac{\text{preparations mean square}}{\text{regression mean square}}\right)}\right\} \quad \text{(Eq. 31)}$$

with all quantities as previously defined.

Numerical Example.—To illustrate the identification of the required quantities the 4-point assay of oestrin by Bülbring and Burn (5) as used by Finney (3) is taken. In that example $L_p = -42$, $L_r = 448$ and the ANOV was essentially

	d.f.	m.s.
Preparations	1	63
Regression	1	7168
Divergence	1	240
Residual error	13	551.15

where the term "divergence" is introduced in preference to "parallelism" or, uglier, "antiparallelism." Hence, for 95% limits,

$$F_c = F_{1,13}(0.05) = 4.67$$

and

$$g = (4.67)(551.15)/7168 = 0.3591$$

The ratio of upper to lower doses was 2 so that, with $d = \log 2$, from Eq. 29,

$$\left(\frac{dL_p}{L_r}\right)_L, \left(\frac{dL_p}{L_r}\right)_U = \frac{0.30103}{0.6409} \times \left\{-\frac{42}{448} \mp \sqrt{\left(0.3591(0.6409 + \frac{63}{7168}\right)}\right\} = -0.2709, 0.1828$$

The basic doses were 0.2 mcg. and 0.0075 ml., for the standard and test preparations, respectively, so that the limits for the actual relative potency estimate $\hat{\rho}_p$ are,

$$(\hat{\rho}_p)_L, (\hat{\rho}_p)_U = \frac{0.2}{0.0075} \text{ antilog } (\bar{1}.7291, 0.1828) = 14.29, 40.60 \text{ mcg./ml.}$$

PRECISION

The formulas derived above are useful in discussions of the precision of bioassays. For example, from Eqs. 29 and 30 the squared length of the confidence interval for 4- and 6-point parallel line assays is seen to depend on the quantity,

$$\frac{1}{(1 - g)^2}\left\{g\left(1 - g + \frac{\text{preparations mean square}}{\text{regression mean square}}\right)\right\} = \frac{g}{1 - g} + \frac{g}{(1 - g)^2}\left\{\frac{\text{preparations mean square}}{\text{regression mean square}}\right\} \quad \text{(Eq. 32)}$$

From the definition of g in Eq. 28, it therefore simply follows that the basic requirements are small values for F_c, s^2, and the mean square for preparations, and a large value for the regression mean square.

CONCLUSION

Detailed presentations of the above formulas have been made because of the assurance [Schultz (6)] that they give considerable computational advantage. In particular, the gain arises because values in the necessarily computed ANOV do double duty. The exact rather than the approximate formulas may be recommended, for parallel line assays at least, because once F_c/F_r has been calculated for arbitration, its retention involves little extra labor.

APPENDIX

Slope Ratio Assay.—Using the values for a_u, a_v, and a_{uv} for a slope ratio assay as given in Eq. 10, the expression $a_u v^2 - 2a_{uv}uv + a_v u^2$ under the radical of Eq. 3 can be written as

$$a_u v^2 - 2a_{uv}uv + a_v u^2 = v(a_u v - a_{uv}u) + u(a_v u - a_{uv}v)$$
$$= \frac{1}{\Delta}\{b_S(b_S\Sigma' x_S^2 + b_T\Sigma' x_S x_T) + b_T(b_T\Sigma' x_T^2 + b_S\Sigma' x_S x_T)\}$$
$$= \frac{1}{\Delta}\{b_S\Sigma' x_S y + b_T\Sigma' x_T y\} \quad \text{(Eq. 33)}$$

This follows from the normal equations of which Eq. 5 is the solution. Further, the numerator on the right of Eq. 33 is the sum of squares for regression in the ANOV. Also,

$$a_u a_v - a_{uv}^2 = \frac{1}{\Delta^2}\{(\Sigma' x_S^2)(\Sigma' x_T^2) - (\Sigma' x_S x_T)^2\} = \frac{1}{\Delta} \quad \text{(Eq. 34)}$$

Substituting from Eqs. 33 and 34 into Eq. 3, the confidence limits can therefore be written as

$$R_L, R_U = \frac{1}{b_S^2 - s^2 F_c a_v}[Rb_S^2 - s^2 F_c a_{uv} \mp \sqrt{s^2 F_c(\text{regressions sum of squares} - s^2 F_c)/\Delta}] \quad \text{(Eq. 35)}$$

Hence, with F_r and F_S defined as in Eqs. 11 and 12, the confidence limits can be expressed as

$$R_L, R_U = \frac{RF_S - F_c(a_{uv}/a_v) \mp \sqrt{F_c(2F_r - F_c)/\Delta a_v^2}}{F_S - F_c} \quad \text{(Eq. 36)}$$

as given in Eq. 13.

Parallel Line Assay.—For a parallel line assay, Eq. 3 with $a_{uv} = 0$ reduces to

$$R_L, R_U = \frac{Rv^2 \mp \sqrt{s^2 F_c(a_u v^2 + a_v u^2 - s^2 F_c a_u a_v)}}{v^2 - s^2 F_c a_v}$$
$$= \frac{1}{\left(\frac{v^2}{a_v s^2} - F_c\right)} \times \left\{\frac{Rv^2}{a_v s^2} \mp \sqrt{\frac{a_u}{a_v} F_c\left(\frac{u^2}{a_u s^2} + \frac{v^2}{a_v s^2} - F_c\right)}\right\}$$
$$= \frac{1}{(F_v - F_c)} \times \left\{RF_v \mp \sqrt{\frac{a_u}{a_v} F_c(F_u + F_v - F_c)}\right\} \quad \text{(Eq. 37)}$$

where F_u and F_v are defined as

$$F_u = u^2/a_u s^2 \text{ and } F_v = v^2/a_v s^2 \quad \text{(Eq. 38)}$$

For the general parallel line assay, comparison of Eqs. 1 and 22 shows that the above result can be applied to obtain the confidence interval for $M - \bar{x}_S + \bar{x}_T$ if

$$u = (\bar{y}_S - \bar{y}_T) \text{ and } v = b \quad \text{(Eq. 39)}$$

If n_S and n_T are the total number of responses to the standard and test preparations, respectively, the variance of $(\bar{y}_S - \bar{y}_T)$ is estimated as

$$V(u) = V(\bar{y}_S - \bar{y}_T) = \left(\frac{1}{n_S} + \frac{1}{n_T}\right)s^2 = a_u s^2 \quad \text{(Eq. 40)}$$

so that, from Eq. 38,

$$F_u = \frac{(\bar{y}_S - \bar{y}_T)^2}{\left(\frac{1}{n_S} + \frac{1}{n_T}\right)s^2} \quad \text{(Eq. 41)}$$

which itself is the F-ratio for preparations in the ANOV. Similarly

$$F_v = \frac{b^2}{s^2}\left\{\sum_S (x - \bar{x})^2 + \sum_T (x - \bar{x})^2\right\} \quad \text{(Eq. 42)}$$

which is the F-ratio for regression in the ANOV. If then F_p and F_r are defined as in Eq. 25, the confidence interval from Eq. 37 is

$$R_L, R_U = \frac{1}{(F_r - F_c)}\left\{RF_r \mp \sqrt{\frac{a_p}{a_r} F_c(F_p + F_r - F_c)}\right\} \quad \text{(Eq. 43)}$$

which establishes the result given in Eq. 23.

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Identification and Differentiation of Organic Medicinal Agents III

Amine-Containing Antiparkinson Agents and Newer Muscle Relaxants

By LESLIE G. CHATTEN and LYNN A. DOAN*

A series of specific physical criteria, by which four amine-containing antiparkinson agents and a newer amine-containing skeletal muscle relaxant, currently in popular clinical application, can be identified and differentiated, is presented. Twenty-three derivatives of these compounds were prepared using a variety of characterizing agents which included picric acid, ammonium reineckate, sodium tetraphenylborate, chloroplatinic acid, and methyl iodide. By the use of microcrystal tests, and formation of derivatives, it is possible to differentiate biperiden, cycrimine, procyclidine, trihexyphenidyl, and phenyramidol. Photomicrographs are presented as a supplemental and additional parameter for characterization purposes.

VARIOUS techniques for the identification of trihexyphenidyl, cycrimine, and procyclidine hydrochloride salts have appeared in the literature. These include microcrystal tests and color spot tests (1), chromatographic studies (2–4), published infrared spectra (5), and derivatization (6–9).

A few isolated derivatives have been reported for phenyramidol hydrochloride (10–12) and those can be used as an aid to identification. No method for the qualitative estimation of biperiden hydrochloride could be found in the literature.

All of these preparations are potent systemic drugs and are intended for oral or parenteral administration; hence reliable criteria for their identification and differentiation are of great importance to the forensic chemist and toxicologist. It is the purpose of this investigation to develop a comprehensive series of several physical reference criteria which can be utilized to positively identify these compounds in the least amount of time.

EXPERIMENTAL

Apparatus.—Fisher-Johns melting point apparatus; Beckman IR-5A infrared spectrophotometer; Bausch & Lomb biological microscope; Metrohm potentiograph model E336.

Compounds Investigated.—Phenyramidol HCl or 2-β-hydroxyphenethylamino-pyridine HCl; biperiden HCl or [α-bicyclo (2.2.1)-hept-5-en-2-yl-α-phenyl-1-piperidino-propanol HCl]; cycrimine HCl or 1-cyclopentyl-1-phenyl-3-piperidino-1-propanol HCl; procyclidine HCl or 1-cyclohexyl-1-phenyl-3-pyrrolidino-1-propanol HCl; trihexiphenidyl HCl or 1-cyclohexyl-1-phenyl-3-piperidino-1-propanol HCl.

Reagents and Solutions.—All reagents used were A.C.S. grade or the highest grade commercially available. Aqueous and ethanolic (95%) solution of: trihexyphenidyl HCl, 0.25, 0.5, 1%; cycrimine HCl, 0.25, 0.5, 1%; procyclidine HCl, 0.25, 0.5, 1%; phenyramidol HCl, 0.25, 0.5, 1%; biperiden HCl, 0.05, 0.1%; aqueous Reinecke salt solutions, 0.1, 0.25, 0.5, 1.0%; aqueous and ethanolic (95%) picric acid solutions, 0.25, 0.5, 1.0%, and in acetone, 0.5, 1%; aqueous and ethanolic (95%) styphnic acid solutions, 0.16, 0.32, 0.64, and 0.25, 0.5, 1.0%, respectively; aqueous chloroplatinic acid solutions, 0.25, 0.5, 1.0%; ethanolic (95%) solutions of picrolonic acid, 0.25, 0.5, and 1.0%. These solutions were stored in 15-ml. amber dropper bottles.

Formation of Derivatives

All purified derivatives were dried in a vacuum desiccator over phosphorus pentoxide at room temperature for 24 hr. before the final melting point was taken on a Fisher-Johns melting point apparatus. Melting points were corrected by a calibration graph prepared from U.S.P. melting point reference standards.

Picrates.—The general procedure for preparation of picrates was that employed by Shriner *et al.* (13). The crude material was recrystallized from 95% ethanol and the equivalent weights determined by the titration technique of Clark and Wang (14). The data obtained, together with the elemental analyses (C, H, and N) verified the purity and identity of the derivatives.

Reineckates.—The procedure of Chatten and Levi (15) was used. The resulting derivatives were recrystallized by adding approximately 5–10 ml. of methanol and sufficient acetone dropwise to solubilize the derivative if it had not already dissolved. Water was then added dropwise with agitation until

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a turbidity was seen to persist. All recrystallizations were performed without the aid of heat.

Purity and identity of the reineckates was established by elemental analyses (C, H, N, and Cr).

Tetraphenylborates.—The general technique of Koehler and Feldmann (16) was used. Products were recrystallized from methanol or methanol/acetone by adding water dropwise with agitation until a turbidity persisted and then cooled.

Equivalent weights of the tetraphenylborates were determined by the nonaqueous titration procedure of Chatten *et al.* (17). These data, together with the elemental analyses (C, H, N), confirmed the identity and purity of these derivatives.

Methiodides.—The procedure by Cheronis and Entrikin (18) for the preparation of methiodide derivatives was used. Products were recrystallized from isopropanol or acetone–ether.

Purity and identity was confirmed by elemental analyses (C, H, N).

Chloroplatinates.—The method outlined by Wild (19) for the preparation of chloroplatinic acid derivatives was modified and adapted for these amine hydrochloride salts.

Biperiden hydrochloride, being almost insoluble in water, was dissolved in a minimal amount of methanol to which a slight excess of aqueous chloroplatinic acid was added with stirring. The mixtures were allowed to stand for 20 min. in an ice bath and then filtered. The resulting products were purified by washing well with cold water.

Elemental analyses (C, H, N) confirmed the purity and identity of the derivatives.

Infrared Spectra

Infrared spectra of the parent compounds and derivatives were measured by the potassium bromide pellet technique.

Preparation of Photomicrographs

One drop of an aqueous or alcoholic solution of the amine muscle relaxant salt was placed on a microscope slide and 1 drop of a reagent solution was added. These were mixed well and covered with a cover glass. For the potassium iodide test, 1 drop of an aqueous solution of the parent compound was placed on a microscope slide, and a few particles of finely ground potassium iodide were sprinkled over the surface of the drop.

In all instances, the time of crystal formation was noted and the photomicrographs were taken before the slide became dry.

RESULTS AND DISCUSSION

Formation of Derivatives

The melting ranges of the derivatives prepared from the compounds under investigation, together with previously reported literature values, are presented in Table I.

Picrates.—A review of the literature has revealed that relatively few picrates have been reported for the basic amino muscle relaxants. Delaby *et al.* (11) have reported the preparation of the picrate of phenyramidol with a melting point of 151.0–152.0°.

Trihexyphenidyl and biperiden picrate were not obtained in a crystalline state. These products initially formed an oily mass, and repeated crystallization attempts from various solvents (ethanol, methanol, acetone) proved unsuccessful.

The other picrate derivatives were obtained in good yield, were easily purified, and had sharp, well distributed, characteristic melting points.

Reineckates.—In every instance, the amine muscle relaxants studied formed the anhydrous reineckate. This was verified by elemental analyses (carbon, hydrogen, nitrogen) and the gravimetric determination of chromium calculated as Cr_2O_3.

All reineckates prepared in this program were observed to have a reasonably sharp decomposition range. However, some overlap in the melting ranges of the derivatives was evident, thus necessitating the preparation of other derivatives for conclusive identification.

Tetraphenylborates.—It has been stated that washing TPB salts free of excess reagent, and drying under vacuum yields compounds which are sufficiently pure for characterization purposes (20, 21). In this investigation, the TPB derivatives were recrystallized to obtain well-defined crystalline products for infrared spectra. Crane (21) has shown several TPB salts to be heat labile, and for this reason the derivatives were prepared and recrystallized at room temperature.

Since good yields of easily purifiable product were obtained and well distributed melting points were noted, the tetraphenylborate salts proved to be highly desirable derivatives for qualitative identification. When titrated in nonaqueous media, excellent quantitative recoveries were obtained for all derivatives.

Methiodides.—The observed melting point of cycrimine methiodide is radically different from that reported in the literature. No apparent reason for this anomaly could be found other than the failure

TABLE I.—MELTING POINTS OF DERIVATIVES OF THE AMINE COMPOUNDS, °C.

Drug	Picrate	Reineckate	Tetraphenylborate	Methiodide	Chloroplatinate
Biperiden		160.0–164.0(dec.)	208.0–209.5	207.0–208.0	142.0–147.0(dec.)
Cycrimine	145.0–146.5	154.0–158.0(dec.)	157.5–159.0	145.0–146.0 235.0–237.0(9)	179.0–182.0(dec.)
Procyclidine	63.5–65.0	149.0–154.0(dec.)	148.0–150.0	204.0–205.0(dec.) 204.0–205.0(6)	199.0–203.0(dec.)
Trihexyphenidyl		159.0–162.0(dec.)	157.0–159.0	208.0–208.5 203.0–204.0(6,8)	132.0–137.0(dec.)
Phenyramidol	153.5–154.5 151.0–152.0(11)	156.0–158.0(dec.)	124.0–126.0	165.5–166.5 164.0–166.0(10)	156–160.0(dec.)

of the workers (9) to confirm identity of the derivative by elemental analysis.

The methiodides were readily prepared and easily purified derivatives with sharp melting points. Their only apparent disadvantage is the overlap in the melting ranges which precludes their use for qualitative identification without the preparation of additional derivatives.

Chloroplatinates.—All of the amino muscle relaxants studied in this investigation were monobasic and formed the 2:1 derivative with the divalent platinic anion.

The literature reveals no record that chloroplatinic acid has been used previously to characterize any of the amino muscle relaxants investigated in this program. All chloroplatinate derivatives decomposed on heating but the decomposition ranges were reproducible and characteristic. Recrystallization was a factor encountered during the preparation of these derivatives. Generally the chloroplatinates were only sparingly soluble in ethanol (95%) or methanol, and any amount of heating resulted in a partial or complete decomposition of the product. In order to avoid this the chloroplatinates were purified by washing well with cold distilled water prior to submission for elemental analyses. Excellent results were obtained and decomposition ranges were characteristic in each instance with no overlap.

Infrared Spectroscopy

Examination of the spectra of the parent compounds provided an additional parameter for differentiation, although the same functional groups (tertiary amino and alcoholic) are present in all of the drugs.

Medium to weak adsorption in the 3500 cm.$^{-1}$ region (OH stretching vibration) and medium absorption throughout the 3050–2850 cm.$^{-1}$ aromatic and aliphatic CH stretching region is common to the picrates in this investigation. Multiple or broad bands in the 2700–2250 cm.$^{-1}$ region due to NH^+ stretching vibrations, overtones, and combinations are also present. Intense bands at 1560 and 1360 cm.$^{-1}$ represent the nitro asymmetrical and symmetrical stretching vibrations, respectively. In the "fingerprint" region two sharp characteristic bands, common to the three spectra, appear at 785 and 740 cm.$^{-1}$.

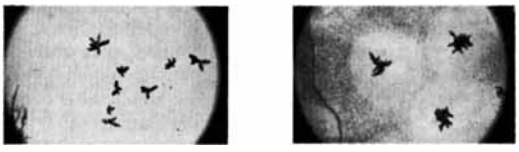
Spectral interpretations appearing in a previous paper of this series (22) are applicable to the reineckates and tetraphenylborates, respectively. However, the methiodide spectra are sufficiently different to merit special mention. These latter spectra exhibit numerous common bands, in keeping with their structural similarity. Strong absorption bands at 3300 cm.$^{-1}$ due to OH stretching vibrations and medium to strong aromatic and aliphatic CH stretching throughout the 3050–2900 cm.$^{-1}$ regions are obvious for all methiodides except that of phenyramidol. In addition, the 8–16 μ region, with its very characteristic CH out-of-plane bending bands in the lower frequencies and especially the 9–14 μ region, is most useful for differentiation of these compounds.

The chloroplatinate spectra were poorly resolved, perhaps due to the damping effect of the anion, and hence were of no value for differentiation of the compounds.

Photomicrography

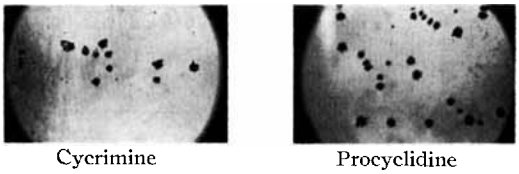
A large number of reagents were employed in an attempt to obtain complete and comparative sets of photomicrographs. However, success was limited to a few of these. All photomicrographs were taken at 50× magnification, and only those depicting distinctive crystal formations are reproduced in Figs. 1–5. It must be understood that the crystalline habits of these compounds are not to be used as the sole criterion for identification, but rather as an adjunct to the other physico-chemical methods previously discussed.

Phenyramidol hydrochloride was the only compound to yield well-defined microcrystals with either picric or styphnic acid and these are presented in Fig. 1. Combinations of these reagents with the other parent compounds yielded oils or oily films which would not crystallize.



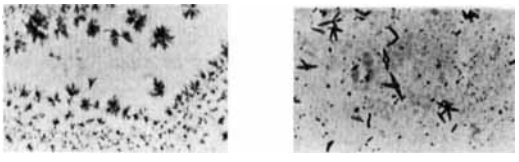
Phenyramidol Picrate — Phenyramidol Styphnate

Fig. 1.—Photomicrographs of the picrates and styphnates.

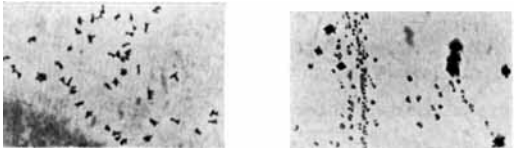


Cycrimine — Procyclidine

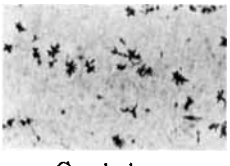
Fig. 2.—Photomicrographs of the chloroplatinates.



Procyclidine — Phenyramidol



Biperiden — Trihexyphenidyl



Cycrimine

Fig. 3.—Photomicrographs of the reineckates.

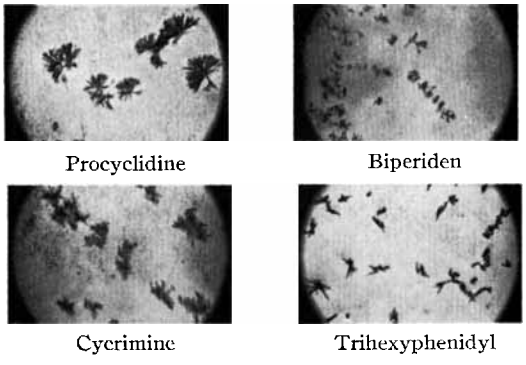


Fig. 4.—Photomicrographs of the picrolonates.

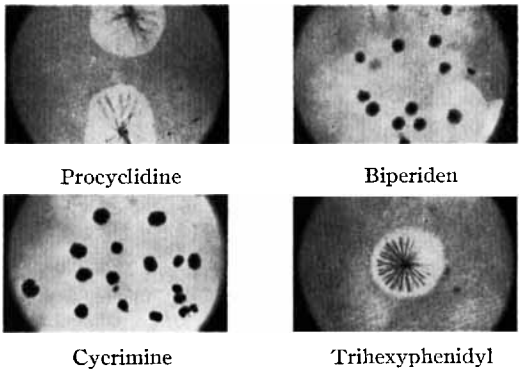


Fig. 5.—Photomicrographs of the hydroiodides.

lonate of phenyramidol could not be obtained in crystalline form under any of the varied conditions employed. The material invariably separated as a golden yellow oil. Clarke (1) has described the morphology of procyclidine picrolonate, and favorable agreement was noted with this investigation.

The method of Hucknell and Turfitt (23) was used for the preparation of the hydroiodides. As illustrated in Fig. 5, satisfactory photomicrographs were obtained for four of the five compounds studied. Phenyramidol yielded a fine amorphous precipitate, but the other compounds gave easily and rapidly prepared microcrystals.

The conditions under which the characteristic microcrystals were formed are summarized in Table II. All solutions are aqueous unless otherwise indicated.

SUMMARY

1. A series of specific physical criteria, by which four amine-containing antiparkinson agents and one amine-containing skeletal muscle relaxant can be positively identified and differentiated, has been presented.

2. Twenty-three derivatives of these drugs have been prepared in a systematic manner, of which 18 have not been reported to date in the literature.

3. The infrared spectra of these derivatives and their parent compounds have been obtained as a further parameter for their qualitative differentiation.

4. A series of photomicrographs have been included in Figs. 1–5, together with a summary of the conditions under which they were formed (Table II).

TABLE II.—AMINE MUSCLE RELAXANTS CHARACTERIZED BY MICROCRYSTALLOGRAPHY[a]

Parent Compd.	Picric Acid	Styphnic Acid	Chloroplatinic Acid	Ammonium Reineckate	Picrolonic Acid	Potassium Iodide
Biperiden HCl	...	...	...	0.1–0.1 (5)	0.1–0.25[b]	0.1 (15)
Cycrimine HCl	...	...	1.0–1.0 (15)	0.5–0.5 (5)	0.5–1.0[b]	0.5 (1)
Phenyramidol HCl	0.5–0.5 (10)	0.5–0.64 (15)	...	0.5–0.5 (20)	...	...
Procyclidine HCl	...	...	0.5–1.0 (3)	0.5–0.5 (5)	0.5–1.0[b] (5)	1.0 (5)
Trihexyphenidyl HCl	...	...	...	0.5–0.5 (10)	0.5–1.0[b] (5)	1.0 (5)

[a] Muscle relaxant concentration (%), reagent concentration (%); numbers in parentheses denote time in minutes when crystals formed. [b] Ethanolic (95%) solution.

Chloroplatinic acid did not prove to be a satisfactory reagent for the identification of all the amine muscle relaxants in this study. Figure 2 shows distinctive, well-defined crystals for only cycrimine and procyclidine. The other compounds formed light amorphous precipitates which were not characteristic. Clarke (1) prepared the chloroplatinate of cycrimine and his description complimented our findings.

Ammonium reineckate proved to be an excellent reagent since characteristic and distinctive crystals were obtained for all five of the amine muscle relaxants. The results are depicted in Fig. 3.

Figure 4 shows that picrolonic acid proved to be a highly satisfactory reagent for characterization of four of the five compounds studied. The picro-

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Some Wax Formulations of Sulfaethylthiadiazole Produced by Aqueous Dispersion for Prolonged-Release Medication

By EVELYN B. DRAPER* and CHARLES H. BECKER

Drug–wax particles were prepared by pouring heated aqueous solutions of surfactants into melted wax which contained dispersed sulfaethylthiadiazole (SETD). The systems then were slowly cooled to room temperature with stirring. The SETD–wax particles were recovered, washed, dried, and sieved into predetermined mesh size ranges. There appeared to be a direct relationship between drug release rate and average theoretical surface area of the SETD–Glycowax S-932 particles *in vitro*. This relationship was not seen in the SETD–beeswax particles. The dissolution rates of all the mesh ranges of particles studied seemed to be pseudo first order after the first 15 min. of testing. SETD–Glycowax S-932, 50- to 60-mesh particles, showed reasonably good prolonged-release properties in an *in vivo* urinary excretion study.

A NUMBER of methods and techniques have been used in the manufacture of oral dosage forms intended to impart prolonged, sustained, or long-acting therapeutic effect. The production of prolonged release of a drug in a wax matrix by means of aqueous dispersion or an emulsification process is mentioned in the literature, but detailed information is not given. Spray-congealing and spray-drying methods using wax with drugs have received quite a bit of attention.

Yamamoto and Baba (1) describe in their patent an aqueous dispersion method for producing wax pearls containing drug for prolonged-release medication. As an example, a melted wax containing dispersed drug is poured into a 2% polyvinyl alcohol aqueous solution, previously heated to 80°, and stirred until cool. The wax pearls that form are strained, washed with water, and dried. In this patent, several suitable dispersing agents are recommended and a number of wax or wax-like dissolution retardants are illustrated.

Kowarski *et al.* (2) describe a similar process in the preparation of prolonged-release sulfamethazine in small size batches. In this method, 2 parts of Japanese synthetic wax was melted with 1 part of sulfamethazine and poured into a running Waring blender containing cold water. After a few minutes of blending, the resulting suspension was filtered and dried. The particles were then washed with hydrochloric acid to remove sulfamethazine embedded on the outside of the granules, which was determined to be about 58% of the total drug in and on the granule.

The purpose of this investigation was to study some wax formulations of sulfaethylthiadiazole (SETD) produced by an aqueous-dispersion method for prolonged action. Bleached beeswax and Glycowax S-932 were used as the dissolution retarding materials. Beeswax was selected because it is a natural product and has plastic properties, whereas Glycowax S-932, a synthetic wax-like product is brittle. Both materials are edible, and the melting points of both are about the same, approximately 63°.

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generalizations pertaining to the preparation of prolonged-release drug–wax particles produced by the aqueous-dispersion method, such as effect of temperature, emulsifier and rate of mixing, as well as to the release pattern of the drug from several specific mesh sizes of drug–wax particles.

The present study involves 3 parts: first, the preparation of the drug–wax particles; second, the study of their *in vitro* release patterns; and third, the *in vivo* evaluation using a urinary excretion study of 1 drug–wax product that proved satisfactory by the *in vitro* screening test.

EXPERIMENTAL

Throughout this investigation official or drug grade materials were employed wherever feasible, except for some chemicals used for test or assay purposes which were usually reagent grade. Sulfaethylthiadiazole (SETD) was obtained from American Cyanamid Co. Wherever the term SETD–beeswax or SETD–Glycowax is employed, this means an approximate ratio of 1 part of SETD to 3 parts of Glycowax S-932 or beeswax. Drug–wax particles designated 16 to 20, 30 to 40, and 50 to 60 mesh mean that the particles passed through the lower-numbered sieve but were retained or did not pass through the higher-numbered sieve.

Preparation of Various Mesh Sizes of SETD–Beeswax and SETD–Glycowax Particles.—Preliminary work showed that many factors had an influence on the production of various mesh sizes of SETD–wax particles as well as the release rate of the SETD from the wax matrix. A combination of sorbitan monooleate[1] and polysorbate 80[2] was found to be a suitable dispersant. To minimize variables as much as possible the following SETD–beeswax formula was used: bleached beeswax, 24.0 Gm.; SETD, 8.0 Gm.; sorbitan monooleate, 1.0 ml.; polysorbate 80, 1.15 ml.; and distilled water, 400.0 ml. The SETD–Glycowax formula employed was: Glycowax S-932, 24.0 Gm.; SETD, 8.0 Gm.; sorbitan monooleate, 1.0 ml.; polysorbate 80, 0.25 ml.; and distilled water, 400.0 ml.

The bleached beeswax or Glycowax S-932 and SETD were placed in a 1000-ml. beaker, heated on a water bath to 75°, and well dispersed. The sorbitan monooleate, polysorbate 80, and distilled water were combined in another beaker and heated to 80°. The aqueous phase was added slowly to the SETD–wax phase while hot with continuous stirring at a predetermined speed, using a Lightnin laboratory type, model F mixer, until the resulting dispersion reached a temperature of about 45°. Cooling the product to this temperature took about 25 min. When the combined hot SETD–wax and aqueous phase was stirred at about 150 r.p.m., the majority of the resulting SETD–wax particles produced were in the range of 12 to 30 mesh. When the mixture was stirred at about 300 to 400 r.p.m., most of the SETD–wax particles were in the range of 30 to 100 mesh.

The SETD–wax particles were separated from the aqueous phase by means of filtration through filter paper and washed with distilled water to remove any free SETD. The SETD–wax particles were then dried.

The air-dried SETD–beeswax and SETD–Glycowax particles were classified into 3 mesh sizes, namely, 16 to 20, 30 to 40, and 50 to 60, using U.S.P. sieves and the U.S.P. XVII method (3) for determining uniformity of fineness.

Assay of SETD Content in Wax Particles.—The SETD–beeswax particles were assayed for SETD by placing exactly 0.5000 Gm. of the air-dried sample in a 25-ml. portion of warm benzene and extracting the SETD with several portions of warm 3.5% HCl using a separator. The SETD–Glycowax particles were similarly treated, except the organic solvent employed was chloroform. In each case, the acid extract was made up to 100 ml. with distilled water in a volumetric flask. An aliquot portion was assayed for SETD content by the Bratton-Marshall colorimetric method (4). A Klett-Summerson colorimeter with a No. 54 filter was employed to determine the color intensity, which was compared to that of standard solutions.

In Vitro Dissolution Studies.—The *in vitro* evaluation of the various mesh sizes of SETD–beeswax and SETD–Glycowax particles for prolonged release was essentially the same as that described by Robinson and Swintosky (5). Using simulated gastric fluid (0.1 *N* HCl, pH 1.1), exactly 0.5000 Gm. of the air-dried SETD–wax samples was employed, 2 for each time interval. Likewise, an equivalent amount of the wax formation particles without SETD was treated similarly to serve as a blank. Three bottles (1 for the blank and 2 for the product) were withdrawn for analysis after intervals of 0.25, 0.5, 1, and 2 hr. and at appropriate times to determine when equilibrium of release was reached. For evaluation of dissolution in simulated intestinal fluid (alkaline pancreatin solution, pH 8.3), exactly 2.000 Gm. of the air-dried SETD–wax samples was used, 2 for each time interval. Similarly, a blank was employed, and test bottles were withdrawn for analysis after intervals of 0.25, 0.5, 1, 3, and 6 hr. and at appropriate times to determine when equilibrium of release of SETD was reached. With all samples, the content of each bottle was filtered and 1 ml. of the filtrate was diluted to an appropriate volume with distilled water in a volumetric flask. Then 1 ml. of the dilution was assayed for SETD content by the Bratton-Marshall colorimetric method (4) as described above. The rotating bottle dissolution apparatus employed was the same as that described by Souder and Ellenbogen (6). The temperature of the water bath was 30 ± 0.5°.

In Vivo Evaluation.—The method used in this study was basically the same as that described by Nicholson *et al.* (7) for the clinical evaluation of sustained-release tablets of SETD using urinary excretion studies. Four healthy adult males were utilized in the clinical evaluation of a 50- to 60-mesh formulation of SETD–Glycowax consisting of approximately 1 part of SETD and 3 parts of Glycowax S-932, which gave a satisfactory *in vitro* release pattern. As a control, 3.9 Gm. of plain SETD was administered in a suspension form in a sweetened, flavored, methylcellulose solution using the following 2-part formula: (*a*) methylcellulose,[3]

[1] Marketed as Span 80 by the Atlas Powder Co., Inc., Wilmington, Del.

[2] Marketed as Tween 80 by the Atlas Powder Co., Inc., Wilmington, Del.

[3] Marketed as Methocel.

400 cps., 3.0 Gm.; methyl salicylate, 0.2 ml.; distilled water, to 200.0 ml.; and (*b*) SETD, 3.9 Gm.; and sucrose, 20.0 Gm. Each subject was told to add the solid ingredients of part (*b*) to solution (*a*), mix well for several minutes, and drink. It was instructed that the bottle be rinsed well with about 2 fluid ounces of water to assure ingestion of all the SETD. Two weeks after the control was run, each subject received the same amount of SETD in a SETD–Glycowax combination, 50 to 60 mesh, in a suspension prepared from the 2-part formula described above.

Concentrations of the free and of the total (free plus conjugated) SETD in urine were determined colorimetrically as described by Bratton and Marshall (4).

Mathematical Calculation of Rates of Dissolution and Excretion.—The rates of dissolution and excretion were determined by use of the Noyes-Whitney equation: $dc/dt = k(C_e - C)$, where C is the concentration at time t, C_e is equilibrium solubility at the experimental temperature, and k is the rate constant.

C_e was determined in the *in vitro* tests to be the concentration at which there was no change with time. C_e in the *in vivo* tests was the cumulative amount excreted at the end of 72 hr.

From plots of log $(C_e - C)$ *versus* time,

$$k = -2.303 \frac{[\log(C_e - C)_2 - \log(C_e - C)_1]}{(t_2 - t_1)}$$

$$k = 2.303 \frac{[\log(C_e - C)_1 - \log(C_e - C)_2]}{(t_2 - t_1)}$$

RESULTS AND DISCUSSION

Preparation of Drug–Wax Particles.—In preliminary work, various nonionic emulsifiers and dispersants were tried. Yamamoto and Baba (1) recommended polyvinyl alcohol (PVA) in a similar process. The partially hydrolyzed PVA,[4] 50–42 grade, was evaluated in this study in varying concentrations, from 0.25 to 4.0%. Regardless of amount used, however, over 50% of the particles produced were of size 40 to 80 mesh. Not enough of the 20- to 40-mesh size could be produced. This investigation required a greater range and a more uniform distribution of particle size than was obtained with PVA.

The effect of the HLB of some surfactants upon particle size was studied. The required HLB values of beeswax (8) are 5 for a w/o emulsion and 10 to 16 for an o/w emulsion. Various proportions of sorbitan monooleate and polysorbate 80 were used with beeswax; and the combination which produced bead-like particles of satisfactory texture and the most desirable range and distribution of particle size had a HLB of 10 and was 1 part of sorbitan monooleate to 1.15 parts of polysorbate 80. The required HLB values of Glycowax S-932 were not available. However, the most satisfactory combination was 1 part of polysorbate 80 to 4 parts of sorbitan monooleate, having an HLB value of 6.44. The proportion of emulsifier to the total volume of dispersion made was less than 1% for both waxes because too much emulsifier would have had a pronounced effect on dissolution of SETD from the wax matrix.

[4] Marketed as Elvanol.

The temperature of the drug–wax phase and of the dispersion solution, the rate of cooling and the speed of mixing of the combined phase during the cooling period, and the ratio of dispersion medium to dispersed phase were all important factors in influencing the size of particles produced. The higher the temperature, the smaller the particles. Slower rates of cooling, increased amounts of dispersion medium in relation to dispersed phase, and higher speeds of mixing yielded smaller particles. Optimum conditions were determined and were kept constant thereafter.

In Vitro Dissolution Studies.—One of the objectives of this investigation was to study the effect of particle size upon rate of release of SETD from the wax matrix. For this reason the drug–wax particles were classified into size ranges of 16 to 20, 30 to 40, and 50 to 60 mesh. The average diameter of these would be 1050, 505, and 230 μ, respectively, assuming spherical shape, which is a ratio of approximately 4, 2, 1. Since specific surface area is inversely proportional to diameter of the particles, S_w = surface of particles/weight of particles = $\pi\Sigma nd^2/(\pi/6)_p\Sigma nd^3 = 6/pd$ (9), the ratio of specific surface area for the samples in all 3 groups is 1, 2, and 4.

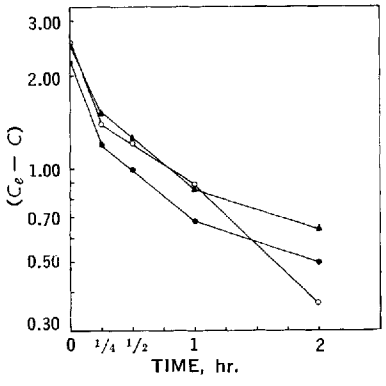

Fig. 1.—*In vitro* dissolution rates of SETD from various mesh sizes of SETD–beeswax particles in 0.1 *N* HCl. Key: ●, 16 to 20 mesh; ▲, 30 to 40 mesh; ○, 50 to 60 mesh.

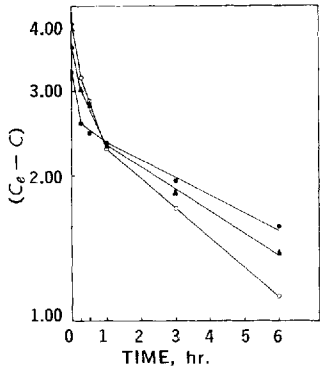

Fig. 2.—*In vitro* dissolution rates of SETD from various mesh sizes of SETD–beeswax particles in alkaline pancreatin solution. Key: ●, 16 to 20 mesh; ▲, 30 to 40 mesh; ○, 50 to 60 mesh.

It was theorized that the rate of dissolution of drug from the particles during the first 15-min. period in the test solutions would be in direct relation to specific surface area, since the particles were assumed to have a uniform distribution of drug on the surface. This theory proved to be correct for just the SETD–Glycowax product (Figs. 3 and 4). The SETD–beeswax particles showed a slower rate of release of SETD for the medium size range than for the smaller or larger (Figs. 1 and 2). The fact that this was slower than the smaller size range follows the theory. The fact that it was slower than the larger sized particles seemed very likely to be due to the aggregation of smaller particles to form some larger ones. Microscopic examination revealed that most of the larger particles were aggregates of smaller ones.

After the first 15-min. period, the rate of dissolution of SETD from the wax matrix varied in all cases (Figs. 1–4). The rate was constant from the end of the first hour to the termination of the test period, and was pseudo first order. The rate of dissolution should change as surface area changes. Some of the groups of particles showed dissolution rates of different ratio to apparent particle size during the last period of dissolution than during the first period. This could be due to the fact that some of the particles were more porous than others, the porosity being due to the speed of stirring causing some air to be trapped in the particles during preparation.

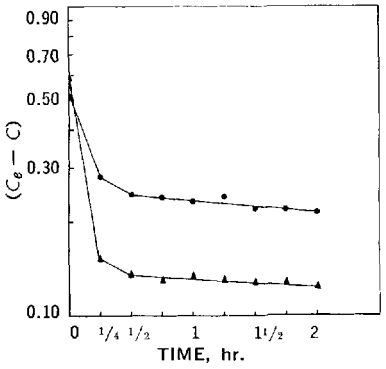

Fig. 3.—*In vitro* dissolution rates of SETD from various mesh sizes of SETD–Glycowax particles in 0.1 *N* HCl. Key: ●, 16 to 20 mesh; ▲, 30 to 40 mesh; ○, 50 to 60 mesh, $(C_e - C) = 0$.

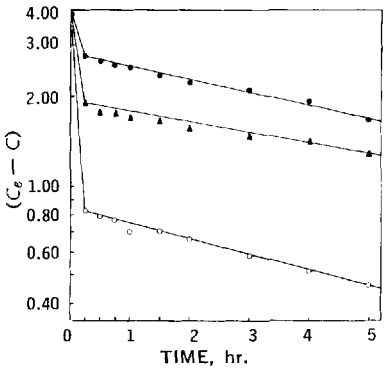

Fig. 4.—*In vitro* dissolution rates of SETD from various mesh sizes of SETD–Glycowax particles in alkaline pancreatin solution. Key: ●, 16 to 20 mesh; ▲, 30 to 40 mesh; ○, 50 to 60 mesh.

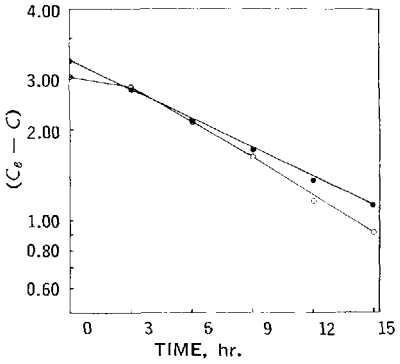

Fig. 5.—Average urinary excretion rates of free SETD for 4 humans receiving a 3.9-Gm. oral dose of SETD in (*a*) plain form and (*b*) SETD–Glycowax combination. Key: ●, plain SETD; ○, SETD–Glycowax combination.

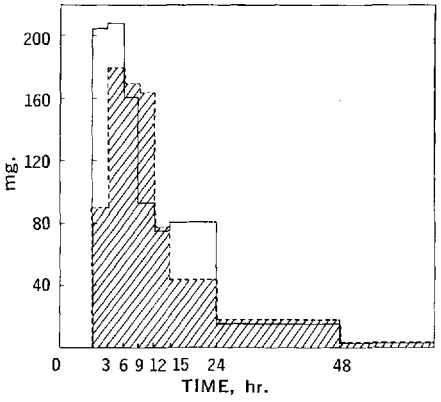

Fig. 6.—Average urinary excretion pattern of free SETD/hr. for 4 humans receiving a 3.9-Gm. oral dose of SETD in (*a*) plain form and (*b*) SETD–Glycowax combination. Key: □, plain SETD; ▨, SETD–Glycowax combination.

The per cent of SETD released in 0.1 *N* HCl (Figs. 1 and 3) was less than that in alkaline pancreatin solution (Figs. 2 and 4) for the same type sample and for the same time period. The rate of release was greater in the 0.1 *N* HCl, however. In alkaline pancreatin solution the peripheral portion of drug was apparently released at about the same rate as in the 0.1 *N* HCl. The increase in amount of release of SETD in the alkaline pancreatin solution could most likely be due to the fact that the wax matrix is partially solubilized, emulsified, and disintegrated by the surfactants and alkalinity of this solution.

It was theorized that the first part of dissolution of SETD from the drug–wax particle in both test solutions involved the simultaneous dissolution of drug by 2 first-order rates, $c = a_1e^{-k_1t} + a_2e^{-k_2t}$ (10). Dissolution data for 30- to 40-mesh SETD–beeswax particles were used in an attempt to test

this theory. From the semilog plot of $(C_e - C)$ *versus* time, the difference in $(C_e - C)$ values as obtained by the data during the first 0.75 hr. in the solution and that which would be obtained if the rate of dissolution was the same as that for the last period of the test was plotted semilogarithmically *versus* time. Since this gave a straight line, one rate appears to be that of the dissolution of the drug on the surface of the particle and the other that of the drug within the particle. Of course, the

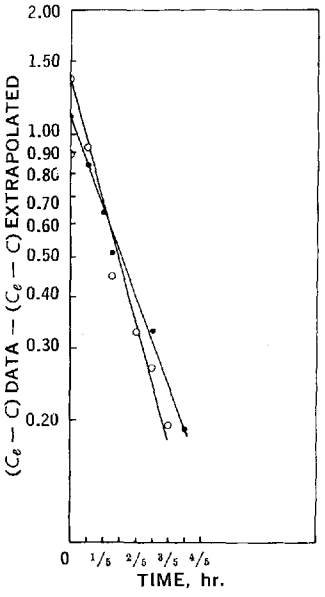

Fig. 7.—$(C_e - C)_{data}$ minus $(C_e - C)_{extrapolated}$ *vs.* time in hr. for 30- to 40-mesh SETD–beeswax particles in 0.1 *N* HCl and in alkaline pancreatin solution. Key: ○, in 0.1 *N* HCl; ●, in alkaline pancreatin solution.

rate during the last period of the test is also that of the drug within the particle. The rate calculated in Fig. 7 and theorized to be that of the surface SETD was 3.44 in 0.1 *N* HCl and 2.51 in alkaline pancreatin solution. It appears logical that this difference in rate may be due to the decrease in rate of diffusion in alkaline pancreatin solution caused by a partially emulsified layer, and possibly a concentration of colloidal particles around the SETD–wax beads.

In Vivo Study.—The SETD–Glycowax product of 50- to 60-mesh size was chosen because the *in vitro* results were most similar to the *in vitro* data of a well-known commercial prolonged-release preparation of SETD (11). Although *in vivo* release does not follow that of *in vitro*, it can be predicted from it by various patterns which can be correlated by experienced diagnosis.

From Figs. 5 and 6, it can be seen that the SETD–Glycowax particles as compared with plain SETD did show a decrease in the rate of release of SETD by about 50% during the first 3 hr. The rates were about the same for the second 3-hr. period. After this time, the rate increased for the SETD–Glycowax particles so that at the end of 24 hr. 71% of the total SETD had been excreted from the SETD–Glycowax particles as compared to 85% from plain SETD. The total free plus conjugated SETD excreted within 72 hr. after ingestion of SETD–Glycowax was 85%, whereas that released *in vitro* at equilibrium was 81%, a fairly close relationship. Since the ideal prolonged-release preparation maintains a proper therapeutic blood level for a 12- or 24-hr. period, the 50- to 60-mesh SETD–Glycowax product evaluated in this study appears to be a reasonably good prolonged-release preparation. Previous work (7) using SETD in urinary excretion studies showed the biological half-life to be about 6 hr. The present work showed a similar half-life period for SETD.

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Pharmacological Studies of Norphenyl Hemicholinium 3

By S. N. THAMPI, FLOYD R. DOMER, VERNON B. HAARSTAD, and F. W. SCHUELER†

Norphenyl HC-3 (NP-HC-3), an analog of HC-3 in which the biphenyl nucleus is replaced by a monophenyl moiety, has been synthesized. NP-HC-3 has been shown to resemble the parent compound in most of its pharmacological properties. Its LD_{50} in mice was found to range from 45–680 mcg./Kg. NP-HC-3 in low doses induced a slowly progressive respiratory paralysis. In higher doses the neuromuscular blocking action was antagonized by choline. Neostigmine reversed only the effects observed with low doses of NP-HC-3. NP-HC-3 did not have adrenergic, antihistaminic, analgesic, or local anesthetic activity.

STRUCTURE-ACTIVITY relation studies concerning analogs of hemicholinium number 3 (HC-3) have been carried out by many investigators (1–4). The structure-activity relationships and actions on the peripheral nervous system of some of these analogs have been reviewed by Long (5). The hemicholiniums are characterized by a high toxicity which is slow in onset at minimal lethal doses. They interfere with cholinergic transmission at such sites as nerve-skeletal muscle junctions, autonomic ganglia, and postganglionic parasympathetic endings (6). To date HC-3 remains the most active of the analogs studied for the over-all hemicholinium-like action. The earlier modifications of the HC-3 molecule involved alterations in the cationic moiety and/or the biphenyl nucleus. Schueler (1) found that the phenacyl analog (*i.e.*, half of the parent compound) was inactive. The purpose of this paper is to discuss the synthesis and pharmacology of an analog of HC-3 in which the biphenyl nucleus has been replaced by a monophenyl moiety. This compound hereafter will be referred to as norphenyl-HC-3 (NP-HC-3).

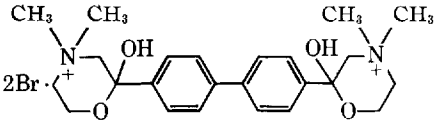
METHODS

Chemical Procedure

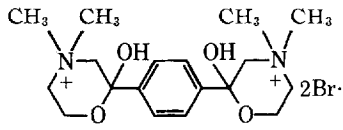
p-Bis(bromoacetyl)benzene was prepared according to the method described by Krohnke and Vogt (7) from 1,4-diacetylbenzene. The product was recrystallized from tetrahydrofuran in the form of stout hexagonal prisms, m.p. 178° (lit. value m.p. 177–178°).

1,4-Bis(1,1-dimethyl-3-hydroxy-3-morpholinyl) Benzene, Dibromide (NP-HC-3).—*p*-Bis-(bromoacetyl) benzene, 10 Gm. (0.031 mole), was dissolved in 200 ml. of boiling tetrahydrofuran. The resulting solution was treated with activated carbon[1] and filtered hot under suction, through diatomaceous earth supported on a sintered-glass funnel. β-Dimethylaminoethanol, 57 ml. (0.05 mole), was added to the hot filtrate, and the mixture was allowed to cool to room temperature. The resulting white precipitate was collected on a filter, washed well with tetrahydrofuran, and recrystallized from a mixture of absolute ethanol and methanol (3:2). The product, collected on a filter, washed well with diethyl ether, and dried over phosphorus pentoxide in a vacuum desiccator, afforded 10.50 Gm. (67.2%) of white crystals, m. p. 228° dec.

Anal.—Calcd. for $C_{18}H_{30}Br_2N_2O_4$: C, 43.39; H, 6.06; N, 5.62. $C_{18}H_{30}Br_2N_2O_4 \cdot H_2O$: C, 42.17; H, 6.37; N, 5.47.[2] Found: C, 42.52; H, 6.34; N, 5.54.[3]



HC-3



NP-HC-3

Pharmacological Procedures

Toxicity Studies in Mice.—Two strains of albino mice (Swiss Webster and $1CR^{CD-1}$) of both sexes, each weighing 20 ± 2 Gm. in groups of 20 were used for each dose of NP-HC-3. The drug was administered intraperitoneally in a volume of 0.01 ml./Gm. body weight in normal saline. In one experiment two LD_{50} determinations were made on the same day, one in the morning and the other in the late afternoon. The LD_{50} was computed by

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† Deceased.

[1] Marketed as Norit by American Norit Co., Inc., Jacksonville, Fla.

[2] VBH and FWS (unpublished data) HC-3 has been found to crystallize in variable hydration states. NP-HC-3, as well as HC-3, is very hygroscopic before recrystallization. The water of crystallization is very difficult to remove from HC-3. Thus, it is felt that the NP-HC-3 could have picked up water before and retained it during the recrystallization.

[3] The chemical analysis was performed by Alfred Bernhardt Microanalytical Laboratories, Mulheim, Germany.

means of the probit-log dose method (8). The animals were observed for signs which differed from those of control animals, and the viscera were examined post-mortem in a few cases.

Antagonism Studies in Mice.—Swiss Webster albino mice of both sexes (20 ± 2 Gm. each) were used. All drugs were injected intraperitoneally in a volume of 0.01 ml./Gm. body weight in normal saline. Three groups of 10 mice received 1 mg./Kg. of NP-HC-3 followed 1 min. later by a second injection of saline, 10 or 20 mg./Kg. of choline. Three groups of 5 mice were given 1–3 mg./Kg. NP-HC-3 followed 1 min. later by saline, 150 or 300 mcg./Kg. of neostigmine.

Blood Pressure-Neuromuscular Preparation.—In this series of experiments, 5 mongrel dogs (9–15 Kg.), 5 albino rabbits (2–3 Kg.), and 2 chickens (2–3 Kg.) of either sex were used. All the animals were anesthetized with sodium pentobarbital (35 mg./Kg., i.v.). The blood pressure was recorded from cannulated arteries connected *via* a Statham pressure transducer to a Grass model 5-DWC polygraph. Heparinized saline (50 u./ml.) was used as an anticoagulant in this system. The Achilles tendon was isolated and tied with a thin copper wire, about an inch above the joint, sectioned distally, and attached to a force-transducer (model FT-03). The muscular contractions were recorded on the polygraph. The ipsilateral sciatic nerve was isolated, sectioned proximally, and the peripheral stump stimulated with a bipolar, shielded, platinum electrode, using a Grass stimulator (model S4G) so as to obtain maximal muscular contractions. The leg was immobilized with an iron pin which passed through the knee joint while the foot was clamped to a rigid frame. The trachea was exposed, cannulated, and artificial respiration was used whenever necessary. The femoral vein of the dog, the jugular vein of the chicken, and the ear vein of the rabbit were cannulated to permit drug administration. In the dog and the rabbit, blood pressure recordings were obtained from the right carotid artery, whereas in the chicken the femoral artery was used for this purpose. In both the dog and the rabbit the left vagus was isolated, ligatured, sectioned centrally, and the distal portion stimulated with a Mallory model 12 Rs6D inductorium.

Two chickens (2.5 Kg. male) were prepared as described above. In addition, the branch of the femoral artery feeding the gastrocnemius muscle was isolated, and a loose loop of thread was placed around it. A side branch was cannulated in the direction of blood flow to permit the injection of acetylcholine.

TABLE I.—BLOOD PRESSURE–NEUROMUSCULAR PREPARATION

No.	Wt., Kg.	Frequency of Stimulation/sec.	Dose, mg./Kg.	Onset of Actions, min.	% Neuromuscular Blockade	Duration of Blockade, min.	Remarks
				Dogs			
1	14.1	2	0.1		0	...	
			1.0	15	100	34	Died of respiratory failure at this time
2	10.0	2	0.3	10	10	30	
			0.7	1.5	50	55	
			1.0	1.0	90	80	
			1.3	1.0	95	95	
			1.5	0.5	97.5	Indefinite	Blockade not reversed even after 2 hr.
3	12.0	2	0.7	20	<10	<10	
		2	1.0	25	37	60	
		4	1.0	3	76	120	
		2	1.0	6	42	65	
4	9.1	2	0.7	10	90	45	
		4	0.7	1.5	90	>180	
5	10.0	2	0.7	20	50	30	
		4	0.7	...	...		Died of respiratory failure after this dose
				Rabbits			
1	3.2	0.4	1	13	100	105	
2	2.5	0.4	0.6				
			0.6	10	30	35	
3	2.5	0.4	0.6	4	100	60	Died before recovery
4	2.6	2.0	0.6	15	98	120	
5	2.0	2.0	0.3	...	0	...	
			0.5	...	0	...	
			0.7	10	50	45	
				Chickens			
1	2.5	0.4	0.3	...	0	0	
			0.6	...	0	0	
			1.2	2	65	60	
2	2.6	0.9	1.0	15	100	30	Died during total blockade

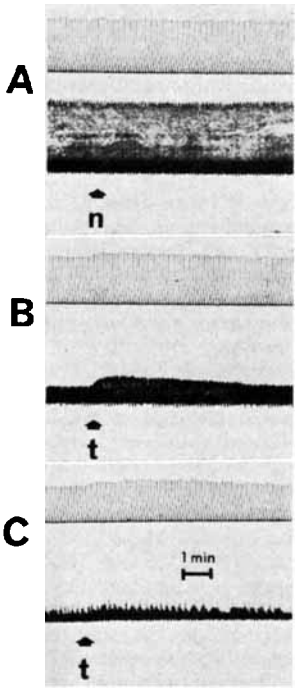

Fig. 1.—Bilateral contractions of the gastrocnemius muscles of the chicken. In the upper tracing the rate of supramaximal electrical stimulation of the ipsilateral sciatic nerve was 0.2/sec. The rate of stimulation in the lower tracing was 0.8/sec. In A, NP-HC-3 (1 mg./Kg.) was given intravenously (n). In B, the first injection of TEA (3 mg./Kg. i.v.) was given. In C, the fourth dose of TEA (3 mg./Kg., i.v.) was given.

Bilateral sciatic-gastrocnemius preparations were done with 3 rabbits and 2 chickens. The arrangement of the apparatus was the same as previously described except that the contractions of both the gastrocnemius muscles of each animal were recorded. One of the muscles was stimulated at a low frequency (0.2–0.4/sec.), and the other was stimulated at varying, higher frequencies. The blood pressure of the chickens was not recorded.

RESULTS

Toxicity Studies in Mice.—About 5 min. after the administration of a 700 mcg./Kg. dose of NP-HC-3 the animals exhibited dyspnea, exophthalmos, and ataxia. Some of the animals exhibited a Straub tail reaction. Then clonic convulsions developed and they stood on their hind limbs. Nearly half of them fell on their sides and died within 15–20 min. Survivors were apparently normal after 2 hr.

Irrespective of the increment of the doses above the lethal levels, a minimum of about 5 min. elapsed before the observable onset of action. At autopsy the heart was still beating, and the intestinal motility appeared to be stimulated slightly more than in control animals.

The LD_{50} (i.p.) in two different batches of ICR-CD-1 albino mice was 680 mcg./Kg. and 320 mcg./Kg. The LD_{50} (i.p.) of NP-HC-3 in a strain of Swiss Webster albino mice was 45.5 mcg./Kg. in the morning and 46.3 mcg./Kg. in the late evening of the same day. This is in contrast to the large variation which previously has been found with HC-3.

Antagonism Studies.—All mice receiving 1 mg./Kg. of NP-HC-3 died within 30–35 min. Three of ten given 10 mg./Kg. of choline died at 10–15 min. Only one of ten treated with 20 mg./Kg. of choline died in 15 min.

A dose of 150 mcg./Kg. of neostigmine protected all mice treated with 1 mg./Kg. of NP-HC-3. All mice died which were given 2 or 3 mg./Kg. of NP-HC-3. In these animals neostigmine produced no marked parasympathomimetic activity. Mice given 300 mcg./Kg. of neostigmine died within 3 min., and exhibited excessive salivation, lacrimation, urination, and defecation (SLUD syndrome). Animals which received this dose of neostigmine 1 min. after a 1 mg./Kg. of NP-HC-3 were slightly ataxic after 20 min. and did not exhibit the SLUD syndrome.

Blood Pressure-Neuromuscular Preparation.—Doses of NP-HC-3 from 0.1–1.5 mg./Kg. did not have any effect on the blood pressure of the dog, rabbit, or chicken. Also, it did not modify the control responses produced by vagal stimulation and double carotid occlusion of injections of acetylcholine, epinephrine, or histamine.

The results found with the neuromuscular preparations may be seen in Table I. Doses of 1 mg./Kg. or less exerted varying degrees of blockade. Increasing the rate of stimulation increased the amount of blockade as well as its duration, *e.g.*, dog 3. Choline (5–20 mg./Kg. i.v.) reversed the neuromuscular blockade either partially or completely. Four 3 mg./Kg. (i.v.) doses of tetraethylammonium bromide given at 3-min. intervals to a rabbit during partial neuromuscular blockade with NP-HC-3 caused a partial reversal of the blockade with the initial dose. The amount of reversal

TABLE II.—EFFECTS OF INTRAVENOUS NP-HC-3 (1 mg./Kg.) ON BILATERAL SCIATIC NERVE-GASTROCNEMIUS PREPARATIONS IN RABBITS AND CHICKENS

Animal	Wt., Kg.	Side	Frequency of Stimulation/sec.	Onset of Action, min.	% Neuromuscular Blockade	Duration of Blockade, min.	Choline, 10 mg./Kg.	Neostigmine, 1–3 mg./Kg.
Rabbit 1	2.6	Left	0.8	15	100	30	Completely reversed	No change
		Right	0.4	25	10	...	No change	Potentiation
Rabbit 2	4.0	Left	3.0	30	50	48	Partially reversed	No change
		Right	0.4	40	10	20	No change	Potentiation
Rabbit 3	3.4	Left	0.4	9	70	70	Partially reversed	...
		Right	0.8	4	90	85	Partially reversed	...
Chicken 1	3.4	Left	3.0	7	90	30	Partially reversed	...
		Right	0.4	20	50	25	No change	...
Chicken 2	3.0	Left	0.2	40	50	60	Partially reversed	...
		Right	0.8	25	95	120	Partially reversed	...

decreased with successive doses until no effect was observed (Fig. 1).

The effect of frequency of stimulation on the intensity and duration of neuromuscular blockade and the action of choline and neostigmine are listed in Table II. Here again, increasing the frequency of stimulation increased the rate of onset of neuromuscular blockade as well as the strength and duration of the blocking action. In rabbits, neostigmine (0.1 mg./Kg.) potentiated the strength of contraction on a side being stimulated at 0.4/sec. and had almost no effect on the side being stimulated at 3/sec. (Fig. 2, B). The same effect was seen with edrophonium (Fig. 2, C), in which case the potentiated height of contraction was more than twice that of the control. Following these 2 drugs, choline administration reversed completely the neuromuscular blockade in the leg being stimulated more rapidly and had no effect on the other side (Fig. 2, D). The contraction produced by a close-arterial injection of 6 mcg. of acetylcholine on the unstimulated muscle of a chicken was completely abolished 30 min. after the i.v. administration of 1 mg./Kg. of NP-HC-3 (Fig. 3 A–C). This was at the onset of the neuromuscular blockade. When the electrical stimulation was resumed following the administration of acetylcholine, there was an initial increase in the strength of the muscular contraction (Fig. 3, D). Ten micrograms of acetylcholine caused a slight depolarization which lasted about 0.5 min. During complete neuromuscular blockade either 6 or 10 mcg. of acetylcholine administered close-arterially during the electrical stimulation resulted in a burst of weak contractions. A dose of 10 mg./Kg. of choline administered i.v. resulted in a series of contractions which were 14% of control and which lasted for a period of 6 min. (Fig. 3, F).

Miscellaneous Studies.—NP-HC-3 did not have any effect on the rat or guinea pig ilia or on responses produced by test doses of acetylcholine, epinephrine, and histamine. It did not exhibit any non-narcotic analgesic activity in mice studied according to the method of Koster *et al.* (9) or any narcotic analgesic activity in mice tested according to the method described by D'Amour and Smith (10). There was also no topical or infiltrative local anesthetic activity found using the rabbit.

DISCUSSION

NP-HC-3 was synthesized in an attempt to investigate the minimum requirements for the hemicholinium-type of activity and also to gain more insight into the mechanism of action of the hemicholiniums. NP-HC-3 ranks next to HC-3 itself in potency among the compounds previously studied for the hemicholinium-type of activity. The primary toxic manifestations of NP-HC-3 are a slowly progressive respiratory depression and muscular weakness. As in the case of HC-3 the LD_{50} in mice varied widely with different batches of mice used, and the observed effects occurred after a latent period which was independent of the dose. On a molar basis the LD_{50} of NP-HC-3 was about 3 times that of the parent compound, HC-3.

The toxicity of both NP-HC-3 and HC-3 is antagonized by choline and neostigmine. As with HC-3 (11) there was a rough correlation among the lethal dose with NP-HC-3, the size of the animal, and the time required for the onset of action.

The neuromuscular blockade produced in the sciatic nerve-gastrocnemius muscle of the rabbit, chicken, and dog was slow in onset and could be augmented by increasing the rate of stimulation. The duration of the blockade was from 30 min. to 2 hr. With successive doses, the time of onset decreased, and the duration of the neuromuscular blockade increased. Similar effects were observed with curarization in humans by Bush and Baraka (12). The apparent potentiation of a second dose of tubocurarine had been discussed by Levy (13). He established a quantitative relationship between the duration of action and the concentration of the drug in successive doses. The observations made with NP-HC-3 are compatible with Levy's hypoth-

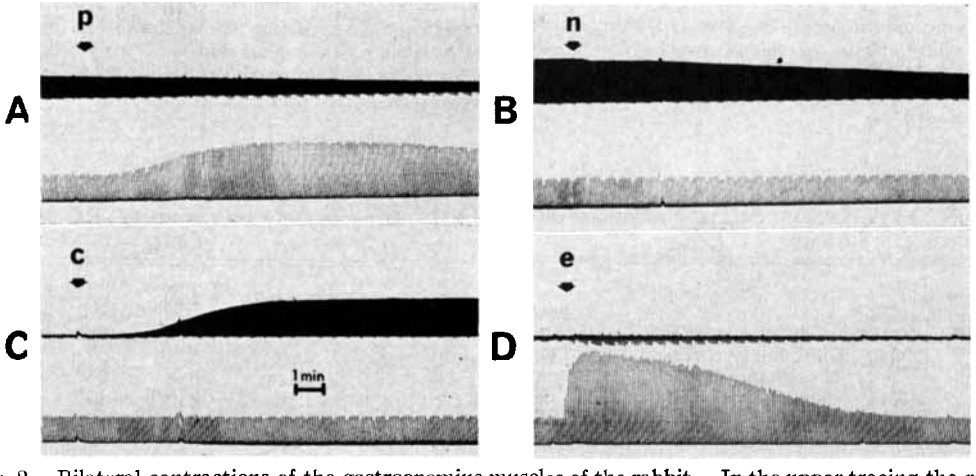

Fig. 2.—Bilateral contractions of the gastrocnemius muscles of the rabbit. In the upper tracing the rate of supramaximal electrical stimulation of the ipsilateral sciatic nerve was 3/sec. The rate of stimulation in the lower tracing was 0.4/sec. In A, NP-HC-3 (n) 1 mg./Kg., i.v. was given. In B, neostigmine (p), 0.05 mg./Kg., i.v. was given. In C, edrophonium (e), 0.2 mg./Kg., i.v. was given. In D, choline (c), 10 mg./Kg., i.v. was given.

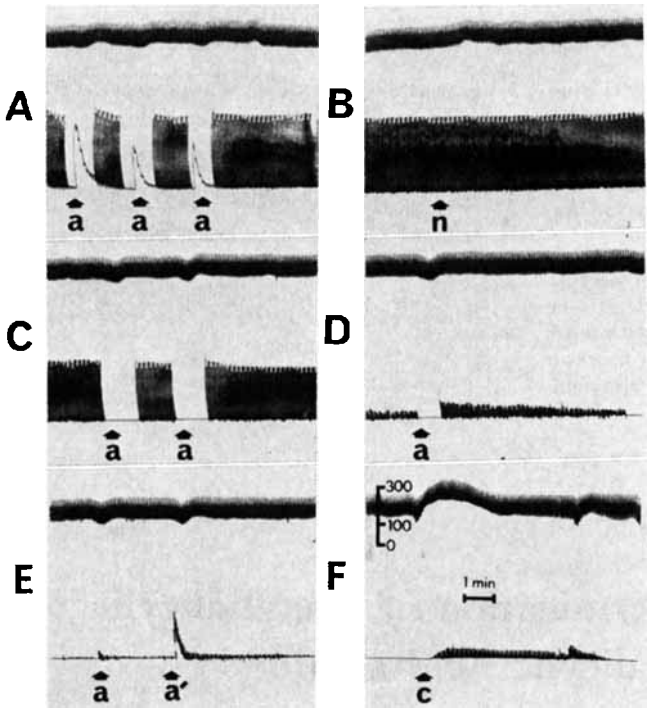

Fig. 3.—Femoral arterial blood pressure and gastrocnemius muscular contractions of the chicken. In A, three close-arterial injections of acetylcholine (a) 0.006 mg./Kg., i.v. In B, NP-HC-3 (n), 1 mg./Kg., i.v. was given. In C, D, and E acetylcholine was given in doses of 0.006 mg./Kg. (a) and 0.01 mg./Kg. (a^1) i.v. In F, choline (c), 10 mg./Kg., i.v. was given.

esis. The neuromuscular blockade produced by high frequency stimulation could be reversed partially, and often completely, with choline and not with neostigmine. However, with muscles being stimulated at a lower frequency, there was no antagonism with choline, whereas neostigmine caused a considerable augmentation in the height of contraction. This may be explained by assuming that during the total blockade seen during high frequency stimulation, all of the acetylcholine stores had been depleted. Thus, even relatively high concentrations of the indirect-acting neostigmine were ineffective in increasing the acetylcholine concentration to an effective level. On the other hand, choline, being the precursor of acetylcholine, replenished the reserves. During low frequency stimulation the acetylcholine stores were only partially depleted.

The temporary reversal observed with initial doses of tetraethylammonium (TEA) during total neuromuscular blockade with high frequency stimulation, and the ineffectiveness of subsequent doses of TEA, has been observed previously by Volle (14) with HC-3. TEA has been shown to be a releaser of acetylcholine from presynaptic nerve endings (15). This is suggestive of a situation in which NP-HC-3 or HC-3 inhibit the synthesis of acetylcholine in presynaptic nerves, and the effect observed with the initial doses of TEA was a facilitated release of the residual acetylcholine. The effect of TEA on the neuromuscular blockade produced by NP-HC-3 and HC-3 should also be reconsidered in the light of recent findings by Bhatnager *et al.* (16). They found that TEA and a variety of other quaternary ammonium compounds acted as inhibitors of acetylcholine synthesis in nervous tissue. The apparent paradox posed by the observations of Volle, Bhatnager, and ourselves may be resolved by pointing out that quaternary ammonium compounds such as HC-3, NP-HC-3, and TEA, may act in at least two ways: (*a*) they may either increase the fragility of the synaptic vesicles, or (*b*) they may inhibit formation of acetylcholine.

It is possible that these quaternary ammonium compounds act like detergents and cause an emulsification of the vesicular membranes. Under such conditions the acetylcholine formed would be exposed to immediate hydrolysis by acetylcholinesterase. In addition, the rate of transport of these quaternary ammonium compounds to the site of acetylcholine synthesis may be altered during varying levels of nervous activity, *e.g.*, resting *versus* high frequency stimulation. Perhaps this may be the reason why the increase in frequency of stimulation lowered the effective neuromuscular blocking doses and accelerated the onset and increased the duration of action.

The lack of response to the close-arterial injections of acetylcholine in the unstimulated gastrocnemius muscle of the chicken during the course of complete neuromuscular blockade indicates a curare-like effect of NP-HC-3. However, the temporary reversal of the neuromuscular blockade seen when acetylcholine was administered close-arterially during or immediately prior to electrical stimulation suggests an additional mechanism of action, *i.e.*, the injected acetylcholine is taken up by the nerve and is then available for release in response to stimulation. The release of the newly acquired acetylcholine from the nerve ending may require extraneous stimulation such as electrical impulses or TEA. The lack of effect of neostigmine during total neuromuscular blockade in preparations

stimulated at high frequencies could reflect either a deficiency of acetylcholine release or that the end plate receptors are already occupied. Observation that the blockade is reversed by acetylcholine injected close-arterially during stimulation indicates that the latter situation does not obtain. Therefore, since the receptors can still respond to administered acetylcholine, the evidence is in favor of a presynaptic, rather than a postsynaptic, mechanism of action.

NP-HC-3 may be assumed to act, at least in part, by decreasing the presynaptic supply of acetylcholine. The exact mechanism by which this is caused has not been elucidated. Replacement of the biphenyl by the monophenyl nucleus has not altered the hemicholinium-like activity, although there is a decrease in the toxicity.

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Fluorometric Determination of Acetylsalicylic Acid and Salicylic Acid in Blood

By WINTHROP E. LANGE and SAUL A. BELL*

A useful micro-method has been developed for the paper chromatographic separation of acetylsalicylic acid and salicylic acid with subsequent determination of each compound by fluorometry from a 0.1-ml. sample of capillary blood. This method permits frequent determinations of both acetylsalicylic acid and salicylate blood levels on the same subject. The accuracy and precision of the micro-method has been studied. *In vivo* studies utilizing the method emphasized the validity of results obtained by a rapid micro-method of analysis.

MANY METHODS for the determination of salicylates in blood and in plasma have been described in the literature. The generally accepted methods of Brodie (1) and Routh (2) involve the extraction of the salicylate from the blood sample and the determination of the concentration colorimetrically by measuring the absorbance of an iron complex. However, Saltzman (3) and Chirigos (4) have described the determination of salicylate in biological tissues by measurement of the characteristic fluorescence of the salicylate ion on exposure to ultraviolet light.

The quantity of acetylsalicylic acid in the biological tissues was estimated from the difference between "free" salicylate and "total" salicylate, and conjugated salicylate being considered to be acetylsalicylic acid (5). Mandel (6) has reported a paper chromatographic procedure for the separation of acetylsalicylic acid from salicylic acid in a plasma sample, and their separate determination fluorometrically. Recently, Nikelly (7) described the gas chromatographic determination of acetylsalicylic acid in the presence of salicylic acid.

Although the information obtained from any of the above methods is valuable, there does not seem to be general agreement among investigators about the levels of acetylsalicylic acid in the blood after taking aspirin. Since the time span of the analgesic effect of aspirin (2–4 hr.) appears to be more closely related to the time that the acetylsalicylic acid persists in the blood, the blood level of acetylsalicylic acid would seem to be a critical measure of the potential analgesic effectiveness of an aspirin formulation. Thus, for the *in vivo* study of acetylsalicylic acid blood levels it is desirable to have a micro-method requiring small volumes of blood for the repeated sampling required to observe simultaneously sustained blood levels of acetylsalicylic acid and salicylic acid. The method should also be fast, practical, and accurate.

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Although the information obtained from any of the above methods is valuable, there does not seem to be general agreement among investigators about the levels of acetylsalicylic acid in the blood after taking aspirin. Since the time span of the analgesic effect of aspirin (2–4 hr.) appears to be more closely related to the time that the acetylsalicylic acid persists in the blood, the blood level of acetylsalicylic acid would seem to be a critical measure of the potential analgesic effectiveness of an aspirin formulation. Thus, for the *in vivo* study of acetylsalicylic acid blood levels it is desirable to have a micro-method requiring small volumes of blood for the repeated sampling required to observe simultaneously sustained blood levels of acetylsalicylic acid and salicylic acid. The method should also be fast, practical, and accurate.

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METHODS AND PROCEDURES

The fluorometric assay described below is based on the characteristic chromatographic separation of acetylsalicylic acid and salicylic acid and the ultraviolet emission of alkaline salicylate ion at 415 mμ when activated at 325 mμ.

Blood Sampling.—To 100 μl. of blood in a 3-ml. centrifuge tube was added 250 μl. of ethylene dichloride (redistilled). The sample was stirred vigorously with a glass rod for 10 sec., 50 μl. of 6 *N* hydrochloric acid was added, and the mixture thoroughly mixed for 1 min. The sample was then centrifuged at 6000 r.p.m. for 15 sec., thoroughly mixed for 1 min., and centrifuged again for 1 min. It was found that for reproducible results the finger before puncture had to be pretreated with isopropyl alcohol and the blood sample representative of the capillary circulation.

Paper Chromatography.—The ethylene dichloride layer in the centrifuge tubes was withdrawn by means of a 250-μl. transfer pipet. The contents of the pipet were carefully streaked in a narrow band across a $1^1/_2 \times 15^5/_8$ in. strip of S & S 589 White Ribbon chromatography paper. The strips were dried and developed by ascending technique in chromatographic tanks containing a 0.75% nitric acid developing solution. The strips were developed at 25° until the solvent front reached 21 cm. (about 1 hr.), and then were removed from the tanks and air dried. Two 4-cm. wide rectangular pieces were cut from the strips at R_f 0.6 and 0.8 corresponding to the salicylic acid and acetylsalicylic acid, respectively. The pieces from the chromatographic strip were placed in 50-ml. conical flasks and exactly 5.0 ml. of 5 *N* sodium hydroxide was added. The alkali was allowed to remain for exactly 8 min. in contact with the papers. The flasks were gently swirled during the hydrolysis and extraction period.

Fluorescent Measurements.—The alkaline solutions were poured into 12 × 75 mm. cells and read in a fluorometer (Turner model 110) containing a "sandwich" excitation filter consisting of two 7-54 filters and a Wratten No. 34A filter; for the emission filter a half-thickness Corning No. 5-58 filter was used. A 4-cm. rectangle cut from the lower portion of a developed chromatograph strip was treated with exactly 5 ml. of 5 *N* sodium hydroxide to give an instrument blank.

Preparation of Standard Curves.—Known amounts of acetylsalicylic acid in the range of 0–1.0 mcg./100 μl. and 0–100 mcg./10.0 μl. of salicylic acid were added to whole citrated blood. These samples were carried through the complete chromatographic separation and fluorometric assay. The meter readings from the fluorometer were plotted against concentration to give standard curves for the salicylic acid and acetylsalicylic acid. To provide assurance of the continuing validity of the standard curves, standard samples were carried through the complete procedure periodically. So long as precautions were taken to control the temperature and drafts of the chromatographic room, the standard deviation reported in the discussion was not exceeded.

The drug concentration in plasma can be estimated from the whole blood concentration either by assuming a 50% hematocrit and using a factor of 2, or by using the equation 1/1 − (hematocrit × 0.01) if the subject's hematocrit has been determined. A comparison of results as calculated by the 2 methods is shown in Table I. The failure to consider the actual hematocrit or binding of the drug by plasma proteins has no effect on the validity of results comparing different dosages or different dosage forms in the same group of individuals.

TABLE I.—COMPARISON OF SALICYLIC ACID BLOOD LEVELS BY THE MICRO-METHOD AND THE BRODIE PROCEDURE

Sampling	Salicylic Acid, mg. %: Micro-Method, Subj. A[a]	Micro-Method, Subj. B[b]	Brodie, Subj. A	Brodie, Subj. B
Whole blood (venipuncture)	1.8	6.7	2.0	8.5
Whole blood (fingertip)	1.8	6.6	...	...
Plasma	3.3	12.5	4.2	15.8
Plasma (from whole blood)[c]	3.5	12.7	...	...
Plasma (from whole blood)[d]	3.6	13.2	...	...

[a] Dosage was 10-gr. aspirin; blood samples taken 2 hr. after dosage; hematocrit, 49%. [b] Dosage was 10 gr. aspirin every 4 hr. for a total of 70 gr.; blood samples taken 2 hr. after last dose; hematocrit, 48%. [c] Calculated from whole blood by applying a correction for hematocrit as follows: mg. % plasma = mg. % whole blood × 1/1 − (hematocrit × 0.01). [d] Calculated from whole blood by applying a correction factor of 2.

Comments on Analytical Procedures.—All of the procedures in the assay were studied to eliminate unnecessary steps and variables and to reduce background fluorescence to a minimum. All glassware was scrupulously clean, cells were selected to have low fluorescence readings, and only reagents with low native fluorescence were used. Special care was taken to prevent contamination by dust that might produce fluorescence. Variability of subjects was reduced by requiring the dose of medication to be taken in a fasting condition with a fixed quantity of water. When repeated dosage studies were conducted the subsequent doses were taken at fixed times in relation to meals. Subjects were cautioned regarding the use of topical preparations because of the possibility of their containing salicylates or ultraviolet absorbers.

The time required for acidification and extraction of the blood samples was found to be critical as is well known in order that hydrolysis of the acetylsalicylic acid might be minimized and that the extraction could be as complete as possible. The method of application of the sample to the chromatographic paper by streaking was critical because of the large volume to be spotted and the need for well-separated sharply defined developed zones of acetylsalicylic acid and salicylic acid. The spotting of the large volume of sample was facilitated by passing a current of cool air over the paper during the operation to speed the volatilization of the ethylene dichloride. The selection of the chromatographic paper was dependent on its purity and separation characteristics. Whatman No. 1 paper gave the same separation of acetylsalicylic acid and salicylic acid as the paper used, but had to be pretreated with dilute nitric acid solution due to a high and variable background fluorescence. With the

S & S paper the R_f for the salicylic acid was approximately 0.6 while the acetylsalicylic acid R_f was 0.8 at room temperature which agreed with the values reported by Mandel (6). The R_f values were established by visual examination of a paper strip containing a reasonable concentration of salicylic acid and acetylsalicylic acid under ultraviolet light. The salicylic acid appears as a blue fluorescent spot while the acetylsalicylic does not fluoresce. However, its R_f was determined by its hydrolysis to salicylic acid on the paper strip in the presence of ammonia vapors. Hydrolysis of the acetylsalicylic acid on the paper strips with alkali or ammonia vapors was found to be unnecessary. Kinetic studies showed complete conversion of the acetylsalicylic acid to salicylic acid and complete elution from the paper in from 5–8 min. in the 5 *N* sodium hydroxide solution.

The fluorescence of salicylic acid and acetylsalicylic acid was somewhat greater in solutions of higher sodium hydroxide concentration, but solutions stronger than 5 *N* disintegrated the paper substrate too rapidly and gave solutions difficult to handle due to their high viscosity. The filter combination used produced excitation at 318–327 mμ and emission fluorescence in the range of 350–480 mμ (maximum at 410 mμ). Other filter combinations were investigated but in most cases these combinations (*i.e.*, primary, Corning No. 7-54 and Wratten No. 34A; secondary, Corning No. 7-54) permitted some overlap of transmitted wavelengths. Fluorescence was found to be linearly proportional to the concentration of acetylsalicylic acid and salicylic acid up to 12 mg. % in 5 *N* sodium hydroxide and reproducible for a period of at least 1 hr.

In the preparation of standard curves for the salicylic acid and acetylsalicylic acid it was found that the recovery of either drug was higher from an aqueous solution than from whole blood. Thus, if aqueous solutions were used to prepare the standard curves one would obtain low concentration values during actual *in vivo* blood level studies. In a check of the accuracy and precision of the method it was found that 90 to 110% of acetylsalicylic acid and salicylic acid was recovered from whole citrated blood to which known amounts of the drugs had been added in the following ranges: acetylsalicylic acid—0.20, 0.50, and 1.20 mg. %; salicylic acid—2.00, 5.00, and 12.00 mg. %. In these same ranges, pairs of duplicate determinations made by the same analyst showed standard deviations of the pairs to be 0.015 to 0.021 mg. % for acetylsalicylic acid, and 0.07 to 0.14 mg. % for salicylic acid.

The micro-method was also studied in a comparison with the procedure of Brodie (1). Two subjects were given aspirin in different doses, and blood samples were assayed for salicylic acid by the 2 methods. Because the Brodie procedure is proposed for plasma separated from a sample of venous blood while the micro-method uses a sample of whole blood, subjects were sampled for both and each method was employed to estimate salicylic acid in both whole blood and in the separated plasma. The results are summarized in Table I. The data for the micro-method show good agreement between the venous whole blood and the capillary whole blood in both subjects. Also by applying a correction value for the hematocrit in each case the plasma salicylate values when calculated from whole blood agree well with that actually recovered from plasma by the micro-method. The higher results obtained by the Brodie method, which determines only salicylate, are possibly due to acetylsalicylic acid which hydrolyzed during the assay to salicylic acid.

APPLICATION OF THE PROCEDURE

This technique is not suitable for routine clinical use because of the critical time factor between collection of the blood sample and extraction of the acetylsalicylic acid. No more than 4 min. should elapse before completion of the extraction procedure, and no more than 6 min. should elapse before the extracted sample is ready for chromatography.

Past methods of analysis have, in general, depended on making the assumption that the difference between "total" and "free" salicylates in the plasma constituted the acetylsalicylic acid. Because of failure by some investigators to take into account the very rapid hydrolysis of acetylsalicylic acid by the serum esterase, investigators rarely agreed about the plasma levels. As a consequence, they were misled in drawing conclusions about the mechanism of absorption.

The authors have observed that absorption of acetylsalicylic acid is very rapid from the stomach, and therefore it can survive long enough to be absorbed into the blood stream. The authors have observed that absorption of acetylsalicylic acid from the intestines is much slower, and that, therefore, the rate of entry into the blood is too slow to permit an appreciable level to be built up. These observations are consistent with the conclusions of Schanker (8) and Smith (9). They both have shown that an increase in pH reduces the rate of absorption of acetylsalicylic acid and salicylic acid to a lesser extent.

TABLE II.—PLASMA SALICYLATE LEVELS[a]

Subj.	Drug Detd.	Dose Given	0.5	1	2	4	4.5	5	6	8
Av. (15 males)	ASA[b]	20r[c]	1.27	0.69	0.17	0.13	1.10	0.79	0.08	0.02
Av. (20 females)	ASA	20r[c]	1.20	0.76	0.28	0.17	1.61	0.89	0.14	0.03
Av. (15 males)	TS[d]	20r[c]	3.8	4.7	4.8	3.4	6.1	7.5	7.3	5.8
Av. (20 females)	TS	20r[c]	3.9	5.3	6.3	5.0	9.2	10.4	10.3	7.9
Av. (11 males)	ASA	20[e]	2.17	1.30	0.29	0.07	...	...	0.04	0.01
Av. (13 females)	ASA	20[e]	2.30	1.42	0.55	0.14	...	...	0.09	0.01
Av. (11 males)	TS	20[e]	6.4	8.6	9.5	8.4	...	...	6.8	6.4
Av. (13 females)	TS	20[e]	6.6	8.5	11.7	10.1	...	...	8.0	6.9

[a] mg. % in plasma was calculated from whole blood with assumed hematocrit of 50% in all cases. [b] ASA = acetylsalicylic acid. [c] 20r = two 5-gr. aspirin tablets at time zero and two 5-gr. tablets again at 4 hr. [d] TS = total salicylate. [e] 20 = four 5-gr. aspirin tablets taken at time zero.

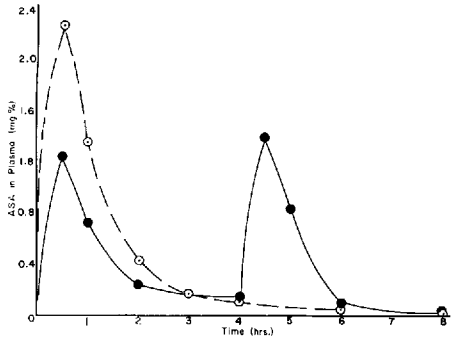

Fig. 1.—Comparison of average plasma levels of acetylsalicylic acid (ASA) after taking single and divided doses of aspirin at equal total dose of 20 gr. Key: ⊙, aspirin, single dose (4 × 5 gr.) 20-gr. dose at time 0; ●, aspirin, divided dose (2 × 5 gr.) 10 gr. at time zero and (2 × 5 gr.) 10 gr. after 4 hr. for a total 20-gr. dose.

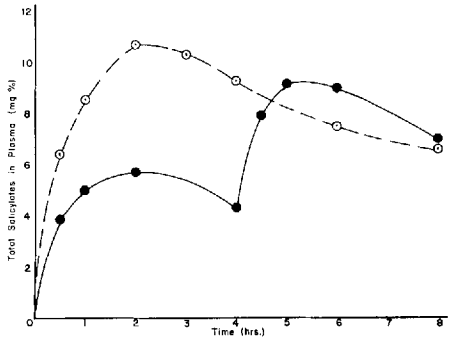

Fig. 2.—Comparison of average plasma levels of salicylates after taking single and divided doses of aspirin at equal total dose of 20 gr. Key: ⊙, aspirin, single dose (4 × 5 gr.) 20-gr. dose at time zero; ●, aspirin, divided dose (2 × 5 gr.) 10 gr. at time zero and (2 × 5 gr.) 10 gr. after 4 hr. for a total 20-gr. dose.

To provide a picture of the levels of acetylsalicylic acid and total salicylate in the blood after normal doses of aspirin, 35 subjects took 10 gr. of aspirin at time zero (8:00 a.m.) and a second 10 gr. exactly 4 hr. later. Blood samples were taken at various time intervals and assayed for acetylsalicylic acid and salicylic acid. Twenty-four of the above panel, at a later date, also took single 20-gr. doses of aspirin. The results of the 2 studies are summarized in Table II. Figure 1 is a graphic comparison of average plasma levels of acetylsalicylic acid after taking 10 gr. of aspirin repeated after 4 hr. for a total of 20 gr., and after taking 20 gr. of aspirin in a single dose. Figure 2 compares average total salicylate plasma levels after the same 2 dosage regimens.

The levels of acetylsalicylic acid and of the total salicylates in the plasma resulting from typical dosage regimens for aspirin provide a framework of reference for this new method of analysis of the content of drug and its principal degradation product in the blood. The peak values provide guidance in product formulation with respect to what levels in the plasma may be considered safe for a new aspirin dosage form. They also provide information which may be considered related to effectiveness. For instance it can be seen that a 20-gr. dose of aspirin, compared with a 10-gr. dose, gives a peak plasma level of acetylsalicylic acid almost twice as great (2.24 *versus* 1.25 mg. %). However, doubling the dose only slightly prolongs the plasma level of acetylsalicylic acid.

It will also be noted from Table II that differences are apparent in the average plasma levels for males and females. These are especially prominent in the total salicylate levels. A portion of this difference can be accounted for by the assumption made in calculating plasma levels that the hematocrits were all 50%. In the case of the females this would generally lead to a 9% high result. Also no consideration was given to differences in blood volumes of the individual subjects. This also on the average would show female plasma levels to be about 11% high.

A more detailed discussion of the clinical aspects of the micro-method and its implications in regard to the pharmacology of aspirin will be published at a later date.

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Thyroxin–Triiodothyronine Concentrations in Thyroid Powders

By W. F. DEVLIN and H. WATANABE

Eleven thyroid powders were assayed for their respective goiter prevention activities in thiouracil treated rats. These activities were compared with the corresponding triiodothyronine and thyroxin levels of the powders as determined by a chemical method. A comparison between pronase, enzyme P, and an erepsin–trypsin mixture was made with respect to their effectiveness in releasing thyroxin and triiodothyronine from commercial thyroid. Pronase was demonstrated to be preferable. There was a consistent relationship between the triiodothyronine levels of the powders and the goiter prevention activities. This relationship was independent of the thyroxin level. Bioassay of diets containing measured amounts of thyroxin and triiodothyronine confirmed the validity of the chemical analyses and the preponderant contribution of the triiodothyronine component to goiter prevention by this assay.

OF ALL the iodine-containing substances in thyroid powders, it is believed that triiodothyronine (T_3) and thyroxin (T_4) account for the total biological activity (1). Reported discrepancies in activity between thyroid preparations (2, 3) might therefore be attributed to varying amounts of T_3 and T_4 even though the total iodine remains constant. Correlation between the iodothyronine content and biological activity has been hampered mainly by difficulties in chemical procedures which must take into account such factors as (*a*) iodothyronine instability in solution (4–6), and (*b*) the slowness of iodothyronine release from thyroid protein by most proteolytic agents (7, 8). In this report, the hydrolytic actions of pronase, enzyme P, and an erepsin–trypsin mixture have been compared with respect to their effectiveness in releasing T_4 and T_3 from thyroid powders. The T_4 and T_3 concentrations of thyroid powders were compared with their corresponding goiter prevention activities. The activities of diets containing measured amounts of T_4 and T_3 were employed to confirm the analyses.

METHODS

Iodoamino Acid Determination.—A quantity of the thyroid powder, containing 50–200 mcg. of iodine, was placed in 2 ml. of 0.05 *M* Tris buffer at pH 8.5. One milligram of pronase[1] or enzyme P[2] was added, and the mixture was incubated at 39° for periods of time ranging from 6–96 hr. For the erepsin–trypsin[3,4] digests, 10 mg. of each enzyme was added at the beginning of the digestion time and 5 mg. of erepsin was added daily thereafter. Occasional gentle shaking during the first hour of incubation was helpful in dispersing the powder. Butanol extraction, paper chromatography, and measurement of the thyronines were performed as previously described (7). U.S.P. reference standard triiodothyronine (liothyronine) and thyroxin (B grade, Calbiochem, Los Angeles, Calif.) were employed throughout as thyronine standards. The iodothyronine values reported were the average of 4 separate determinations.

Goiter Prevention Assay.—The biological activities of the thyroid samples were determined by the goiter prevention assay employing thiouracil-treated adult female rats as the test animals (9). For the preparation of diets containing T_3 and T_4, solutions of each thyronine were made up in acid ethanol (90 ml. of ethanol plus 10 ml. of 2 *N* H_2SO_4) at a concentration of 100 mcg./ml. The required volumes of the thyronine solutions were mixed in a mortar with 2-Gm. amounts of casein, then dried in a stream of nitrogen and dispersed in ground Fox Cubes.[5]

RESULTS AND DISCUSSION

Rate of Release of the Iodothyronines, T_3 and T_4, During Proteolysis in Tris Buffer.—In Fig. 1 the effectiveness of pronase and enzyme P in releasing iodothyronines from commercial thyroglobulin (sample 6, Table 1) is compared with that of erepsin and trypsin in combination. Similar peaks of recovery of T_3 and T_4 were obtained in both enzyme P and pronase digests. The largest amount of T_3 was recovered following 6 hr. digestion, and the amount of T_4 recovered reached a near maximum level at 24 hr. Pronase was considered to be superior because a greater quantity of the iodothyronines was recovered. The reduced recoveries of the T_3 and T_4 with longer digestion periods in enzyme P and pronase digests were apparently due to iodothyronine instability in this buffer medium. A similar decline in recoverable T_3 and T_4 from enzymatic hydrolysates as the period of digestion was lengthened, has been reported by others (10, 11). Since the stability of T_3 relative to T_4 in this medium is not known with precision, it is not possible to say that pronase favors the release of T_3 over

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[1] Calbiochem, Los Angeles, Calif.
[2] Biddle-Sawyer Co., New York, N. Y.
[3] Erepsin, Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] Trypsin, Difco 1:250.
[5] Maple Leaf Mills, Toronto, Ontario, Canada.

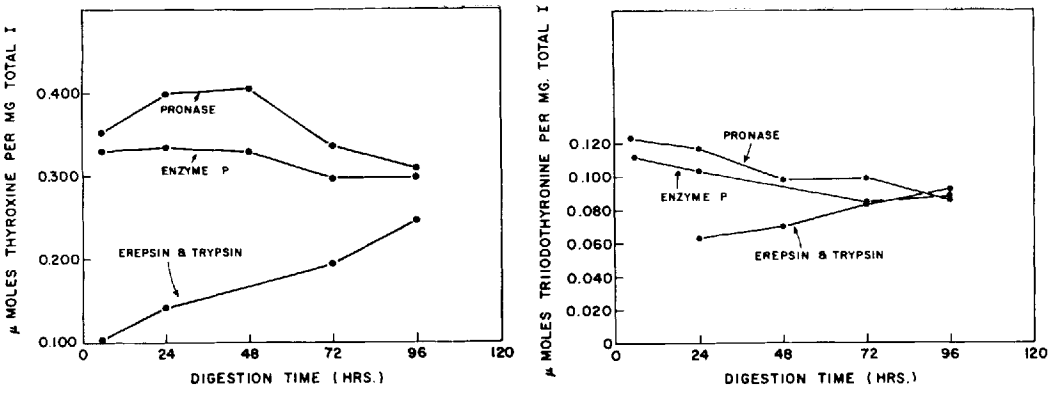

Fig. 1.—The recovery of thyroxin and triiodothyronine from enzymic digests of commercial hog thyroglobulin (1.02% iodine). Digestion was performed in 0.05 *M* Tris (hydroxymethyl aminomethane) buffered at pH 8.5. Butanol extracts of the hydrolysate were taken to dryness and the residue redissolved in ammoniacal alcohol immediately before application to paper chromatograms.

TABLE I.—IODOTHYRONINE CONTENT OF COMMERCIAL THYROID POWDERS

Sample	Species of Origin	Geographic Origin	Total Iodine, %	mcg./mg. Iodine T_4	mcg./mg. Iodine T_3	$\frac{I T_4}{I \text{ Total}} \times 100$	$\frac{I T_3}{I \text{ Total}} \times 100$	*M* Ratio $T_4:T_3$
1	...	...	0.63	114	41	7.5	2.4	2.3
2	Beef	U.S.	0.62	226	49	14.8	2.9	3.9
3	Beef	U.S.	0.55	294	50	19.2	2.9	4.9
4	Beef	...	0.44	348	60	22.7	3.5	4.8
5	Sheep	U.K.	0.48	280	73	18.3	4.3	3.2
6	Hog	U.S.	1.02	274	75	17.9	4.4	3.1
7	Hog	U.S.	0.73	268	78	17.5	4.6	2.9
8	Beef	U.K.	0.35	340	83	22.2	4.9	3.4
9	Hog	U.S.	0.20	210	87	13.7	5.1	2.0
10	Hog	U.S.	0.86	268	95	17.5	5.6	2.4
11	Hog	U.K.	0.60	256	100	16.7	5.9	2.1

T_4 in the early stages of hydrolysis as might be deduced from Fig. 1. There was a steady increase in iodothyronine recoveries up to 96 hr. in the erepsin–trypsin digests which indicated that the rate of their release from the thyroid protein under these conditions exceeded the rate of degradation. The greater bulk constituted by the erepsin–trypsin mixture in the hydrolysate medium may have conferred a measure of stability to the free iodothyronines in solution. In this connection, Rosenberg has observed that the rate of disappearance of T_4 is less in Viokase thyroid hydrolysates when compared with pronase hydrolysates (11). Although the recoveries of T_3 and T_4 continued to increase during the digestion period with the erepsin–trypsin mixture in Tris buffer, the final amounts of T_3 and T_4 actually obtained were less than the peak levels found with the other enzymes.

In a previously reported investigation (7) comparable peak recoveries of T_3 and T_4 were achieved in 96-hr. erepsin–trypsin digests when performed in borate buffer. However, the short digest periods required by pronase action reduced risk of losses by degradation or microbiological contamination. Also Tris buffer provided a better buffering capacity than the borate medium. Therefore, optimum conditions for the proteolytic removal of T_3 and T_4 from commercial thyroid powders has been defined as overnight digestion (15–18 hr.) in the presence of 1 mg. of pronase per 2 ml. of Tris buffer.

Iodothyronines in Commercial Thyroid Powders.—Table I summarizes the results of analyses of the T_3 and T_4 content in 11 thyroid powders obtained from 4 different laboratories. Sample *9* was diluted with filler of unspecified composition to meet the requirements of the "United States Pharmacopeia" (12). Sample *1* possessed an exceptionally low iodothyronine level which was reflected in bioassay experiments here and elsewhere. Samples comparable in quality and activity to sample *1* have been judged "clinically defective" in human therapy trials.[6] In general, the beef thyroid powders had a lower per cent of total iodine than the hog powders.

Excluding the defective sample, the per cent of the total iodine accounted for by the T_3 component ranged from 2.9–5.9. The per cent of the total iodine accounted for by the T_4 component ranged from 13.7–22.7. The molar ratios ranged from 3.4–4.9 in the beef powders and from 2.0–3.1 in the hog powders. This species difference in $T_4:T_3$ *M* ratios seems to be a characteristic which results from simultaneous elevated T_4 levels and reduced T_3 levels in the beef thyroids when compared with those originating from the hog. Pileggi *et al.* (13) have reported essentially the same range of T_3 concentrations as listed in Table I but slightly lower T_4 levels in a number of commercial thyroid powders of unspecified species origin. The single sheep sample

[6] Private communication from Dr. L. Meister, Veteran's Administration Hospital, Long Beach, Calif.

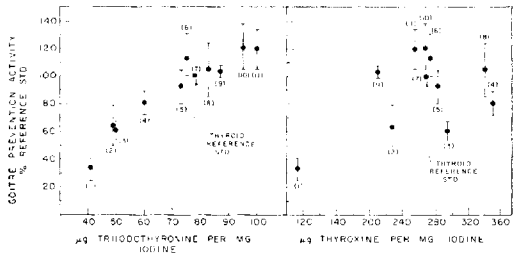

Fig. 2.—The relation between triiodothyronine and thyroxin concentrations in thyroid powders and goiter prevention activity in thiouracil-treated rats. The numbers in parentheses correspond with thyroid sample numbers listed in Table I. Vertical bars represent the 95% confidence limits for each estimate of potency.

reported in Table I had a molar ratio of 3.2; 22.6% of the total iodine was accounted for by the T_3 and T_4 components. In these respects, it was therefore intermediate between the hog and beef thyroid powders.

Goiter Prevention Versus T_3 Content in Orally Administered Thyroid.—In the goiter prevention assay the dose of desiccated thyroid was adjusted according to the total iodine content. Experiments employing this bioassay suggested that the T_3 component probably accounted for most of the response (14). To test this hypothesis, the individual T_3 and T_4 concentrations expressed as mcg./mg. total iodine in the thyroid powders were plotted against the respective goiter prevention activities (Fig. 2). All activities were expressed as a percentage of that possessed by the reference standard (sample 7, Table I). It was noted that, with the exception of sample 8, all thyroids possessing activity of more than 100 by this system of comparison originated from the hog. The T_4 levels within this group varied from 210–340 mcg./mg. iodine, and did not correlate well with the observed activities. However, in spite of the fluctuating T_4 levels, there remained a consistent relationship between the T_3 concentrations and the respective goiter prevention activities.

It was desirable also to compare the level of goiter prevention activity of thyroid powder with that of a synthetic mixture containing similar amounts of T_3 and T_4. If the same level of activity could be demonstrated in this manner, it would, in addition to lending support to the validity of the chemical analysis, show that neither the union of the hormones with thyroid protein at the time of administration nor the presence of other components normally present in thyroid powders contribute to the response. Consequently, the 3 diets listed in Table II were prepared. Diet *A* contained the reference thyroid powder while diet *B* contained a mixture of T_4 and T_3 in the same proportions as diet *A*. Diet *C* had the same content of T_3 as diets *A* and *B* but had no T_4. Diet *D* had T_4 only in a concentration which would reduce the gland weight in the assay to a suitable level for comparison with the other diets. For purposes of calculating the relative potency it was supposed that the added thyronines provided the same fraction of the total iodine content as the reference powder. The activities of the the powder and the corresponding thyronine mixture were approximately the same. The artificial mixture of T_4 and T_3 in diet *B* possessed only slightly higher activity than that of the reference powder. If the accuracy of the chemical analysis is accepted, this small discrepancy may be due to incomplete intestinal hydrolysis of the orally ingested powder and consequent reduced absorption of T_4 and T_3. This possibility has been raised by Levy and Knox (15) who observed a discrepancy of similar magnitude between the activities of a thyroid powder and its hydrolysate. Diet *C* which contained the same amount of T_3 as in the reference thyroid produced essentially the same activity as that of the powder itself. Diet *D*, with more than 5 times the thyroxin present in the reference powder diet mix, was only about one-seventh as active. The minor contribution of T_4 relative to T_3 under these conditions of assay is therefore confirmed. These findings are consistent with the relationships illustrated in Fig. 2.

It is acknowledged that the metabolic effectiveness of T_4 may be impaired in thiouracil-treated animals (16), and therefore in this assay the contribution of T_3 relative to T_4 will be somewhat exaggerated. However, the essential agreement between the activity of the powder and its corresponding T_3–T_4 mix suggests at least the validity of the T_3 measurement. It has now been deduced that the T_3 component of many thyroid tablets when administered orally during clinical therapy probably accounts for the greater part of the response (25).

The widely varying levels of T_4 and T_3 which have been reported for thyroid (17, 18) have often been attributed to the physiological state of the

Table II.—The Goiter Prevention Activities of Thyroxin–Triiodothyronine Mixtures Relative to a Corresponding Thyroid Powder

Diet	Material Added to Diet	Dose/100 Gm. Diet[a] μm. T_3	Dose/100 Gm. Diet[a] μm. T_4	Relative Potency with 95% Confidence Limits
A	Thyroid powder	0.004	0.013	1.0
		0.007	0.022	
B	$T_4 - T_3$	0.004	0.013	1.10 (0.99–1.25)
		0.007	0.022	1.11 (1.00–1.23)
C	T_3	0.004	...	1.03 (0.84–1.28)
		0.007		
D	T_4	...	0.096	0.14 (0.12–0.21)
			0.145	

[a] Two dosage levels of the thyroactive diets were administered in all bioassays. The amounts listed indicate the ratio present. Each dose group comprised 8 animals.

TABLE III.—T_4 AND T_3 LEVELS IN 1-gr. U.S.P. THYROID (65 mg. 0.2% IODINE)

Species	Sample[a]	mcg./gr. U.S.P. Thyroid T_4	T_3	*M* Ratio T_4:T_3
Beef	*2*	29	6.4	
Beef	*3*	38	6.5	
Beef	*4*	45	7.8	
Beef	*8*	44	10.8	
Av.		39	7.9	4.0
Hog	*6*	36	9.8	
Hog	*7*	35	10.1	
Hog	*9*	27	11.3	
Hog	*10*	35	12.4	
Hog	*11*	33	13.0	
Av.		33	11.3	2.5

[a] Sample numbers correspond to those appearing in Table I.

animals from which the glands were taken (19). Thyroid powders prepared on the North American continent for pharmaceutical use usually originate from gland pools representing large numbers of healthy animals. In those instances where the thyroid preparation had obviously low clinical activity (20), the possibility of losses or degradative changes during processing must be considered as a cause. Fresh thyroid tissue is known to possess autolytic activity (21) which could bring about selective losses of iodine-containing constituents, if, for example, the tissue were not processed or frozen immediately following collection. Also the unusual prospect of a thyroid powder acquiring a gain in activity during processing operations has been raised by Braverman and Ingbar (22). They have treated patients with commercial thyroid powder standardized to U.S.P. specifications which gave P.B.I. levels and other clinical responses indicative of markedly increased amounts of T_3 or some other equally potent thyroactive substance. Alternatively, this phenomenon might also be caused by a selective loss of T_4 during processing operations.

U.S.P. thyroid is required to contain 0.2 ± 0.03% iodine in thyroid combination (12). In Table III the levels of T_3 and T_4 have been calculated for U.S.P. thyroid as they would exist when prepared by dilution of the powders listed in Table I. From the average values, 1 gr. of a composite sample of the 4 beef thyroid samples would contain 39 mcg. of T_4 and 7.9 mcg. of T_3. Similarly, 1 gr. of U.S.P. thyroid prepared from the hog powders would have 33 mcg. of T_4 and 11.3 mcg of T_3. There is significantly less T_3 in the beef thyroid and this would account for previously reported lower levels of activity in beef thyroid when assayed by this method (14).

The present study suggested that the oral administration of desiccated thyroid reduced thiouracil-induced goiter in rats to an extent which was comparable to that brought about by an equivalent mixture of T_3 and T_4. In view of such factors as diet and species which are known to affect the availability of orally ingested T_4 (23, 24) the fraction of activity contributed by the T_4 component in a T_4–T_3 mixture may be influenced by the conditions of the experiment. Lavietes and Epstein (25) have demonstrated the difficulties in following the course of thyroid therapy from observation of serum P.B.I. levels when the T_4:T_3 ratio of the administered U.S.P thyroid is abnormally low. Clinicians have noted also some qualitative differences between the responses of T_3 and T_4 when administered separately (26, 27). In any event, an expression of potency for a thyroid powder will depend not only on its T_3 and T_4 content but also on the physiological effect selected as the response metameter.

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Effect of Complex Formation on Drug Absorption III

Concentration- and Drug-Dependent Effect of a Nonionic Surfactant

By GERHARD LEVY, KAREN E. MILLER, and RICHARD H. REUNING*

The effect of various concentrations of the nonionic surfactant polysorbate 80 on the absorption of a number of alcohols and barbiturates by goldfish has been studied. The absorption rate of the barbiturates was increased significantly in the presence of low concentrations of polysorbate 80, and decreased by higher concentrations of the surfactant. The absorption rate of the alcohols studied was not affected significantly by the surfactant. The retardation of barbiturate absorption at higher polysorbate 80 concentrations, which occurred also during mechanical agitation of the solution (when diffusion of drug to the absorbing membranes is definitely not absorption rate limiting), is interpreted as being indicative of the absence of a dissociating effect of the biologic membranes on the drug–micelle complex. The drug–micelle complexes differ in this respect from the nonmicellar dye complexes studied previously, apparently due to the greater exposure of substances in simple 1:1 complexes. Equilibrium dialysis and surface tension determinations have been carried out in an attempt to elucidate the mechanisms of the effects of polysorbate 80 on drug absorption. It is shown by kinetic analysis that the modification of barbiturate absorption by polysorbate 80 represents the net effect of enhanced absorption and decreased thermodynamic activity of the drug due to micellar complexation.

NUMEROUS studies of the effect of surfactants on drug absorption have shown that these agents can either increase, decrease, or exert no apparent effect on the transfer of drugs across biologic membranes (1). Some of the complexities and biopharmaceutical aspects of this problem have been reviewed recently (2). It is now appreciated that the type and magnitude of effect can be a function of the concentration (3) and chemical nature of the surfactant and that a given surfactant also may have certain specific pharmacologic properties of its own (2). It has been suggested by one of the authors that the observed effect of a surfactant on absorption may represent, in a certain concentration range, the net result of both enhancement and retardation of absorption (2).

Most of the studies of surfactant effects on drug absorption have been carried out on microbial systems. The results thus obtained may have limited applicability to multicellular organisms, since the latter are able to maintain homeostasis much more effectively. Moreover, the presence of enzymes and other vital cell constituents in the cell membrane makes unicellular organisms particularly sensitive to direct effects of surfactants. Absorption studies using small animals, isolated intestines, or man present other difficulties—dilution effects and interaction with mucus and/or other components of intestinal fluids and tissues make it practically impossible to maintain a constant known concentration of surfactant and drug. The results of such experiments, therefore, can be interpreted only qualitatively or, at best, semiquantitatively. These difficulties are not encountered when using fish for absorption studies (4–6). The major advantage of the fish system is due to the large volume of drug solution which may be used; this permits the maintenance of an essentially constant concentration gradient of drug and surfactant across the biologic membrane, despite continuous absorption of the drug and possible binding of some of the surfactant to mucus and/or membrane constitutents. Previous studies have shown that the drug absorption characteristics of fish membranes are similar to those of rats (5), while the latter yield results similar to those obtained in man (7).[1]

The investigation described here is part of a continuing study of the effect of complex formation of drug absorption (8, 9). The purpose of the presently described investigation was to determine the effect of various concentrations of a representative nonionic surfactant, polysorbate 80, on the absorption of certain noninteracting and interacting drugs by goldfish. It was desired also to elucidate the mechanism of the observed effects, and to examine their relationship to certain physico-chemical characteristics of the drug–surfactant system.

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Previous paper: Levy, G., and Matsuzawa, T., *J. Pharm. Sci.*, **54**, 1003(1965).

* Fellow of the American Foundation for Pharmaceutical Education.

[1] This statement refers to absorption by passive diffusion; there are appreciable differences between species in active transport characteristics.

EXPERIMENTAL

Goldfish, *Carassius auratus*, common variety, weighing from 15 to 25 Gm., were used. All fish utilized in a given set of experiments were from the same lot.

Drug Solutions.—All solutions were prepared in bulk on the day of the experiment from reagent, U.S.P., or N.F. grade chemicals. The drugs were dissolved in 0.05 *M* tris(hydroxymethyl)aminomethane (Tham) buffer, adjusted to pH 5.9 at 20° with hydrochloric acid.

Determination of Absorption Rate.—Single goldfish were placed in 250-ml. capacity beakers containing 175 ml. of drug solution at 20 ± 1°. The time of death, evidenced by cessation of gill and mouth movements, was noted. Absorption rate constants were calculated as previously described (5).

All determinations of time of death were carried out by the same individual. Prior to the experiment, all beakers containing the various drug solutions were labelled with code numbers by an individual not otherwise associated with the study. The codes were broken only after completion of the experiment.

Effect of Stirring on Time of Death.—Single goldfish were placed in 250-ml. capacity beakers containing 100 ml. drug solution at 28 ± 1°. Half of the solutions were stirred with a magnetic stirrer at about 500 r.p.m. The dimensions of the stirring bar were: diameter 0.25 cm., length 1.7 cm. The solutions could not be coded in this experiment since foaming on stirring made the surfactant-containing solutions readily recognizable.

Determination of Micellar Complexation.—The possible binding of ethanol by polysorbate 80 was investigated by equilibrium dialysis, using a method similar to that described previously (8). Ten milliliters of 2% w/v polysorbate 80 in 0.05 *M* Tham, pH 5.9, was placed in nylon dialysis bags.[2] Each bag was suspended in a 125-ml. conical flask containing 110 ml. of 2% v/v ethanol in 0.05 *M* Tham, pH 5.9. The solutions were equilibrated for 14 to 20 days at room temperature. The concentration of ethanol inside and outside the dialysis bag was then determined by the method of Hoult and Pawan (10).

The binding of secobarbital by polysorbate 80 was determined also by equilibrium dialysis. A number of different drug and surfactant concentrations were employed. The solvent system was 0.05 *M* Tham at pH 5.9. Due to the very poor permeability of the nylon membrane to secobarbital, cellulose dialyzer tubing[3] was used. In agreement with observations by others (11), this material was found to be impermeable to polysorbate 80. Equal concentrations of secobarbital were placed initially inside and outside the dialysis bag, and the solutions were equilibrated for 2 to 4 days at 20 ± 1°. The concentration of secobarbital on each side of the dialysis bag then was determined spectrophotometrically at 255 mμ, using 0.5 *M* sodium hydroxide solution as the diluent and blank. Polysorbate 80 interference in the secobarbital assay was additive; analytical results from polysorbate 80-containing solutions were corrected appropriately. The binding data yielded Freundlich-type isotherms (8) which permitted determination of the degree of binding at various total secobarbital concentrations.

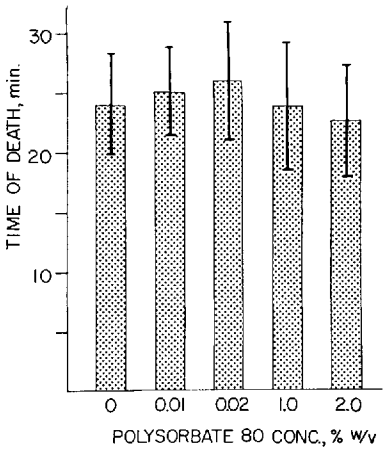


Fig. 1.—Effect of polysorbate 80 on the time of death of goldfish immersed in 5% ethanol solution (pH 5.9, 20°). Mean of 10 fish each. Vertical bars indicate ±1 standard deviation.

Surface Tension Determinations.—Surface tensions were determined with a Du Nouy tensiometer[4] at 20 ± 1°, using standard procedures for cleaning of the ring and for correction of the instrumental readings (12). To reduce surface aging effects, the solutions were swirled and agitated moderately immediately before each reading. The reported values are therefore dynamic rather than equilibrium values.

RESULTS AND DISCUSSION

It was found in preliminary experiments that immersion of goldfish in 0.01 to 2.0% polysorbate 80 solutions for 24 hr. had no apparent deleterious effect on the fish. This is consistent with the very low acute systemic toxicity of polysorbate 80 in other animals; for example, Nissim (13) reported that subcutaneous injection of up to 8 Gm. of polysorbate 80 per Kg. body weight did not produce any untoward effects in mice.

Polysorbate 80 had no significant effect on the rate of absorption of ethanol by goldfish, as judged by the time of death of the fish after immersion in 5% ethanol solution containing up to 2.0% of the surfactant (Fig. 1). Equilibrium dialysis showed that there was no binding of ethanol by polysorbate 80.

The surfactant had a pronounced, concentration-dependent effect on the absorption rate of secobarbital (Fig. 2). The barbiturate was absorbed more rapidly in the presence of low concentrations of polysorbate 80; higher concentrations of the surfactant decreased the rate of absorption significantly. Equilibrium dialysis indicated that there was considerable binding of secobarbital by polysorbate 80 when the concentration of the surfactant was in the range which caused retardation of secobarbital absorption. Since a previous study

[2] Tomac Nylon bags, American Hospital Supply Corp., Evanston, Ill.
[3] Fisher Scientific Co Model 70545, Central Scientific Co. Chicago Ill.

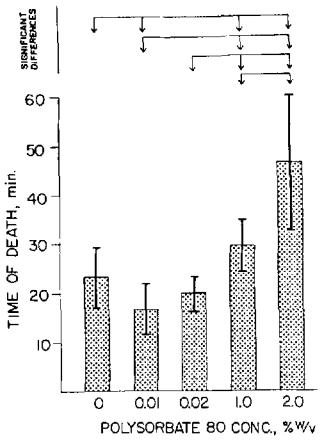


Fig. 2.—Effect of polysorbate 80 on the time of death of goldfish immersed in 0.020% sodium secobarbital solution (pH 5.9, 20°). Mean of 10 fish each. Vertical bars indicate ±1 standard deviation. Arrows connect values which differ significantly ($p < 0.05$) from one another.

(9) has shown that biologic membranes can have a dissociating effect on certain types of complexes, an experiment was designed to determine if the absorption-retarding effect of polysorbate 80 was due to the decreased thermodynamic activity of secobarbital as a consequence of its partial micellar complexation, or if the effect is due to a decreased rate of diffusion of the drug in micelles to the biologic membranes. For this purpose, the effect of polysorbate 80 on secobarbital absorption from intensively stirred solutions was compared to the effect obtained in unstirred solutions. There was no difference in the respective ratios of the times of death in solutions with polysorbate 80 to those without polysorbate 80 (Table I). Since the absorption-retarding effect of the surfactant was present also during rapid stirring, where diffusion of drug molecules and molecular aggregates is definitely not absorption rate limiting,[5] it may be concluded that fish membranes do not have a dissociating effect on secobarbital–polysorbate 80 micellar complexes. The difference in the effect of biologic membranes on micellar complexes and certain simple 1:1 complexes (9) is due probably to the greater exposure of drugs in the latter. This permits better contact and facilitates the interaction between the drug and the biologic membrane.

Differences in the times of death listed in Table I and those shown in Fig. 1 reflect the effect of temperature on the absorption rate and the lethal dose of secobarbital, and on the binding of the drug by polysorbate 80. Technical difficulties made it impossible to carry out the stirring experiments at 20°. Stirring itself apparently enhanced drug absorption; this was due probably to the more rapid flow of drug solution through the mouth and thereby across the gills. The gills are responsible for about 50% of the total drug absorption (6).

In view of the apparent lack of dissociating effect of the biologic membranes on the micellar complex

[5] It is believed that this is true even in unstirred solutions, due to the constant movement of the fish. The described experiment was carried out to establish this fact under more rigorous conditions.

Table I.—Secobarbital Absorption by Goldfish from a Stirred and an Unstirred Medium[a]

Concn. of Polysorbate 80, w/v	Stirring	Time of Death,[b] min.	Ratio of Times of Death, With Polysorbate: Without Polysorbate
0	no	19.0 ± 5.7	1.5
2	no	28.6 ± 7.4	
0	yes	13.8 ± 2.8	1.5
2	yes	20.8 ± 3.9	

[a] 0.020% sodium secobarbital in 0.05 *M* Tham buffer, pH 5.9. [b] Mean of 5 animals ±1 standard deviation; determined at room temperature (28 ± 1°).

of secobarbital and polysorbate 80, and the absorption enhancing effect of polysorbate 80 itself, the model shown in Scheme I is believed to represent the over-all effect of the surfactant.

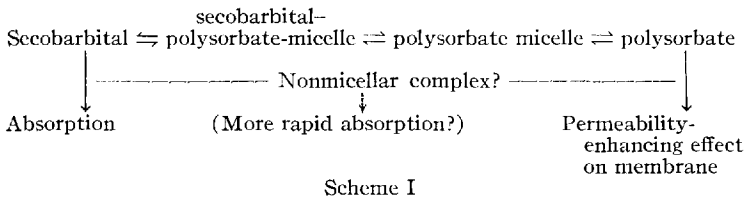
The possibility that the more rapid absorption of secobarbital in the presence of low concentrations of polysorbate 80 is mediated by a nonmicellar secobarbital–polysorbate 80 complex will be considered in a subsequent paragraph. Disregarding this possibility, a kinetic model based on an equation derived previously (4, 5) would consist of the following relationships.

No polysorbate present: $k = L/(CT_L)$,
Polysorbate concentrations below CMC: $k' = L/(CT_L)$
Polysorbate concentrations above CMC: $k' = L/(C_f T_L)$

where k and k' represent the "normal" and the "enhanced" absorption rate constant, respectively; L is the lethal dose of the drug; C and C_f are the concentrations of total and free drug, respectively; T_L is the time of death, and CMC is the critical micelle concentration. This model may be verified by showing that the value of k' remains constant over a wide concentration range of polysorbate 80.

The apparent and corrected constants for secobarbital absorption in the presence of various concentrations of polysorbate 80 are listed in Table II. The corrected constants are based on the concentration of free secobarbital as determined by equilibrium dialysis. The low concentration of polysorbate (0.01%) is approximately the critical micelle concentration (14). Binding of secobarbital at this concentration was either absent or very slight (<5%). The values of k' were reasonably constant at 0.01, 1.0, and 2.0% polysorbate 80 concentrations and were appreciably greater than k. These results verify, within the limits of experimental accuracy, the kinetic model presented in the preceding paragraph. The data show also that the effect of the surfactant above the CMC represents the net result of absorption-enhancing and retarding effects, as has been suggested previously by one of the authors (2).

The question arises why polysorbate 80 enhances the absorption of secobarbital but has no such effect on the absorption of ethanol. It was thought possible that ethanol itself decreases the surface tension of the solution so much that addition of polysorbate 80 would have no appreciable additional effect. This possibility was ruled out experimentally (Table III). Moreover, it was found that



Scheme I

TABLE II.—RATE CONSTANTS[a] FOR SECOBARBITAL ABSORPTION IN GOLDFISH

Concn. of Polysorbate 80, % w/v	Concn. of Sodium Secobarbital, % w/v	Free Drug, %	Time of Death,[b] min.	Apparent[c] k L. Gm.$^{-1}$ min.$^{-1}$ × 10^6	Corrected[d] k L. Gm.$^{-1}$ min.$^{-1}$ × 10^6
0	0.020	100	23.2		8.19
0.010	0.020	>95	16.6		11.4
1.0	0.020	50	29.3	6.48	13.0 } k'
2.0	0.020	36	46.2	4.11	11.4

[a] $k = L/(CT_L)$, where L = 0.038 mg./Gm. body weight. [b] Mean value, based on 10 animals; determined at 20 ± 1°. [c] Based on total secobarbital concentration. [d] Based on free secobarbital concentration.

the surface tensions of secobarbital–polysorbate 80 and ethanol–polysorbate 80 solutions were quite similar at any given surfactant concentration. Alexander and Trim (3) have suggested that the enhanced absorption of a drug in the presence of surfactant concentrations below the CMC may be due to formation of a nonmicellar complex of increased interfacial activity which augments the amount of drug on the surface of the biologic membranes. No evidence for an association of secobarbital and polysorbate 80 at concentrations of 0.005 and 0.01% of the latter was obtained from solubility, equilibrium dialysis, and ultraviolet absorption data. However, it is possible that such interactions may yet be found by other, more sensitive methods.

Another possible reason for the difference in the effect of polysorbate 80 on the absorption of ethanol and secobarbital, respectively, can be related to the different routes of absorption of these 2 drugs. Ethanol can diffuse across membranes through pores, while secobarbital diffuses through the lipoid barrier (4). Polysorbate 80 in 0.01% concentration had no significant effect on the absorption of other low molecular weight alcohols but increased significantly the absorption of another barbiturate (Fig. 3). This lends support to the theory that polysorbate 80 could have a specific effect on the permeability of the lipoid barrier portion of the biological membrane. There is some direct and biochemical evidence which suggests such an effect (15).

TABLE III.—EFFECT OF POLYSORBATE 80 ON SURFACE TENSION OF DRUG SOLUTIONS

Drug[a]	Polysorbate 80 Concn., % w/v	Surface Tension,[b] dynes/cm.
0.02% Sod. secobarbital	none	62.9
same	0.010	40.5
same	0.020	39.5
same	1.0	38.7
same	2.0	38.4
5% Ethanol	none	55.4
same	0.010	41.5
same	0.020	40.8
same	1.0	38.7
same	2.0	39.2

[a] Dissolved in 0.05 M Tham, adjusted with HCl to pH 5.9 at 20°. [b] Mean of 4 determinations, obtained at 20 ± 1°.

Figure 3 shows also the results of another experiment with ethanol on a larger number of fish. The purpose of this experiment was to establish definitely that 0.01% polysorbate 80 had no effect on ethanol absorption. The results obtained with *n*-octanol, also depicted in Fig. 3, cannot be interpreted readily because the data were quite variable and since there was some evidence of formation of mixed micelles.

In summary, it appears that the absorption-enhancing effect of polysorbate 80 may be due to the formation of a nonmicellar drug–surfactant complex, or that it may represent a direct action of the surfactant on the lipoid barrier portion of the biologic membrane. The present data do not permit distinction between these possibilities. It is reasonable to assume that an effect of the surfactant on biologic membranes should be time-dependent, yet the kinetic analysis (which assumes a time-independent effect) gives no such indication. However, the time course of the permeability-

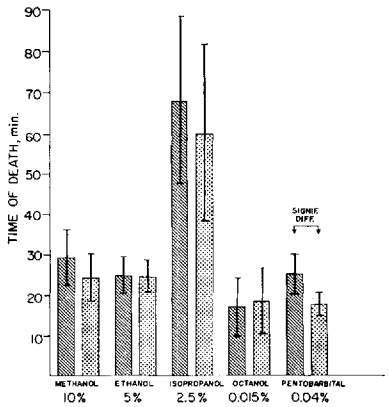


Fig. 3.—Effect of 0.010% polysorbate 80 on the time of death of goldfish immersed in solutions of various drugs in 0.05 M Tham (pH 5.9, 20°). Ethanol data are mean of 26 fish each, all others are the mean of 10 fish each. Vertical bars indicate ±1 standard deviation. Key: ▨, without polysorbate; ▩, with polysorbate 80, 0.01%.

enhancing effect could be such that most of the effect is elicited within a few minutes. Alternatively, the surfactant may promote better interfacial contact and thus increase the effective surface area of the membrane. Studies are now being initiated to determine if immersion of the fish in surfactant solutions for various periods of time will affect the rate of absorption of secobarbital upon subsequent immersion of the fish in secobarbital solutions without surfactant. This should establish whether or not the surfactant promotes drug absorption by modifying the barrier properties of the biologic membranes. However, the present investigation has shown already that the effect of polysorbate 80 on drug absorption is a function of the drug and of surfactant concentrations, and that an effect of polysorbate 80 concentrations above the CMC can represent the net result of absorption enhancement and retardation.

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Comparative Absorption of Micronized and Nonmicronized Medroxyprogesterone Acetate in Man

By DAVID L. SMITH, ALBERT L. PULLIAM, and ARLINGTON A. FORIST

A specific, sensitive method has been developed for the analysis in urine of microgram quantities of the principal urinary metabolite of medroxyprogesterone acetate. The method consists essentially of the following steps: hydrolysis with β-glucuronidase, extraction with chloroform, Florisil column chromatography, thin-layer silica gel chromatography, and measurement of either ultraviolet absorption or fluorescence resulting from sulfuric acid treatment. The method has been used to compare the gastrointestinal absorption of medroxyprogesterone acetate from tablets containing either 10 mg. of micronized or nonmicronized medroxyprogesterone acetate. The 8-hr. excretion of metabolite following oral ingestion of the tablets by normal adult humans was employed as the measure of absorption. The increased metabolite output, resulting from the tablet prepared from micronized medroxyprogesterone acetate, was very highly significant ($p < 0.001$). Ten subjects in a crossover study excreted an average of 2.23 ± 0.19 (S.E.M.) times as much metabolite in 8 hr. after ingesting the micronized formulation as they did after ingesting the nonmicronized one.

THIS STUDY was undertaken to develop an analytical method for the purpose of determining whether a tablet prepared from 10 mg. of micronized medroxyprogesterone acetate[1] (I) would afford a significant increase in absorption compared to a tablet prepared from nonmicronized material. Since medroxyprogesterone acetate has very low solubility in water (~0.3 mg./100 ml. at 37°), its gastrointestinal absorption may be limited by its gastrointestinal dissolution rate; reducing its particle size, therefore, might be expected to increase its physiologic availability (1–4). Helmreich and Huseby (5), who employed doses of 50–200 mg. of medroxyprogesterone acetate, have already noted that particle size reduction might influence its absorption efficiency.

Helmreich and Huseby (6) identified the principal urinary metabolite of medroxyprogesterone acetate as 6β,17α,21-trihydroxy-6-methyl-pregn-4-ene-3,20-dione,17-acetate (II). Others (7) have reported it to be the 21-acetate (III). This metabolite, which is excreted in the human as a glucuronide, accounts for approximately one-half of the total drug-related material excreted in the urine (5). The 24-hr. urinary output in the human was found to range from about 4–8% of a 200-mg.

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dose with more than 80% of medroxyprogesterone acetate-related material excreted in the feces (5). These data alone do not indicate poor absorption, however, since biliary excretion of medroxyprogesterone acetate and/or its metabolites is extensive even after intravenous administration of medroxyprogesterone acetate (8).

The approach of Helmreich and Huseby (5), who measured the amount of the major urinary metabolite excreted in the urine, seemed to be the most practical measure of absorption. A comparison of the absorption of medroxyprogesterone acetate at the 10-mg. dose level by measurement of the metabolite excreted in the urine required a very sensitive analytical method. The method developed is an extension of previously described procedures (5, 9, 10) and consists essentially of the following steps: (*a*) hydrolysis with β-glucuronidase, (*b*) extraction with chloroform, (*c*) Florisil[2] column chromatography, (*d*) thin-layer silica gel chromatography, and (*e*) measurement of either the specific ultraviolet absorption or fluorescence resulting from sulfuric acid treatment.

EXPERIMENTAL

Test Tablets.—Both tablets contained 10 mg. of medroxyprogesterone acetate and 0.05 mg. of ethinyl estradiol,[3] except that in one of them the medroxyprogesterone acetate was micronized. The micronized medroxyprogesterone acetate, which was used in the preparation of the compressed tablet, had a size distribution so that 99.9% of the particles were smaller than 10 μ diameter. Micronized and nonmicronized medroxyprogesterone acetate possessed specific surface areas, prior to preparation of the tablets, of approximately 7.4 $M.^2$/Gm. and 1.2 $M.^2$/Gm., respectively. Both of the formulations contained the other excipients of the commercial tablet.

Subjects and Conduct of Absorption Tests.—The tablets were ingested orally by normal female (postmenopausal) volunteers after an overnight fast; no food was taken until 2 hr. after administration of the tablet, and fluid intake was controlled. The ages and weights of the subjects are given in Tables II and III. Two tests were conducted: 1 tablet nonmicronized *versus* 1/2 tablet nonmicronized and, several months later, 1 tablet micronized *versus* 1 tablet nonmicronized. Both tests were of the crossover type, and required 2 weeks each. On day 1 of each week, control urine (8 a.m. to 4 p.m.) was collected and on day 2 one of the test tablets was ingested at 8 a.m. and urine collected from 8 a.m. to 4 p.m. The urine samples from each subject were randomly coded, but were assayed in parallel to avoid complicating the results with possible day-to-day assay variations.

Analytical Procedure.—The procedure used for the determination of the 6,21-dihydroxy metabolite of medroxyprogesterone acetate differs from the Helmreich and Huseby procedure (5) primarily in that it employs thin-layer chromatography.

Six milliliters of 0.5 *M* pH 4.7 acetate buffer was added to 60 ml. of the urine to be examined in a 100-ml. volumetric flask, and 15 ml. of 5000 units β-glucuronidase/ml.[4] added. The flask was placed in a constant-temperature bath at 47° for 18 hr. The hydrolyzed urine was transferred to a 250-ml. separator and extracted twice with 100 ml. of $CHCl_3$ as follows. The funnel was shaken vigorously for 1 min. and then allowed to stand for 1 hr.; the remaining emulsion was broken by gently swirling the separator. The $CHCl_3$ extracts were drained into a second 250-ml. separator, and the combined extracts washed with 20 ml. of 0.1 *N* NaOH and 20 ml. of 0.1 *N* HCl. Five minutes were allowed for each wash. The washed extract was drained into a 250-ml. beaker and evaporated to dryness under nitrogen at temperatures below 45°. The residue was dissolved in 5 ml. of $CHCl_3$ and transferred quantitatively to a previously prepared Florisil column (see below). The column was eluted with 25 ml. of $CHCl_3$, 25 ml. of 2% CH_3OH in $CHCl_3$, and 50 ml. of 25% CH_3OH in $CHCl_3$. The 25% CH_3OH–$CHCl_3$ fraction was collected in a 50-ml. wide-mouth centrifuge tube and evaporated to dryness under nitrogen at temperatures below 45°. The centrifuge tube was washed down with 5 ml. $CHCl_3$, 3 ml. $CHCl_3$, and 1 ml. $CHCl_3$, evaporating to dryness after each wash. The residue was dissolved in a small volume of $CHCl_3$ and quantitatively transferred to a fluorescent, 20 × 20 cm. Silica Gel G thin-layer plate of 250 μ thickness. The plate was developed to its full length with $CHCl_3$–C_2H_5OH (17:3), and the metabolite zone located by fluorescence quenching. The silica gel corresponding to the location of the metabolite was removed from the plate and transferred to a 35-ml. centrifuge tube. The metabolite was eluted from the silica gel by shaking with two 10-ml. portions of absolute methanol; after centrifuging, each eluate was decanted into a 50-ml. beaker. The combined eluates were evaporated to dryness under nitrogen at temperatures below 45°. Exactly 0.5 ml. of absolute methanol was added to the residue followed by exactly 4.5 ml. of 70% H_2SO_4. After the solution had stood for 30 min., the ultraviolet spectrum was recorded from 340–380 mμ with a Cary spectrophotometer.

If the fluorescence end point was to be used, the sulfuric acid solution was allowed to stand for 3 hr., and then with a precalibrated spectrophotofluorometer the fluorescence intensity at 535 mμ[5] resulting from activation at 465 mμ[5] was recorded using an Aminco-Bowman spectrophotofluorometer. The metabolite concentration in the urine and the total output were calculated from the appropriate standard curve, taking into consideration the total urine volume and dilution and yield factors. In the present study, an assay yield of 65.7 ± 2.2% was obtained. Correction for the "blank" was

[2] Florisil is a magnesium silica gel adsorbent manufactured by the Floridin Co., Tallahassee, Fla.

[3] This combination of medroxyprogesterone acetate and ethinyl estradiol is marketed as Provest by The Upjohn Co., Kalamazoo, Mich.

[4] Marketed as Ketodase by Warner-Chilcott.

[5] The fluorescence and activation wavelengths are uncorrected. (*Cf. Reference 15*, Chap. 4, p. 121.)

TABLE I.—FLUOROMETRIC AND ULTRAVIOLET RESPONSE OF THE 21-ACETATE (III) UPON TREATMENT WITH 63% SULFURIC ACID

Concn., mcg./ml.	Florescence at 535 mμ (F)[a] × Scale Rdg. × Sens. Factor	F/mcg./ml.	Absorbance 340 mμ	Absorbance 360 mμ	Absorbance 380 mμ	A_{Allen}	A_{Allen}/mcg./ml.
0.000	0.0000		0.000	0.000	0.000	0.000	
0.005	0.0075	1.50					
0.010	0.0155	1.55					
0.025	0.0369	1.48					
0.035	0.0556	1.59					
0.050	0.0769	1.54					
0.100	0.174	1.74					
0.250	0.435	1.74					
0.350	0.568	1.62					
0.500	0.747	1.49					
1.070	1.77	1.65	0.043	0.068	0.030	0.031	0.0290
3.250	5.35	1.65	0.135	0.202	0.082	0.093	0.0286
5.370	8.59	1.60	0.220	0.340	0.140	0.160	0.0298
7.490	11.0	1.47	0.305	0.470	0.190	0.222	0.0296
10.700	16.5	1.54	0.438	0.670	0.272	0.315	0.0294
	Mean	1.58					0.0293
	S.D.	±0.09					±0.00048
	% S.D.	±5.7%					±1.6%

[a] Activation: 465 mμ. Corrected for a blank of 0.015.

made by subtracting the result obtained with a pretreatment urine sample.

Preparation of the Florisil Column.—The Florisil was washed and activated as follows: 1 Kg. of Florisil (60–100 mesh) was washed by shaking sequentially with 2 L. of 95% ethanol for 1–2 hr., 2 L. of 25% CH_3OH—$CHCl_3$ for 2 hr., and 2 L. of fresh 25% CH_3OH–$CHCl_3$ overnight. The Florisil was then filtered and washed twice with 1-L. portions of absolute ethanol, dried, and then activated at 600° for 4 hr. The Florisil columns were prepared by packing 25-ml. burets (11 mm. diameter), plugged with glass wool, to a height of 7 cm. with gentle tapping. The columns were eluted with 50 ml. of $CHCl_3$ before adding the samples.

RESULTS

Analytical End Points for the 6,21-Dihydroxy Metabolite.—Since the 17-acetate (II) was not available for calibration at the outset of this study, the 21-acetate (III) was used. When II was synthesized, it was shown to have a response equivalent to III and IV in the end points used. This is in agreement with the results of Zaffaroni (11), who found that free steroids and their acetates gave identical sulfuric acid-induced absorption spectra.

Sulfuric Acid-Induced Ultraviolet Absorption.—Like many steroids (12), II, III, and their hydrolysis product (IV) exhibit an induced ultraviolet absorption upon treatment with concentrated sulfuric acid. Sulfuric acid-induced absorption has comparable sensitivity to the method of Porter and Silber (13), and is more specific for II and III. The 63% sulfuric acid-induced ultraviolet absorption of III at 360 mμ reaches a maximum value within 30 min. and is stable for at least 2 hr. When corrected for background by the Allen method (14), the ultraviolet absorption is linear from 1–10 mcg./ml. with a relative standard deviation of 1.6% (Table I). The Allen absorbance is defined by the equation:

$$A_{Allen} = A_{360m\mu} - {}^1/_2 (A_{380m\mu} + A_{340m\mu}) \quad \text{(Eq. 1)}$$

This correction, which is designed to eliminate the effects of linear background absorption due to impurities, yields a blank which is essentially independent of the urine volume and increases the selectivity of the end point. The induction of ultraviolet absorption by concentrated sulfuric acid in a solution of the metabolite, which was isolated from urine of the medroxyprogesterone acetate-treated subject (*vide infra*), was found to have a relative standard deviation of 1.7% at the 3 mcg./ml. level.

Sulfuric Acid-Induced Fluorescence.—Concentrated sulfuric acid also induces in compounds II, III, and IV an intense fluorescence maximum at 535 mμ when activated at 465 mμ. In this respect, they are similar to corticosterone (15). The 63% sulfuric acid-induced fluorescence response of III is linear from 0.005–10.7 mcg./ml. with a relative standard deviation of 5.7% (Table I). Replicate determinations of the production of fluorescence by the metabolite, which was isolated from the urine of a medroxyprogesterone acetate-treated subject, showed a relative standard deviation of 4.9% at the 3 mcg./ml. level. The fluorescence reaches a maximum in about 2 hr. and is stable for at least an additional 2 hr. The Allen correction could not be employed using the fluorescence end point because the impurities showed a nonlinear wavelength response.

Isolation of the Metabolite from Urine.—Various amounts of III were added to the urine of a normal, nontreated, female subject and carried through the procedure of Helmreich and Huseby (5). III could not be detected in the 25% CH_3OH–$CHCl_3$ fraction from the Florisil column; instead, all of it appeared in the 2% CH_3OH–$CHCl_3$ fraction in which the fluorescence response was directly proportional to the amount of III added to the urine. This result indicated that the metabolite was not III, as reported by Castegnaro and Sala (7), since the 25%

CH_3OH–$CHCl_3$ fraction contains by far the major portion of urinary metabolite.

Since III did not behave like the metabolite in the Helmreich and Huseby isolation procedure, it obviously could not be used to test or modify this procedure. Consequently, a 24-hr. urine collection from a female patient, who had been receiving 200 mg. of medroxyprogesterone acetate daily for 1 week, was obtained as a source of the metabolite.[6] This urine will be referred to as the "medroxyprogesterone acetate urine." Aliquots (0, 1, 2, 4, 6, and 10 ml.) of this urine were added to aliquots of a urine sample from a normal, nontreated female to give a total volume of 30 ml. Using the Helmreich and Huseby procedure, both fractions from the Florisil column were examined for the metabolite. By both the fluorescence and ultraviolet end points, the response of both the 2 and 25% CH_3OH–$CHCl_3$ fractions were proportional to the volume of medroxyprogesterone acetate urine added. The major portion of the responding material (90% by ultraviolet; 80% by fluorescence) was found in the 25% CH_3OH–$CHCl_3$ fraction. The results of these preliminary experiments with the Helmreich and Huseby procedure were so encouraging it was decided to test the procedure by assaying the 8-hr. urine samples from 5 subjects who had received either 1 or one-half tablet of the nonmicronized formulation. Employing duplicate assays, the increased urinary output of the metabolite resulting from 1 tablet over and above that from one-half tablet was just significant at the 95% confidence level. Although these results were encouraging, the Allen absorbance averaged only 0.022 at the 10-mg. medroxyprogesterone acetate dose level. These results indicated that although the Helmreich and Huseby procedure was applicable to the determination of the 6,21-dihydroxy metabolite at the low levels resulting from 10 mg. of medroxyprogesterone acetate, its sensitivity would have to be increased in order to statistically demonstrate small absorption differences.

Addition of Thin-Layer Chromatography (TLC) to the Helmreich and Huseby Procedure.—In an attempt to reduce the response of the blank, TLC was added to the procedure following the Florisil chromatography. After the 25% CH_3OH–$CHCl_3$ eluate was evaporated to dryness, the residue was dissolved in a small volume of $CHCl_3$, quantitatively transferred to a thin-layer plate, and developed with $CHCl_3$–C_2H_5OH (17:3). With this developing system the metabolite has an R_f of about 0.7 and moves ahead of most of the urinary components in the extract. In order to locate the position of the metabolite on the TLC plate and to study the effect of TLC on the procedure, aliquots of the medroxyprogesterone acetate urine were added to aliquots of a urine sample from a normal, nontreated female to give a total volume of 30 ml. These spiked samples were assayed essentially as before but with the addition of TLC to the procedure. Although at least ten TLC zones were observed in the 25% CH_3OH–$CHCl_3$ fraction, the density of one zone clearly increased with the volume of the medroxyprogesterone acetate urine added. Sections of the TLC plate were eluted with methanol, and assayed by measuring the ultraviolet absorption at 360 mμ following addition of 63% sulfuric acid. The metabolite zone produced 94% of the total absorption at 360 mμ. Another zone, whose R_f corresponded to the alcohol (IV), accounted for the remaining 6%. The addition of TLC to the procedure increased its specificity and lowered the background considerably, but decreased the over-all yield by about 20%.

Further Efforts to Increase Sensitivity and Assay Yield.—Further experiments were carried out with the medroxyprogesterone acetate urine in an attempt to improve the sensitivity of the procedure, *e.g.*, the extracting solvent, its volume, extraction time, number of extractions, and agitation method were varied in an attempt to increase yield and precision. It was found, for example, that an extraction method employing vigorous shaking by hand followed by allowing the emulsion to stand for 1 hr. gave higher yields and better precision than the use of an automatic wrist-action shaker.

Florisil chromatography decreases considerably the blank contribution from nonspecific background material (determined by the fluorescence end point or total ultraviolet absorption at 360 mμ) but does not remove any of the fluorescence-quenching TLC zones, *i.e.*, the chloroform extract and the 25% CH_3OH–$CHCl_3$ fraction show the same TLC pattern.

The yield of metabolite was increased by decreasing the contact time between the extracting solvent and the NaOH and HCl wash solutions. In the final procedure, the contact time of these wash steps was strictly controlled and minimized.

A relatively high concentration of β-glucuronidase was used in the present studies to insure complete hydrolysis. The 18-hr. incubation with β-glucuronidase at 47° was sufficient for complete hydrolysis, *e.g.*, 18 and 42 hr. incubation gave the same end point response.

The use of the total ultraviolet absorption at 360 mμ was investigated in an attempt to increase sensitivity. As shown by the data of Table I, the use of the Allen correction decreases the sensitivity by one-half. Because of variation in the absolute ultraviolet absorbance of the blanks at 360 mμ, however, the ultraviolet results were poor when the Allen correction was not used.

Washing and activating the Florisil decreased the total ultraviolet absorption of the blank considerably, but did not affect the assay yield.

The addition of TLC to the procedure and the optimization of other variables resulted in a three to fourfold increase in the sensitivity of the procedure. The assay yield was determined by adding the metabolite to water and performing the entire procedure. The yield determined in this manner was 65.7 ± 2.2%. Considering the complexity of the procedure, the yield and precision were considered quite satisfactory.

Stability of the Metabolite.—Originally, it had been intended to determine the yield every day that a set of assays were run by the use of a parallel yield determination, *i.e.*, by adding aliquots of a "standard solution" of the metabolite in methanol to control urine. In fact, this procedure was followed for some time until it was discovered that the yield was actually decreasing with time. TLC of the methanol solution of metabolite, which had been standing for some time, showed three zones,

[6] Dr. R. A. Huseby, American Medical Center, Denver, Colo., supplied this sample.

TABLE II.—8-hr. URINARY EXCRETION OF 6,21-DIHYDROXY METABOLITE OF MEDROXYPROGESTERONE ACETATE FOLLOWING INGESTION OF EITHER 1 OR 0.5 TABLET OF THE NONMICRONIZED FORMULATION (BOTH END POINTS USED)

Volunteer Code	Week 1 Tablet	Week 1 mcg. Excreted/8 hr. U.V.	Week 1 mcg. Excreted/8 hr. Fluor.	Week 2 Tablet	Week 2 mcg. Excreted/8 hr. U.V.	Week 2 mcg. Excreted/8 hr. Fluor.	Ratio 1 Tablet/0.5 Tablet U.V.	Ratio 1 Tablet/0.5 Tablet Fluor.
G(49, 80)[a]	1	247	204	0.5	79	63	3.13	3.24
J(63, 57)	1	325	298	0.5	134	134	2.43	2.22
C(61, 83)	1	286	357	0.5	115	124	2.49	2.88
S(60, 55)	0.5	87	180	1	298	570	3.43	3.17
L(51, 56)	0.5	192	170	1	270	257	1.41	1.51
						Mean	2.58	2.60
						S.E.M.	±0.35	±0.32
						95% C.I.	±0.97	±0.89

[a] Age and weight (Kg.), respectively.

whereas the fresh solution had shown only one. In four TLC systems the decomposition products of the metabolite possessed R_f values identical to III and IV. The sum of the absorbances of the three zones, as determined by sulfuric acid-induced ultraviolet absorbance at 360 mμ, remained constant, *i.e.*, the appearance of the zones corresponding to III and IV was accompanied by a corresponding decrease in II. The half-life of disappearance of the metabolite in methanol at room temperature is estimated to be about 3 days. No significant decomposition of II takes place during the analytical procedure used in the present study.

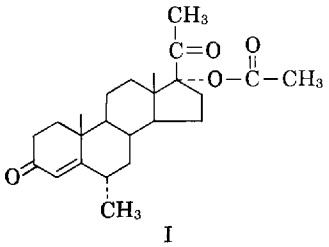
Evaluation of Metabolite Excretion as a Measure of Absorption (One Tablet versus One-Half Tablet).—To test the modified analytical procedure and the hypothesis that the amount of metabolite excreted in the urine is directly related to the amount of medroxyprogesterone acetate absorbed, urine samples were assayed from 5 subjects who had received 1 tablet and one-half tablet of the nonmicronized formulation in a crossover design (Table II). The assay easily distinguished between no drug, one-half tablet and one tablet. Both fluorescence and ultraviolet absorption were employed as the end points. With the modified procedure and using only single determinations, both the ultraviolet and fluorescence end points gave an 8-hr. output of metabolite for every subject in the order: $0 < 0.5 < 1$ tablet. The increased urinary output of the 6,21-dihydroxy metabolite resulting from 1 tablet over and above that from one-half tablet was statistically significant at the 99% confidence level by both end points, when calculated by averaging the ratios obtained for each subject. The average amount of metabolite excreted in the urine in 8 hr. following one-half tablet of the nonmicronized formulation was 121 ± 20 mcg. and 134 ± 64 mcg. (±S.E.M.) by the ultraviolet and fluorescence end point, respectively. The average amount of metabolite excreted in the urine in 8 hr. following 1 tablet of the nonmicronized formulation was 285 ± 13 and 337 ± 21 mcg. (±S.E.M.) by the ultraviolet and fluorescence end points, respectively.

Comparison of Excretion of Metabolite after Ingesting the Micronized and Nonmicronized Formulations.—Each of the 10 subjects excreted a larger amount of metabolite after ingestion of the micronized tablet (Table III). Only the ultraviolet end point was employed, since the sensitivity of the fluorescence end point was not required. There was a very highly significant difference ($p < 0.001$) between the nonmicronized and micronized formulations. The average micronized/nonmicronized ratio ±95% confidence limits of metabolite excreted was 2.23 ± 0.43, when calculated by averaging the individual ratios. The micronized/nonmicronized ratio ±95% confidence limits, calculated from the average amount of metabolite excreted in 8 hr., was 2.12 ± 0.57. The average amount of metabo-

TABLE III.—8-hr. URINARY EXCRETION OF 6,21-DIHYDROXY METABOLITE OF MEDROXYPROGESTERONE ACETATE FOLLOWING INGESTION OF MICRONIZED AND NONMICRONIZED TABLETS

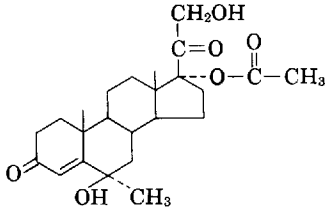
Volunteer Code	Week 1 Tablet	Week 1 mcg. Excreted/8 hr.	Week 2 Tablet	Week 2 mcg. Excreted/8 hr.	Ratio Micronized/Nonmicronized
A(64, 66)[a]	Nonmicronized	244	Micronized	778	3.19
B(61, 54)	...	351	...	915	2.61
C(60, 59)	...	278	...	757	2.72
D(63, 89)	...	176	...	468	2.66
E[62, 80)	...	243	...	596	2.45
F(59, 77)	Micronized	643	Nonmicronized	283	2.27
G(46, 77)	...	532	...	414	1.29
H(52, 65)	...	634	...	515	1.53
I(60, 68)	...	796	...	472	1.69
J(55, 72)	...	478	...	248	1.93
				Mean	2.23
				S.D.	±0.60
				S.E.M.	±0.19

[a] Age and weight (Kg.), respectively.



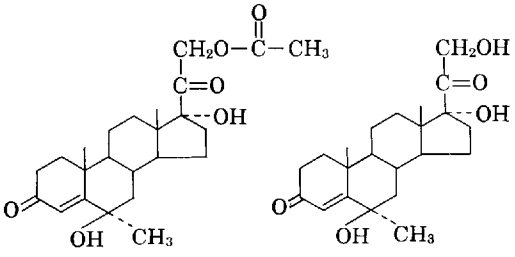
I

Medroxyprogesterone Acetate



II

(17-Acetate)



III IV

(21-Acetate)

lite excreted by the 10 subjects in 8 hr. was 312 ± 30 mcg. (S.E.M.) and 660 ± 47 mcg. following the nonmicronized and micronized formulations, respectively (3.1 and 6.6% of the dose, respectively).

DISCUSSION

Ten subjects were employed in a crossover design in order to minimize the effect of possible week-to-week variables, *e.g.*, a change in the percentage of drug which is converted to the measured metabolite or a change in the percentage of drug absorbed independent of formulation differences. The increase, therefore, in the quantity of metabolite excreted in the urine in 8 hr. after ingesting the micronized formulation undoubtedly results from increased absorption. The applicability of the procedure used was demonstrated by showing that an average of about twice as much metabolite was excreted in 8 hr. after ingesting 1 tablet as after one-half tablet.

The greater urinary excretion of the metabolite during the first 8 hr. following ingestion of micronized medroxyprogesterone acetate must result from the increased rate of absorption of medroxyprogesterone acetate from this formulation. Because of the finite transit time of drug through the gastrointestinal tract, this increased 8-hr. excretion of metabolite very likely reflects an increase in the eventual total amount absorbed. The latter interpretation is based on the data of Helmreich and Huseby, who found that even at doses 20 times greater than used in the present study, an average of about 70% of the 24-hr. excretion of metabolite occurred during the first 8 hr.

The metabolite is not the 21-acetate (III), as reported by Castegnaro and Sala (7), since the latter possesses different TLC R_f values from the metabolite in four systems and, unlike the metabolite, is eluted from the Florisil column in the 2% CH_3OH–$CHCl_3$ fraction. When the 17-acetate (II) was subsequently synthesized by an unambiguous method (16), it was shown to undergo the 17- to 21-acetate migration in methanol and to have the same R_f values as the metabolite in several systems, thus substantiating the 17-acetate assignment of Helmreich and Huseby (6). The observed conversion of II to III probably explains why Castegnaro and Sala identified the metabolite as the 21-acetate. Since the latter workers also found the metabolite in the 25% CH_3OH–$CHCl_3$ fraction of the Florisil column, the acetate group migration must have occurred after Florisil chromatography. This acetate group migration is not unique with the metabolite. Similar behavior has been noted by Gardi *et al.* (17), who observed that corticosteroid 17-monoesters could be rearranged quantitatively to the corresponding 21-monoesters.

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Direct Spectrophotometric Determination of Salicylic Acid, Acetylsalicylic Acid, Salicylamide, Caffeine, and Phenacetin in Tablets or Powders

By A. W. CLAYTON and R. E. THIERS*

A simple ultraviolet spectrophotometric method has been developed for the simultaneous determination of up to 5 different common analgesic compounds. The technique can be applied to mixtures of the compounds or to tablets or powders containing common excipients. A water–isopropanol mixture capable of dissolving chloroform is used as spectrophotometric solvent at 3 different wavelengths and under 3 different conditions of acid or base content. One absorbance measurement per constituent is required.

STANDARD analytical methods for the determination of commonly used analgesic and antipyretic compounds in tablets or powders generally involve multiple steps, and often include special separation methods. Thus in the method of the Association of Official Agricultural Chemists (1) for analysis of mixtures containing phenacetin, caffeine, and acetylsalicylic acid the compounds are separated by column chromatography prior to measurement. Other techniques of separation have been used, such as solvent extraction (2, 8), and many methods of measurement have been proposed, including infrared spectrophotometry (3), ultraviolet spectrophotometry (4, 5), titration (2), phosphorimetry (6), and nuclear resonance spectroscopy (7).

Of these approaches ultraviolet spectrophotometry seems to offer the greatest promise of accuracy combined with simplicity and the ability to measure a number of constituents. Mixtures of compounds have been analyzed in this fashion by several workers, in some cases after preliminary separation from each other (4, 5, 8–11). However, common excipients employed in the formulation of actual analgesic powders or tablets also possess ultraviolet absorption and can interfere.

A simple method has been developed which can simultaneously determine up to 5 common analgesic compounds together, in simple mixtures or in tablets and powders containing the common excipients used in manufacture. Separation of the 5 compounds is unnecessary. A mixture of isopropanol and water is used as solvent for spectrophotometry under 3 different conditions of acid or base content. Five absorbance measurements are made, and from the 5 values obtained the amounts of acetylsalicylic acid (ASA), salicylamide (SAL), salicylic acid (SAA), caffeine (CAF), and phenacetin (PHE) in the original sample can be calculated easily and accurately.

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EXPERIMENTAL

Apparatus and Reagents.—*Spectrophotometer.*—A Beckman DU 2 spectrophotometer was used, except for the acquisition of the spectra shown in Fig. 1, which were obtained on a Bausch & Lomb model 505 spectronic spectrophotometer. In all cases standard 1 cm. square fused silica cells were employed.

Solvents.—Spectral grade, analytical reagent chloroform and isopropanol (Mallinckrodt Chemical Co.) were used.

Mixed Solvent.—An acid solution of isopropanol and water was made by placing 400 ml. of isopropanol in a volumetric flask, adding about 500 ml. of water, adding 0.5 ml. concentrated hydrochloric acid, mixing, then diluting to volume with water.

Hydrochloric Acid.—Mallinckrodt, analytical reagent grade.

Sodium Hydroxide.—Harleco APHA, ammonia free, 50% solution (Hartman-Leddon Co., Philadelphia, Pa.).

Acetylsalicylic Acid.—Monsanto, U.S.P. grade.

Salicylamide.—S. B. Penick Co., N.F. grade.

Salicylic Acid.—Merck, reagent grade.

Phenacetin.—Monsanto, U.S.P. grade.

Caffeine.—Monsanto, U.S.P. grade.

All 5 analgesic compounds were checked by infrared spectrophotometry.

Spectrophotometric Reference Solution.—A reference or "blank" solution for spectrophotometry was made by pipeting 1.00 ml. of chloroform into a 200-ml. volumetric flask, adding about 80 ml. of mixed solvent, mixing carefully, then diluting to the mark with mixed solvent and remixing.

Procedure.—Weigh accurately about 1 Gm. of the dry powder or tablet to be tested. Place this sample into a 100-ml. volumetric flask. Add chloroform to volume and mix to dissolve all soluble matter. Set aside for 15 min., or until the undissolved material has settled, leaving a clear supernatant fluid.

Pipet 1.000 ml. of this supernatant fluid into each

of three 200-ml. volumetric flasks labeled A (for acidic), B (for basic), and H (for hydrolyzed). Add about 100 ml. of mixed solvent to each and mix. Add 1.0 ml. of 50% NaOH to each of flasks B and H and mix. Wait 15 min. to ensure complete hydrolysis of ASA, then add to flask H 3.0 ml. of 12 *N* HCl to render the solution acid again, and mix. Dilute all 3 to the mark with mixed solvent.

If all 5 components are to be determined, read the absorbance against the reference solution, of solution A at 250, 273, and 301 mμ, of solution B at 333 mμ, and of solution H at 301 mμ. If fewer components are to be measured, appropriate omissions and short cuts may be introduced by inspection of the equations below in the light of the specific problem.

Calculations.—If the absorptivity, *a*, of phenacetin, for example, at 333 mμ is designated a_{333}^{PHE},

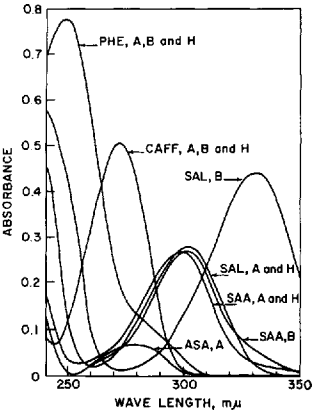

Fig. 1.—Spectra of 0.1 mg./L. solutions of the 5 compounds in mixed solvent.

$$A_{333}^{total} = a_{333}^{ASA}(ASA) + a_{333}^{SAL}(SAL) + a_{333}^{SAA}(SAA) + a_{333}^{CAF}(CAF) + a_{333}^{PHE}(PHE) \quad \text{(Eq. 3)}$$

Each of the 5 measurements made in the procedure provides an A^{total} for one particular set of conditions of wavelength and acid–base treatment. For each set of conditions a unique set of values of the various absorptivities exists. Five simultaneous equations may therefore be set up from which the 5 desired concentrations may be calculated. Other components of the original sample which are insoluble in chloroform do not interfere in the measurement. The chloroform itself is wholly soluble in the mixed solvent and its absorbance at the lower wavelengths is exactly cancelled by the reference solution.

Table I gives the value of *a*, the absorptivity, for each component under each condition and wavelength involved in the calculation. Figure 1 shows the spectra of 0.1 mg./L. solutions of the 5 compounds under the conditions shown.

As can be seen from both the figure and table, (*a*) the difference between the absorbance of solutions H and A at 301 mμ is wholly due to hydrolysis of acetylsalicylic acid to salicylic acid, (*b*) the absorbance of solution B at 333 mμ is predominantly due to salicylamide, (*c*) the absorbance of solution A at 301 is largely due to salicylic acid and the above two, (*d*) and finally, in solution A caffeine is the compound with by far the highest absorptivity at 273 mμ, while at 250 mμ phenacetin has this distinction. These facts permit a simpler but equally accurate approach to the calculations than the conventional solution of 5 simultaneous equations. In this approach a preliminary approximate calculation is made by equating to zero certain small absorptivities. This gives the following equations:

$$A_{301}^{H} - A_{301}^{A} = 2.10\,(ASA) \quad \text{(Eq. 4)}$$

which provides a value for (ASA)

TABLE I.—ABSORPTIVITIES[a]

Compd.	Condition: 333 mμ Flask B	301 mμ Flask A	301 mμ Flask H	273 mμ Flask A	290 mμ Flask A
ASA	0.11	0.05	2.15	0.63	0.52
SAL	4.40	2.81	2.81	0.57	1.73
SAA	0.17	2.75	2.75	0.48	0.96
CAF	0.02	0.05	0.05	4.92	1.57
PHE	0.03	0.28	0.28	1.81	8.02

[a] The absorbance values listed are those observed when 1.000 Gm. of each pure component is treated by the procedure described. Their units are therefore "absorbance units per Gm. in the original weighed sample." The values given are averages of triplicate measurements on each of triplicate weighed dry samples of the pure components repeated 4 separate times over a number of weeks.

and the concentration of phenacetin as (PHE), then the absorbance due to phenacetin at 333 mμ, A_{333}^{PHE}, will be given by:

$$A_{333}^{PHE} = a_{333}^{PHE}(PH) \quad \text{(Eq. 1)}$$

At any given wavelength, *e.g.*, 333 mμ the measured absorbance, A_{333}^{total}, is the sum of the absorbances of the various components.

$$A_{333}^{total} = A_{333}^{ASA} + A_{333}^{SAL} + A_{333}^{SAA} + A_{333}^{CAF} + A_{333}^{PHE} \quad \text{(Eq. 2)}$$

and therefore

$$A_{333}^{B} = 0.11\,(ASA) + 4.40\,(SAL) \quad \text{(Eq. 5)}$$

which provides a value for (SAL)

$$A_{301}^{A} = 0.05\,(ASA) + 2.81\,(SAL) + 2.75\,(SAA) \quad \text{(Eq. 6)}$$

which provides a value for (SAA)

$$A_{273}^{A} = 0.63\,(ASA) + 0.57\,(SAL) + 0.48\,(SAA) + 4.92\,(CAF) + 1.81\,(PHE) \quad \text{(Eq. 7)}$$

$$A_{250}^{A} = 0.52\,(ASA) + 1.73\,(SAL) + 0.96\,(SAA) + 1.57\,(CAF) + 8.02\,(PHE) \quad \text{(Eq. 8)}$$

TABLE II.—MIXTURE NUMBER[a]

Compd.	1 Taken	1 Found	2 Taken	2 Found	3 Taken	3 Found	4 Taken	4 Found	5 Taken	5 Found
ASA	0.200	0.194	0.350	0.353	0.300	0.294	0.250	0.247	0.300	0.301
SAL	0.300	0.297	0.000	0.000	0.300	0.302	0.150	0.149	0.100	0.097
SAA	0.000	0.000	0.000	−0.002	0.000	0.000	0.100	0.096	0.150	0.148
CAF	0.000	0.000	0.050	0.050	0.040	0.042	0.200	0.209	0.050	0.048
PHE	0.250	0.254	0.250	0.253	0.000	−0.001	0.050	0.052	0.100	0.103
Total	0.750	0.745	0.650	0.654	0.640	0.637	0.750	0.753	0.700	0.697

[a] Results are in Gm.

Equations 7 and 8 can easily be solved for (CAF) and (PHE).

The final calculation is made by rewriting Eqs. 5 and 6 to include all terms, as follows, then recalculating in the same manner as above but including the preliminary values for the terms missing in the first approximation.

$$A^{A}_{333} = 0.11\,(ASA) + 4.40\,(SAL) + 0.17\,(SAA) + 0.02\,(CAF) + 0.03\,(PHE) \quad (Eq.\ 5a)$$

$$A^{A}_{301} = 0.05\,(ASA) + 2.81\,(SAL) + 2.75\,(SAA) + 0.05\,(CAF) + 0.28\,(PHE) \quad (Eq.\ 6a)$$

For concentrations usually encountered in practice a third approximation is seldom necessary; and if any prior knowledge of approximate composition exists, it can usually be employed to make the first calculation the only one required.

If fewer than 5 constituents are known to be present, fewer readings and equations are obviously sufficient. The known absence of salicylamide makes the B flask and 333 mμ reading unnecessary, and of acetylsalicylic acid, the H flask and its reading. If salicylic acid is known to be absent it is possible to omit one step, but the omission is not recommended, since this step provides a measure of any hydrolysis of acetylsalicylic acid which might have occurred during storage or manufacture of the product analyzed.

The value $A^{H}_{301} - A^{A}_{301}$ can, of course, be obtained directly by making solution A the reference or "blank" sample at 301 mμ for solution H.

Discussion.—Three features of the method described combine with the known advantages of ultraviolet spectrophotometry to provide a simple and accurate procedure. First, the utilization of isopropanol–water as a solvent (as distinct from chloroform, etc.) permits spectra to be measured under either acidic or alkaline conditions, and permits the utilization of the solitary 333 mμ absorbance of basic salicylamide. This mixed solvent also dissolves sufficient chloroform to enable one to dilute a chloroform solution with an aqueous solution and still have a one-phase system. The lower limit of the concentration of isopropanol in the mixed solvent is in fact set by the amount of chloroform which must be dissolved. An important practicality—the mixed solvent handles like water and is more convenient than organic solvents. Second, the technique of rapid hydrolysis of acetylsalicylic acid to salicylic acid in basic solution followed by a return to acid conditions (solution H) provides a direct assay for the former compound in the presence of any other material unaffected by the treatment. Experiments were performed to determine the optimal concentration of base and time of hydrolysis. The values chosen provide complete hydrolysis in a conveniently short time interval without causing decomposition. Third, it is a fortunate fact that the excipients generally used in the manufacture of tablets and powders are not only insoluble in chloroform but settle out of suspension very rapidly when shaken with chloroform. Centrifugation, originally designed as part of the procedure, proved unnecessary. A variety of excipients were treated by the procedure described and in no case was any absorbance observed in the mixed solvent. These include talc, cornstarch, lactose, sucrose, microcrystalline cellulose,[1] dextrin, gelatin, acacia, tragacanth, fumaric acid, sodium chloride, and potassium chloride.

Ordinarily the spectrophotometric measurement of so many components and the setting up of so many simultaneous equations is neither convenient nor accurate. In this case, however, it has been possible to choose conditions where certain of the absorptivities approach zero, while others are maximal. In addition one set of the chosen conditions has, by hydrolysis, reduced the number of components present. The calculations are thus greatly simplified.

The procedure is inherently simple and accurate. As a test of these factors, 5 different known mixtures were prepared and presented to a technician who, although experienced in spectrophotometry, had never analyzed mixtures by this procedure. The results obtained on the one sample which was analyzed from each mixture are given in Table II. Duplicate absorbance readings were made on each cell of sample, but no replication of samples was permitted. Results were calculated (from the averages of the absorbance readings) exactly as described above with no prior knowledge of sample composition assumed. The total error in 25 determinations was −0.004 Gm.; the average deviation was ±0.002 Gm.

This procedure has been in use for 6 months in routine analysis and quality control of products containing some but not all of the 5 compounds, as well as numerous excipients. It is rapid, complete analysis of one sample requiring about 10 min. of the operator's time when multiple samples are being run, with another 10 min. required for calculation. Its precision, based on repeated analysis of standards during this time, can be expressed as a coefficient of variation of 0.5%. When necessary, this value could easily be decreased further by employment of techniques of ultra high precision spectrophotometry (12).

[1] Marketed as Avicel by the American Viscose Corp.

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Effects of Ionizing Radiation on Two Gelatin Fractions III

Carbonyl Group Analyses and Electron Spin Resonance Studies

By LEONARD P. PRUSAK* and BARTLEY J. SCIARRONE

Two fractions, F-I and F-II, obtained by alcohol fractionation of commercial pigskin gelatin, and having molecular weight values of 173,000 and 86,000, respectively, showed the following relationship with respect to additional carbonyl group content resulting from low-level irradiation under a 3 Mev. Van de Graaff: 1 per cent solution > 5 per cent solution (gel) > film. Irradiated films (F-I and F-II) showed doublets with 25 gauss line separation in electron spin resonance studies. These, together with previously published data, indicate that the gelatin fractions undergo molecular weight changes through free radical mechanisms involving scission, crosslinking, and weak bond formation.

In previous papers (1, 2) the authors showed the isolation of 2 fractions, F-I and F-II, from commercial pigskin gelatin; the irradiation technique used with a 3 Mev. Van de Graaff accelerator; acid-base titration behavior; sedimentation velocity and intrinsic viscosity studies; and molecular weight determinations.

We now give further experimental data which indicate that irradiation of these gelatin fractions produces changes in structure through free radical mechanisms.

The currently accepted concept regarding radiation effects on organic materials is that chain scission is synonymous with molecular weight decrease, and crosslinking is a sign of molecular weight increase. Intermediate variations are explained on grounds that both scission and crosslinking occur. This thinking may be extended to the irradiation of complex polymers, assuming that conditions are employed which lead to these ultimate effects. It is doubtful, on the basis of experimental data available today, that gelatin is susceptible to predictable irradiation behavior.

Some factors which must be considered before rupture or linkage are proposed as explanations for shifts in molecular weight of irradiated gelatin include solvent–solute interaction, absorbed irradiation dose, presence or absence of oxygen, solute concentration, nature of solvent, thermal history, aging, irradiation temperature, etc.

Since the formation of free radicals in solids and liquids exposed to irradiation has been established, explanations of molecular weight shifts in irradiated gelatin require consideration of free radical formation. This, in turn, poses the question as to indirect effects of the solvent if irradiation is carried out on a fluid system. Experimental evidence indicates that irradiation of oxygenated protein solutions, such as gelatin, results in the formation of carbonyl functions (3, 4) and that these are traceable to intermediate free radicals. Radiolytic cleavage of the peptide chain yields an amide and an additional carbonyl group:

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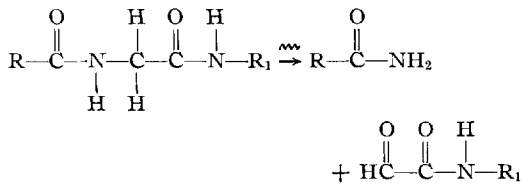
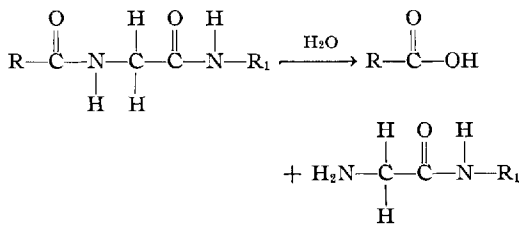


as opposed to hydrolytic fracture, which yields an acid and free amino group:



Proof of additional carbonyl group formation has been obtained (3) by isolation of a number of α-keto acids from irradiated gelatin—namely, oxalacetic, α-ketoglutaric, glyoxalic, pyruvic, and phenylpyruvic. Formation of these is believed to occur through an imino intermediate which yields the acid on hydrolysis.

Numerous details regarding the precise mechanistic sequences and interrelationships of free radical formation, chain scission, and crosslinking are yet to be resolved, but it can be said with reasonable certainty that these events do occur in irradiated gelatin. Data are presented here to show that additional carbonyl group formation affords an excellent means for correlating the degree of scission and/or crosslinking in the gelatin fractions as functions of irradiation dose and physical state (film or aqueous solution).

Since the formation of stable free radicals containing unpaired electrons in irradiated organic solids has been established, the application of electron spin resonance to the determination of the free radical quanta has assumed considerable importance. Because others (5, 6) have shown that the application of this method to proteins gives meaningful results, spin resonance measurements on irradiated gelatin films were included in the present work.

Table I.—Results of Carbonyl Analysis

	F-I μmoles Carbonyl/Gm.	F-II μmoles Carbonyl/Gm.
1% Soln., Mrad.		
0.41	23.4	11.8
0.60	39.5	13.5
1.15	53.1	25.3
2.10	66.6	47.6
5% Soln., Mrad.		
0.41	3.6	1.1
0.60	6.0	1.2
1.15	13.8	1.8
2.10	36.0	4.9
Film, Mrad.		
0.41	0.2	0.06
0.60	0.4	0.15
1.15	0.8	0.65
2.10	1.1	0.95

Table II.—Resonance Curve Values Normalized to Sweep 50, Gain 50

Peak-to-Peak Distance (Arbitrary Scale)	Sweep	Gain	Normalized Value	Dose, Mrad.
		F-I		
8.4	320	200	0.328	0.461
6.1	100	200	0.762	0.679
5.0	50	100	2.5	1.71
8.8	50	50	8.8	3.12
		F-II		
6.7	100	500	0.335	0.461
6.3	100	100	1.575	0.679
6.4	50	100	3.2	1.71
7.9	50	32	12.35	3.12

EXPERIMENTAL

Carbonyl Analysis.—Determination of carbonyl group formation was made according to the procedure of Lappin and Clark (7) using the 2,4-dinitrophenylhydrazone derivative of acetaldehyde as a reference standard. Hydrazone content was determined in terms of μm. carbonyl/Gm. of gelatin at 440 mμ with a Beckman DU spectrophotometer. Additional carbonyl group formation was obtained by difference between controls and irradiated samples. The data are given in Table I.

Electron Spin Resonance Studies.—Film strips of F-I and F-II were submitted to the scanned beam of the 3 Mev. Van de Graaff accelerator as previously described (1), and the resulting average absorbed doses were calculated immediately following irradiation. They were: 0.46, 0.67, 1.71, and 3.12 Mrads, respectively. The films were exposed to these doses at room temperature (24°C.), inserted in heat-sealed aluminum foil packages, and immediately immersed in liquid nitrogen (77°K.). Controls were included to the point of irradiation.

Spin resonance measurements, conducted on all samples on the day of irradiation, were made using a Varian 100 kc. field modulation EPR spectrometer at a microwave frequency of 9000 Mc./sec. Various degrees of attenuation of resonance intensity were used to obtain readable signals. Therefore, in order to have comparable measures of free radical population all resonance intensities, measured on an arbitrary scale, were normalized to the instrument parameters sweep 50 and gain 50. (See Table II.) A log-log plot of normalized resonance signal intensity *versus* dose is shown in Fig. 1.

RESULTS

Carbonyl Analysis.—In the case of 1 and 5% solutions of both fractions, straight line relationships were obtained from plots of dose (Mrad.) *versus*

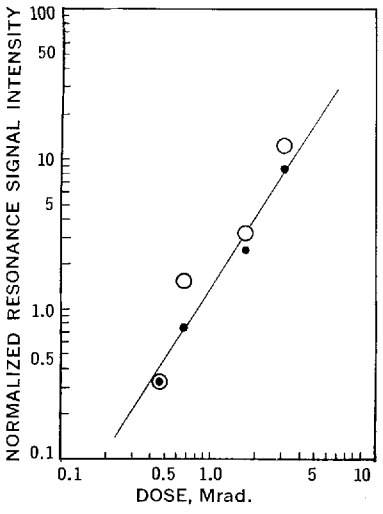


Fig. 1.—Plot of the linear relationship between EPR signal intensity, normalized to the instrument parameters sweep 50 and gain 50, and the dose impinging upon films of F-I (●) and F-II (○).

μm. carbonyl formed per Gm. of protein. Furthermore, some correlation was evident between the same concentration levels of the separate fractions. Figure 2 shows a plot of dose *versus* μm. carbonyl/Gm. of gelatin in 5% solution of both fractions. Data for obtaining the best possible straight line were computed by regression analysis. Slope values for 1% solutions of F-I (4.2×10^{-2}) and F-II (6.2×10^{-2}) were remarkably close, but the values for 5% solutions differed by a factor of 10 (*cf.* Fig. 2). Nonetheless, it can be stated that in irradiated solutions the number of carbonyl groups formed per Gm. of protein is directly proportional to the dose imparted and is concentration dependent.

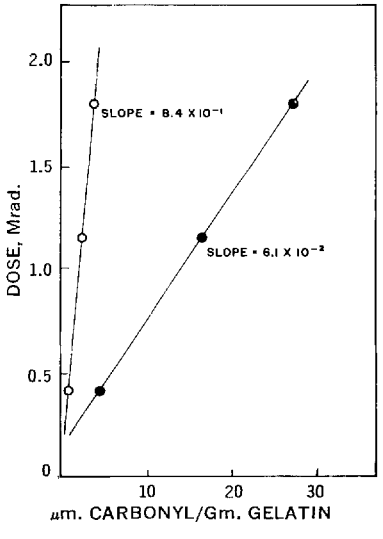


Fig. 2.—Arithmetic relationship between the dose, impinging upon a 5% solution of F-I (●) and F-II (○), and the resulting extra μm. of carbonyl groups produced/Gm. of the gelatin fraction.

Figure 3 shows a semilog plot of dose *versus* μm. carbonyl/Gm. of gelatin in irradiated films of F-I and F-II. In contrast to 1 and 5% solutions, the films showed less carbonyl formation, and proportional relationship to the logarithm of the dose. It would appear that carbonyl groups were formed at the same rate in the 2 gelatin fractions despite widely different average molecular weights.

G values, or the number of events (carbonyl groups formed) per 100 ev. (electron volts) absorbed energy, were derived from the formula:

$$G = \frac{\text{No. of changed molecules} \times 100}{E\ (\text{ev.})}$$

$$= \frac{(\mu\text{m./Gm.})\ (6.06 \times 10^{17})\ (100)}{(\text{Mrad.})\ (\text{ev./Gm./Mrad.})}$$

The expression above applies to materials irradiated in the dry state, *i.e.*, films, since the second term

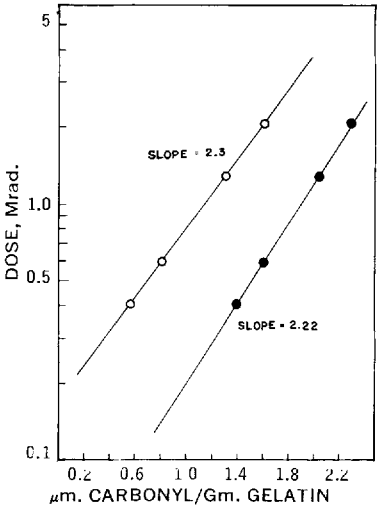


Fig. 3.—Semilog relationship between the dose, impinging upon films of F-I (●) and F-II (○), and the resulting extra μm. of carbonyl groups produced/Gm. of the gelatin fraction.

in the denominator refers to grams. In the case of 1% solutions, the second term was appropriately adjusted to enable calculation of energy absorption (*E*) on the basis of 100 ml. (1 Gm. of protein in 100-ml. vol.). Similarly, with the 5% solutions, another adjustment was made in the second term to permit calculation of energy absorption of a 20-ml. vol. Thus, the second expression in the denominator is, respectively, for

film: 6.37×10^{19} ev./Gm./Mrad.
1% solution: 6.37×10^{21} ev./100 ml./Mrad.
5% solution: $6.37 \times 10^{20} \times 2$ ev./20 ml./Mrad.

The *G* (carbonyl) values for both fractions are given in Table III.

Results for all samples were somewhat lower than the $G = 1.2$ reported by Garrison (3) for gelatin solutions (5 mg./ml.) irradiated with ^{60}Co γ-rays at a dose rate of about 2×10^{17} ev./ml./min. This difference in values can be accounted for by observing that Garrison's rate is equivalent to about 0.2

TABLE III.—EXPERIMENTALLY DETERMINED CARBONYL FORMATION *G* VALUES

Mrad.	F-I	F-II
	1% Soln., G Values	
0.41	0.54	0.27
0.60	0.63	0.21
1.15	0.44	0.21
2.10	0.30	0.22
	5% Soln., G Values	
0.41	0.42	0.12
0.60	0.47	0.10
1.15	0.57	0.08
2.10	0.82	0.11
	Film, G Values	
0.41	0.46	0.14
0.60	0.63	0.24
1.15	0.66	0.54
2.10	0.50	0.43

Mrad./hr. (2×10^{17} ev./ml./min. = 1.2×10^{22} ev./L./hr. Since 1 Mrad./hr. = 6.37×10^{22} ev./L./hr., 1.2/6.37 = 0.188, or 0.2 Mrad./hr.). In the 3 Mev. Van de Graaff, on the other hand, all dose rates were imparted on a "per second" basis. Thus, in the range of doses used in the present experiments (0.4–2.0 Mrad.), the dose rates differed by factors ranging from 7,200 to 36,000 (greater). Since the efficiency of free radical formation is reduced at higher dose rates through recombinations caused by high local concentration, the higher dose rate is responsible for the lower *G* (carbonyl) values.

Of collateral interest in connection with the *G* (carbonyl) value calculations, are the previously referred to Wiederhorn experiments (2), which showed that in parent gelatin (from which F-I and F-II were fractionated) the molecular weight between crosslinks increased as the irradiation dose increased. This implies the formation of thermostable covalent bonds, a requirement for copolymerization. F-I and F-II, on the other hand, did not respond to the crosslinking test at any irradiation level (at least through the crosslinking mechanism), hence failed to polymerize despite carbonyl formation. This, in turn, would suggest that either the number of carbonyl groups formed was inadequate (Table III), or that a crosslinking factor present in the parent source was lacking in the purified fractions, or both.

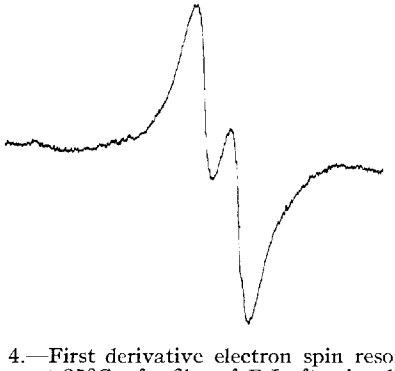


Fig. 4.—First derivative electron spin resonance spectrum at 25°C. of a film of F-I after irradiation with accelerated electrons giving a total dose of 3.12 Mrad. Instrument parameters used were sweep = 50, gain = 50.

Electron Spin Resonance Studies.—Figures 4 and 5 are first derivative resonance curves of F-I and F-II irradiated at 3.12 Mrad., and Figs. 6 and 7 show resonance curves on controls, all observed at room temperature. Figures 4 and 5 also illustrate the typical doublet of 25 gauss line separation obtained for all irradiated samples.

One of the samples, F-I, irradiated at 3.12 Mrad. at 25°C. was observed at 77°K. Total erasure of the doublet occurred when the gain was reduced to 125 and the sweep held at 50. By increasing the gain to 160 and reducing the power by 15 decibels, the same sample (at 77°K.) gave a doublet of 25 gauss line separation, indicating that at low temperature the signal was readily saturated. Shouldering beyond the points of inflection was evident, indicating possible dipolar broadening.

Shifting the sweep and gain to 32 and 200, respectively, retaining the sample at 77°K., and reducing the power by 20 decibels gave a doublet of 20 gauss line separation and retained evidence of shouldering. The sample was then removed from the nitrogen atmosphere and raised to room temperature (25°C.). At sweep 32, gain 500, −15 decibels, the pattern approached that originally obtained at room temperature with a doublet of 25 gauss. Retention of the doublets despite shifts in resonance temperature conditions (following irradiation at room tempera-

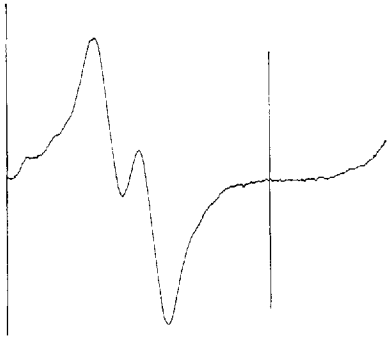


Fig. 5.—First derivative electron spin resonance spectrum at 25°C. of a film of F-II after irradiation with accelerated electrons giving a total dose of 3.12 Mrad. Instrument parameters used were sweep = 50, gain = 32.

ture) from 25°C. to 77°K. to 25°C. shows (5) that free radicals produced at room temperature remain in the same chemical form upon cooling and subsequent warming, and that changes in room temperature patterns following lowering of the temperature to 77°K. are reversible. The increase in signal strength at low temperature is primarily due to $1/T$ dependence of the magnetic susceptibility.

By normalizing the resonance curves to sweep 50 and gain 50 constant values (Table II), and plotting the resulting data as a function of dose (Mrad.) on a log-log scale, linear trends were obtained (Fig. 1) for F-I and F-II. Deviations from the straight line plot can be attributed to variations in sample weight and positions of the films in the cavity.

From Table III one can readily determine the relative increases in free radical formation for F-I and F-II when subjected to identical increases in irradiation dose, keeping the initial free radical and

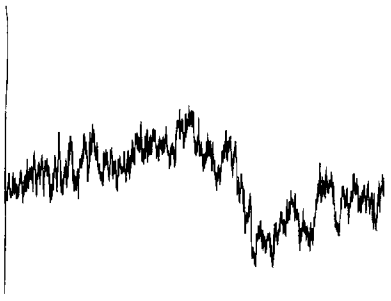


Fig. 6.—First derivative electron spin resonance spectrum at 25°C. of a control (unirradiated) film of F-I. Instrument parameters used were sweep = 500, gain = 800.

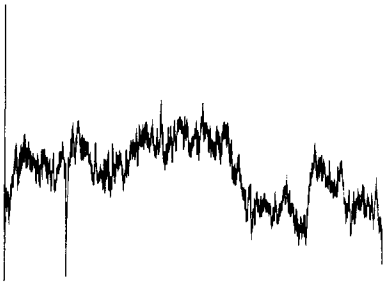


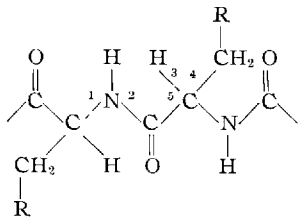
Fig. 7.—First derivative electron spin resonance spectrum at 25°C. of a control (unirradiated) film of F-II. Instrument parameters used were sweep = 500, gain = 800.

dosage norms as bases for comparison. A 32% increase in radiation from 0.461 to 0.679 Mrad. elicited a 56% increase in free radicals in F-I and a 78% increase in F-II. A further increase of 60% in radiation produced 69% more free radicals in F-I, but only a 50% increase in F-II, the latter indicating a downward trend in free radical formation despite a 2.5-fold increase in energy over the previous run. Finally, a further 45% increase in irradiation produced 71% more free radicals in F-I and a 75% increase in F-II. In both films, therefore, the higher the dose absorbed, the greater the excess of unpaired electrons. Contrary to the expectation that radical concentration, in terms of gain strength, should be directly proportional to the absorbed radiation, the data show that radical concentration is proportional to the three-halves power of the radiation dose.

DISCUSSION

Assuming that the predominating amino acid components in gelatin are alanine, glycine, proline, and hydroxyproline with no representation of —S—S— linkages in the absence of cystine (8), the most probable site of radiation damage would appear to be the α-carbon in the polypeptide back bone.

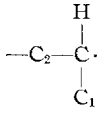
Viewing the protein configuration as:



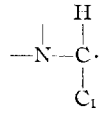
fracture of the C—N bond at 1 would require 25 gauss for the proton, with a possible structure for —CH_2, and a 10 gauss triplet at the nitrogen. Fracture at 2 would require a 25 gauss proton, a 10 gauss triplet at the nitrogen, and a single line for the carbon with a double bonded oxygen. Should fracture occur at 3, the proton would split off, with possible evidence of a 5 gauss triplet at the nitrogen and a —CH_2 triplet. Fracture at 4 would require a 25 gauss triplet to account for the —CH_2—R group, as well as a 25 gauss doublet (carbon) and a 5 gauss triplet (nitrogen). This could occur, for example, in the event of recombination of ·CH_2—R. Finally, fracture at 5 would give a singlet, a 25 gauss doublet, a 5 gauss triplet, and possibly evidence for CH_2.

It would appear that the observed spectra are characteristic of the

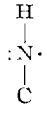
free radical. Formation of



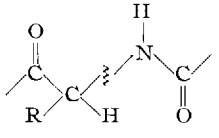
would give a doublet of 25 gauss, but protons on C_1 and C_2 might lead to additional structure, if they were noncoplanar with the carbons. Formation of



also would give a 25 gauss doublet, and a possible triplet, with 5 gauss separation due to the nitrogen. The latter splitting could be broadened by nitrogen quadrupolar interactions. If

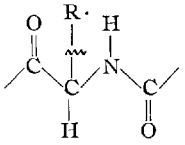


formation occurred, a 25 gauss doublet (H) and a 10 gauss triplet (N) splitting would be evident if quadrupolar broadening did not occur. Of the 2 remaining possibilities



appears to be the less likely since it would involve twice the nitrogen splitting. It seems reasonable to

conclude that formation of the following free radical structure:



is feasible since the data are indicative of spin being on an atom which is bonded to only one hydrogen.

Proof is not available here for postulating with absolute certainty at which of these positions free radical formation occurs. However, it is interesting to note Gordy's (6) conclusions, based on more extensive experimental data, that the peptide free radical resulting from radiation damage to proteins forms at a glycine residue.

It should be observed that the spin-proton interactions did not elicit any qualitative differences in resonance between the 2 protein fractions studied, despite a 2:1 ratio in molecular weight values. Doublets were conspicuously persistent at all irradiation levels, although one fraction showed a successively lesser tendency to increase in molecular weight as the dose was increased, and the other increased in molecular weight as higher doses were applied.

CONCLUSIONS

Low temperature alcohol fractionation of acid-treated pigskin gelatin yielded 2 distinct fractions having different average molecular weights and characteristic acid/base binding properties (1). Dilute solution intrinsic viscosity studies on these fractions (2) showed higher values for the higher molecular weight (173,000) fraction, F-I, than the lower (86,000) fraction, F-II, as expected. The similarity in acid/base binding capacity in the 2 fractions may be best explained on the ground that F-II was constituted of shorter fragments of chains similar to those prevailing in the larger fragments. The virtually superimposable acid-base titration curves over the entire titration range support the proposition that the same N-terminal and C-terminal groups existed in the high molecular weight, F-I, and low molecular weight, F-II, controls.

Of additional importance, particularly with respect to films, is the total absence of water, hence the lack of OH· and H· radicals, to cause alterations in protein structure through secondary effects. Further evidence that more concentrated gelatin is less susceptible to radiation damage is provided in Table I, which shows lower values for carbonyl production in 5% solutions and films of F-I and F-II, than corresponding 1% solutions.

It is *not unreasonable* to suggest that fracture of electrovalent bonds in the gelatin structure occurs on irradiation and that these breaks are responsible for molecular weight reduction. Indeed, evidence that free radicals were formed in films of F-I and F-II contributes to the validity of this proposal, especially when considered in the light of Fig. 3. These data indicate that as the radiation dose is increased, the rate of free radical formation rises. More importantly, at least when considered as a film, the damaged gelatin appears to retain the unpaired electron structure. Thus, retention of high numbers of free radicals reflects minimal recombination, hence absence of crosslinking.

Since the intrinsic viscosity studies and the molecular weights calculated from these data were based on experiments with 1% solutions (2), evidence of disproportionate retention of stable free radicals in films cannot be extrapolated to interpretation of changes in molecular weights predicated on solution experiments. Nonetheless, it seems permissible to reason that free radicals are formed in solutions (as they are in films), but the differences in radiation environment lead to dissimilar results. Irradiated films tend to retain free radicals because the compactness of the molecule locks the radicals into their created positions. Ultimately, after absorption of moisture, the molecular structure becomes loosened, and the radicals are able to shift. Irradiated solutions, on the other hand, do not retain free radicals in abundance because the concurrent high energy impact on solute and solvent creates, in addition to solute free radicals, unpaired electrons in the solvent structure. These are, in the case of water, OH· and H·. The formation of stable free radicals and a structurally less compact solute configuration facilitates (*a*) inactivation by abstraction of hydrogen from another molecule and (*b*) recombination and coupling. Assuming that each of these reactions occurs to a variable degree in irradiated gelatin solutions, it could be suggested for F-I that (*b*) predominates at low radiation levels, causing an increase in molecular weight. As the irradiation levels become greater in intensity, the ability of the free radicals to recombine and couple is progressively decreased and (*a*) controls.

Gelatin in solution answers the description of a polyelectrolyte (9), its isoelectric behavior being dependent upon which groups, the carboxyl or the amine, predominate in the gross structure. Its principal amino acid components are glycine, proline, and hydroxyproline, with lesser amounts of lysine, hydroxylysine, histidine, and arginine. Cystine and tryptophan are lacking.

The acid-base titration curves previously reported (1) tend to confirm the differences in behavior between the high and the low molecular weight fractions. At 1% concentration, irradiation did not appear to affect either the acid or the base functions of either fraction until the dosage was increased to the 2 and 3 Mrad. levels. At that point the basic groups were reduced in number, with a slight increase in bound carboxyls. The midportions of the 1% curves were unaffected in F-II, but F-I showed a decrease in bound imidazole groups at the low radiation level, with a gradual approach to the control value as the energy was increased.

In 5% concentrations, neither F-I nor F-II imidazole groups were affected. However, both fractions suffered carboxyl and amino depletion with the greatest damage imparted to the higher molecular weight protein. Apparently as the concentration of the protein is increased, radiation effects become more pronounced and tend to disrupt drastically the charge organization without affecting the isoelectric point. Irradiation of F-I and F-II in the solid state, on the other hand, caused little deviation in charge.

Carbonyl analyses in terms of μm./Gm. follow an arithmetic progression in approximate proportion to

the absorbed dose (Table I). Conversion of these data to G (carbonyl) values shows somewhat more carbonyl groups in F-I than in F-II per 100 ev. absorbed dose. By taking averages of the G values and drawing comparisons between F-I and F-II within the respective physical states irradiated, it will be seen that in the 1% solutions the averaged G values differed by a factor of 2; in 5% solution the difference increased to a factor of 6; but in films the difference factor fell to about 1.5. Within each group, on the other hand, there appeared to be reasonable consistency in G values and a noticeable lack of deviation, irrespective of variation in water content.

The anomalies may be due, at least in part, to the fact that irradiation was conducted in the absence of low temperature controls. Thus, at room temperature some free radicals tend to recombine to form carbonyl groups. Others enter into crosslinking reactions. The rates of recombination will vary depending on solute concentration, temperature variations, dose imparted, and other factors. In films, the conditions are such that the free radicals are not only deprived of free movement due to the relatively fixed configuration of the molecule, but also of participation in secondary reactions with solvent free radicals.

Notwithstanding this shortcoming, the relative pattern of carbonyl production on a weight basis assigns the lowest quantity to films and the highest to 1% solutions, the 5% solutions being intermediate. The relationship offers at least a partial basis for concluding that a greater number of free radicals are formed in irradiated dilute solutions of proteins than in those more concentrated because of solute–solvent interaction during the ionization event. Although G values in all cases remained below unity, there is no reason to minimize the conclusion that these data are in substantial agreement with Garrison's $G = 1.2$ (3) for carbonyl production in gelatin solutions influenced by ^{60}Co γ-rays. As explained in detail earlier, the lower values obtained in our experiments are attributable to the higher dose rate imparted, which functionally reflects the efficiency of free radical production.

Decarboxylation and deamination occurred in irradiated gelatin solutions in proportion to absorbed dose. Dilute solutions of the low molecular weight fraction were not severely damaged by low energy radiation, but the higher molecular weight fraction appeared to be injured at the imidazole portion. Neither the high nor the low molecular weight fraction was altered in charge groups when irradiated in the dry state, but free radicals were formed in both when irradiation was imparted at room temperature. No alteration in free radical structure occurred when irradiated gelatin films were brought to 77°K. nor when they were subsequently returned to room temperature.

Variations in solvent–solute ratios of gelatin solutions reflected differences in the number of new carbonyl groups formed on a weight basis. Higher carbonyl values per unit weight of protein were obtained in dilute than in concentrated solutions, confirming the secondary effects of water in altering the solute structure on irradiation. Relatively lower G (carbonyl) values were obtained in F-II than in F-I.

It seems permissible to conclude that the end effects of low level irradiation emanating from an electron beam upon fractions of gelatin obtained from the same source are predictable only to the extent that free radical and carbonyl group formation occur. The degree of radiation damage is proportional to the absorbed dose. The presence or absence of water in the system being irradiated also determines the extent of denaturation as reflected in carbonyl group formation. No direct evidence is available that aggregation plays the dominating role in molecular weight increase as a result of irradiation. In view of the extensive free radical formation and the strong indications of solvent–solute interaction during the irradiation event, it is a fair inference that increases in molecular weight in irradiated dilute gelatin solutions can be attributed to crosslinking through covalent and weak hydrogen bonds.

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Cholinergic Anionic Receptors III

Steric Requirements for Quaternary Ammonium Inhibitors of Acetylcholinesterase II

By J. C. KELLETT, JR.*, and W. CLARK DOGGETT†

The inhibitory activity of 36 structurally related quaternary ammonium compounds was undertaken as a logical extension of our earlier report studying the discrete nature of the anionic receptor of a typical cholinergic receptor, acetylcholinesterase. The results permit some sophistication in the discussion of the several mechanisms apparently operative in this system.

IN AN earlier report (1) the authors postulated that the inhibition of acetylcholinesterase (AChE) by simple quaternary ammonium compounds could be ascertained as a function of affinity for the enzyme and that certain relationships existed between the ionic volume of the inhibitory ion and its affinity for the enzyme. Our results prompted a logical extension of the compounds under evaluation in order to clarify the role of ion volume in affinity for a cholinergic receptor.

To meet this end 36 quaternary ammonium compounds were prepared and examined enzymologically. The compounds were: *N*-alkyltrimethylammoniums, *N*-alkyltriethylammoniums, and *N*-alkylquinuclidiniums, where the alkyl group was varied from methyl through decyl plus isopropyl; tetrapropylammonium; tetrabutylammonium; and tetrapentylammonium. These compounds include 3 homologous series, many of which have been examined in other cholinergic systems. Several interesting related compounds are included such as the isopropyl derivatives and the tetraalkyl derivatives. The conformational uniqueness of the quinuclidinium compounds was discussed in an earlier paper (1).

The fundamental rationale for studying competitive inhibition of AChE in a study of the receptor site was similarly established in the first report.

EXPERIMENTAL

Chemistry

Properties and recrystallization solvents for all salts are given in Table I.

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† National Science Foundation Undergraduate Research Participant, supported by grant GE-1929.

Quinuclidine.—The method of Leonard and Elkin (2) as modified in an earlier paper (1) was used. A significant improvement over earlier reports involves an increase in the rate of flow of the vapors of the 4-(2-hydroxyethyl) piperidine by raising the pot temperature to about 200° with the elimination of the air bleed. This reduced the amount of polymer formed in the catalyst bed and the volatile impurities previously obtained in the product. Larger batches could be run before the receiving apparatus became clogged.

N-Alkylquinuclidinium Salts.—One-tenth mole each of the appropriate alkyl halide and quinuclidine were sealed with 100 ml. of absolute ethanol in a citrate bottle for 18 hr. with intermittent shaking. The solvent was then removed by vacuum evaporation, and the salt recrystallized.

N-Alkyltrimethylammonium Salts.—The reaction described for *N*-alkylquinuclidinium salts was used for the preparation of *N*-alkyltrimethylammonium compounds by utilizing water as the solvent and lengthening the reaction time to 1 week.

N-Alkyltriethylammonium Salts.—All *N*-alkyltriethylammonium compounds except *N*-isopropyl[1] were prepared by refluxing 0.1 mole of the appropriate alkyl halide with 0.1 mole of triethylamine in 100 ml. absolute ethanol for 18 hr. The solvent was removed and the solid purified as previously described.

N-Isopropyltriethylammonium Bromide.—Diethylisopropylamine was synthesized according to the procedure of Caspe (3). This compound was subsequently reacted with ethyl bromide according to Robinson (4).

Tetrapentylammonium Bromide.—Tripentylamine, 0.1 mole, and 1-pentyl bromide, 0.1 mole, were reacted together as described for the synthesis of 1-alkylquinuclidinium salts.

Enzymology

pH-stat titrimetric determinations were made of AChE activity using an apparatus and a procedure described previously (1). The conditions used in these experiments (different from those reported earlier) were: enzyme[2] concentration, 0.01 mg./ml.; NaCl concentration, 0.15 *M;* $MgCl_2$ concentration, 0.05 *M*, and acetylcholine (ACh) concentration, variable. ACh perchlorate was used.

The velocity of the hydrolysis reaction determined over a period of 5 min. (after an initial 2

[1] Only triethylammonium bromide could be isolated from attempts to react triethylamine with isopropyl bromide.

[2] Nutritional Biochemicals (bovine erythrocytes AChE).

TABLE I.—CHEMICAL AND BIOLOGICAL DATA OF SIMPLE QUATERNARY AMMONIUM COMPOUNDS

Compd. $(CH_3)_3N^+$—R R =	X^- =	Recrystn. Solvent	Observed M.p.	Reported M.p.	Ionic Vol., cubic Å.[a]	$10^5 K_i$	Anal.[b] Calcd.	Found
Methyl	Bromide		[c]	[c]	58.1	187.0	[c]	
Ethyl	Bromide	[h]	332.0–2.5°		68.5	97.0	N, 8.38	8.26
Propyl	Bromide	[h]	237.0		79.0	57.2	N, 7.69	7.66
Butyl	Bromide	[h]	181.0–4.0		89.4	46.9	N, 7.14	7.06
Pentyl	Bromide	[i]	174.0–5.0	174.0–5.0°[d]	99.9	100.0		
Hexyl	Bromide	[i]	186.0–7.0	186°[e]	110.3	66.7		
Heptyl	Bromide	[j]	182.0–3.0		120.8	37.6	N, 5.88	5.82
Octyl	Bromide	[j]	214.0–5.0	215°[e]	131.3	13.9		
Nonyl	Bromide	[j]	227.0–30.0		141.8	6.6	N, 5.26	5.19
Decyl	Bromide	[j]	239.0–41.0	239.0–42.0°[e]	152.3	4.9		
2-Propyl	Bromide	[i]	311.0–2.0		79.0	41.2	N, 7.69	7.58
$(C_2H_5)_3N^+$—R	X^-							
Methyl	Iodide	[h]	294.0–5.0		89.4	103.0	N, 5.76	5.56
Ethyl	Bromide		[c]	[c]	99.9	83.5	[c]	
Propyl	Bromide	[h]	228.0		110.4	48.8	N, 6.25	6.23
Butyl	Bromide	[i]	207.0–8.0		120.8	37.5	N, 5.88	5.86
Pentyl	Bromide	[j]	145.0–7.0		131.2	57.5	N, 5.53	5.43
Hexyl	Bromide	[j]	103.0–4.0		141.7	45.5	N, 5.24	5.21
Heptyl	Bromide	[j]	109.0–10.0		152.1	31.0	N, 4.98	5.10
Octyl	Bromide	[j]	107.0–8.0		162.6	8.3	N, 4.74	4.76
Nonyl	Bromide	[j]	105.0–6.0		173.1	5.8	N, 4.52	4.45
Decyl	Bromide	[j]	106.0–7.0		183.5	4.4	N, 4.33	4.40
2-Propyl	Bromide	[i]	263.0–4.0	264.0°[f]	110.4	12.0		
 —R	X–							
Methyl	Iodide	[i]	352.0–3.0	357.0–8.0°[g]	88.2	13.8		
Ethyl	Iodide	[i]	273.0–4.0	270.0–1.0°[g]	98.7	9.0		
Propyl	Iodide	[i]	144.0–6.0	144.0–6.0°[k]	109.1	6.7		
Butyl	Bromide	[i]	236.5–238.0	236.5–238.0°[k]	119.6	7.8		
Pentyl	Bromide	[j]	206.0–7.0		130.1	12.0	C, 55.14	54.94
							H, 9.19	9.57
							N, 5.32	5.37
							Br, 30.35	30.28
Hexyl	Bromide	[j]	173.5–5.0		140.6	28.6	C, 56.46	56.24
							H, 9.41	9.51
							N, 5.07	5.14
							Br, 28.92	28.69
Heptyl	Bromide	[j]	165.0–6.0		151.1	32.6	C, 57.87	57.84
							H, 9.64	9.80
							N, 4.82	5.16
							Br, 27.52	27.27
Octyl	Bromide	[j]	155.0–6.0		161.6	4.1	C, 59.15	59.18
							H, 9.86	10.17
							N, 4.60	4.79
							Br, 26.25	26.05
Nonyl	Bromide	[j]	173.0–4.0		172.1	3.4	C, 60.31	60.36
							H, 10.05	9.95
							N, 4.40	4.35
							Br, 25.10	25.12
Decyl	Bromide	[j]	186.0–8.0		182.6	2.8	C, 61.37	61.26
							H, 10.23	10.20
							N, 4.21	4.34
							Br, 24.04	24.06
2-Propyl	Bromide	[i]	320.0–1.0		109.1	4.7	C, 51.47	50.98
							H, 8.58	9.00
							N, 5.96	6.07
							Br, 33.99	33.96
Tetrapropyl-ammonium	Bromide		[c]		141.7	5.3	[c]	
Tetrabutyl-ammonium	Bromide		[c]	[c]	183.5	4.9	[c]	
Tetrapentyl-ammonium	Bromide	[h]	96.0–7.0	100°[l]	225.5	31.0		

[a] Calculated according the procedure in *Reference 1.* [b] Microanalyses performed by Alfred Bernhardt, Max Planck Institute, Mulheim, Ruhr, Germany. [c] Obtained commercially. [d] McDowell, M. J., and Kraus, C. A., *J. Am. Chem. Soc.*, **73**, 2170(1951). [e] Kato, T., Morikawa, T., and Suzuki, Y., *J. Pharm. Soc. Japan*, **72**, 117(1952). [f] *Reference 4.* [g] Mosby, W. L., "Heterocyclic Systems with Bridgehead Nitrogen Atoms, Part II," Interscience Publishers, Inc., New York, N. Y., 1961, p. 1339. [h] Isopropyl alcohol–acetone. [i] Ethyl alcohol–ethyl acetate. [j] Methyl ethyl ketone–ethyl acetate. [k] *Reference 1.* [l] *Footnote d* above, p. 3293.

min. of reaction) was determined in the presence of varying concentrations of inhibitor and substrate. The K_m (the Michaelis constant) for this system, determined graphically from Lineweaver-Burk plots, was 1.82×10^{-4}.

All values for K_i were determined graphically from Lineweaver-Burk plots utilizing at least a fourfold range in [S].[3] Each velocity determination was made at least twice, and a minimum of 4 points were plotted for each K_i determination. It was observed that several of the compounds tested displayed noncompetitive kinetics when employed in concentrations at or above I_{50}. For uniformity, therefore, the [I] values used fall be-

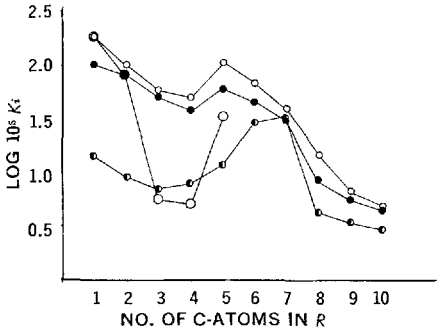

Fig. 1.—The relationship between K_i and length of alkyl chain of ammonium compounds. Key: large circle, R_4N^+; small circle, $(CH_3)_3N^+$—R; ●, $(C_2H_5)_3N^+$—R; ◐, $C_7H_{13}N^+$—R.

tween I_{20} and I_{30}. Under these conditions, all compounds tested display competitive kinetics in this system. The expression used to determine K_i on the $-1/(S)$ intercept was

$$K_i = \frac{K_p}{K_m[I] - \frac{1}{[I]}}$$

where $-1/K_p$ is the value of $1/[S]$ at the intercept. All data are summarized in Table I.

RESULTS AND DISCUSSION

Figure 1 displays a comparison between K_i and the chain length in the 3 homologous series and the R groups of the tetraalkylammonium compounds. A significant difference between the data presented here and the first paper merit comment. This difference relates to the minimum in the line for tetraalkylammonium. Earlier it was found to be at tetrapropylammonium; in this work it is at tetrabutylammonium. The authors have established that this difference (as well as some other quantitative differences) arises from the changed conditions of assay. The present procedure is used more commonly. The point remains that correlation of structure to activity in ACh–AChE systems is quite sensitive to the conditions of assay and should be compared accordingly.

Figure 1 reveals an interesting group of parallels. Note particularly the parallelism shown by the methyl, ethyl, and propyl homologs in the trimethylammonium and triethylammonium series; even more striking is the relationship among the octyl, nonyl, and decyl homologs in the trimethylammonium, triethylammonium, and quinuclidinium series. It is obvious that a parallelism exists between the entire series of triethylammonium and trimethylammonium compounds. These parallels are strongly suggestive of mechanistic parallelism.

The bioisosteric quinuclidinium series fails to display such complete similarity to the other 2 homologous series. The first 3 members and the last 3 members are near parallel, but 2 distinct differences are obvious. First, there is a dramatic quantitative difference in affinity between the first 3 quinuclidinium compounds and their trimethylammonium or triethylammonium bioisosteres. Second, the group butyl, pentyl, hexyl, and heptylquinuclidinium actually form a group of reversed slope from the trimethylammonium or triethylammonium series.

Apparently there is a lack of correlation between the tetraalkylammonium series and any of the N-alkyltrialkylammonium series, in terms of the function alkyl group length.

Figure 2 illustrates the relationship between K_i and the volume of the inhibitory ion. From this figure it is apparent that ion volume data do not offer a general explanation for an inhibitory ion's affinity for the enzyme. However, there is a crude generalization evident from the tendency for all of the compounds tested (*except* the lower members of

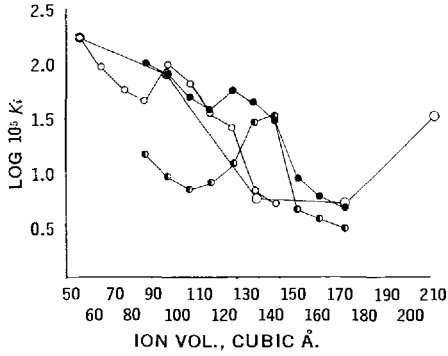

Fig. 2.—The relationship between K_i and ion volume of inhibitory cation. Key: large circle, R_4N^+; small circle, $(CH_3)_3N^+$—R; ●, $(C_2H_5)_3N^+$—R; ◐, $C_7H_{13}N^+$ – R.

the quinuclidinium series) to scatter around a line terminating in a minimum value for K_i at 170 Å.[3] Suggestions on a mechanism parallel between compounds is not evident from this figure. Indeed, much variation in affinity is obvious within a given volume increment which must be due to structural differences among equivolume ions. There are no minima of K_i as a function of volume common to 2 or more homologous series.

Not illustrated in the figures are the data from the isopropyl compounds. Table I reveals that the isopropyl compounds have in every case lower values of K_i than their n-propyl analogs. Simi-

[3] These data are consistent with values obtained from v/v_i *versus* [I] plots.

larly, these compounds represent further equivolume ions with an affinity for AChE which is uniformly enhanced by changing an *n*-propyl group for an isopropyl group. The implications of this observation are several in number, but no conclusion is evident. The fact that adding an alpha-carbon to an ethyl-substituted ammonium ion produces a better inhibitor than adding a terminal methyl group implies that the receptor surface does not contain a simple channel at all but is irregular in nature near the anionic center. The production of a different conformational perturbation by the isopropyl compounds is not deducible from these results, but must be considered. Obviously, the effect of α branching on one or more of these homologous series would constitute an interesting experiment.

According to Belleau (5) the compounds in the *N*-alkyltrimethylammonium series act in inducing 2 different conformational perturbations (with a transitory phase in between) dependent upon the length of the alkyl substituent. The parallelism between the *N*-alkyltrimethylammonium and *N*-alkyltriethylammonium series in this work strongly suggests that the same mechanism is operative for either series. The qualitative similarity between the 3 lowest and 3 highest members of all 3 of the homologous series (as well as the quantitative similarity between the 3 highest members) suggests that a similar mechanism is being invoked in each case. This similarity is independent of ion volume, as adequately demonstrated by Fig. 2.

These suggestions are modified by the lack of parallelism between the "middle region" of the *N*-alkylquinuclidinium series and the other 2 series. This feature, together with the quantitative dissimilarity displayed by the lower members, suggests that an entirely different mechanism is being demonstrated by the quinuclidinium derivatives up to C-7.

It is apparent that several forces are active in determining affinity for AChE. Very closely interdependent are: (*a*) ion volume, (*b*) chain length of longest alkyl chain, (*c*) the conformational possibilities of the ion, and (*d*) the exact mechanism whereby the ion acts (*i.e.*, type of conformational perturbation induced in the receptor).

Specifically excluded are arguments based on relative electron deficiency of the ion. It appears that this area is best thought of in terms of the conformational possibilities of the ion, rather than to attempt to quantify charge. All the compounds are easily dissociated at the concentrations employed. Therefore, the only electrostatic variables anticipated are those arising from the relative availability of the charge center(s). It appears from this approach to the problem that a very significant function of affinity is the shielding of the quaternary nitrogen atom, or the availability of the α-carbon atoms to a plane surface. Some discussion of α plane availability as related to cholinergic activity has been given by Thomas (6, 7).

CONCLUSIONS

No single simple molecular function serves to predict affinity of simple quaternary ammonium ions for the anionic site of AChE. The several features, related above, that obviously affect affinity are interdependent to a high degree. For example, it appears that for optimum affinity, some gross criteria of ion volume must be met; however, if the ion has other unique features enhancing affinity (such as the compact quinuclidinium "head"), the disadvantage conferred by the "wrong" size may be offset. In addition, it is likely that changing one molecular feature changes the mechanism whereby the ion acts. Therefore, the optimum molecular requirements must be stated in terms of a single mechanism to have meaning. The present state of these experiments does not permit this fine a conclusion.

Therefore, it is apparently correct to say that, among simple quaternary ammonium ions (*a*) the affinity of members in a homologous series tends to increase (with significant variations at intermediate positions) to a maximum in the region of 170 Å.^{3} volume of the ion; (*b*) butyl groups (rarely propyl) represent a maximum of affinity (however less than octyl, nonyl, or decyl) within a homologous series; (*c*) restricting the conformational variability of the cationic "head" generally enhances affinity; and (*d*) it is suggested that at least 4 discrete "mechanisms" of action are demonstrated by the compounds in this report. Changes of mechanism refer, in this context, to changes in the conformational perturbation type effected in the protein.

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Effect of Glidants in Tableting

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A method of measuring the fluidity of semifluid powders is described and used to investigate the role of various silica-type glidants in the direct compression of microcrystalline cellulose and a spray-dried lactose/microcrystalline cellulose blend on 2 different tablet presses. All silica-type glidants were found to improve the flow properties of microcrystalline cellulose as reflected in increased tablet weights and decreased weight variations, whereas a reversal of these effects was noted when the filler was the spray-dried lactose blend. While flow enhancement was attributed to a coating of the filler particles by the glidant, the reverse effect noted in the latter case was attributed in large measure to an increase in the bulk volume of the blend. No difference between the 2 tablet presses was found when the coefficients of variation were tested statistically. A pyrogenic silica and a silico-aluminate were found to be the most effective glidants in terms of over-all performance. These glidants were found effective in concentrations as low as 0.1 per cent by weight when added to microcrystalline cellulose and showed an optimum glidant activity at a concentration of about 0.5 per cent by weight.

THE FLOW of particulate solids is involved in many pharmaceutical operations such as tableting, encapsulation, solid-solid blending, tumbling, or fluidized bed drying. However, in none of these operations is flow any more critical, both in terms of product quality and production economics, than in the transport of solids from the hopper of tablet machines to and into the die cavities of a tablet press.

The limiting factor in the tableting of powders is often the flow properties of the material to be tableted. Uniform tablet weights and uniform doses of active ingredients, as well as production rate, are dependent on the ability of particulate matter to feed into the dies in a reproducible manner. In the past, poor flow properties have been overcome by granulating the powder or blend to be compressed. Unfortunately, granulation involves a series of steps which consumes a great deal of time and utilizes additional men, materials, and equipment. Recent advances in pharmaceutical technology and engineering have eliminated the need for granulation in many cases by making direct compression possible. Direct compression has been made commercially feasible with the advent of induced die feeding units which force-feed the die cavities, and the production of more fluid fillers by means of such engineering processes as spray-drying, instantizing, and controlled crystallization. The use of glidants has also been advocated for improving the flow properties of divided solids (1).

Glidants are defined as substances which improve granulation flow in the hopper or into the die cavity. The study of the usefulness of adjuvants in improving the flow properties of materials to be tableted has been largely confined to substances more properly defined as "lubricants" (2–4). The only "glidant" studies that have been reported in the pharmaceutical literature have been concerned with talc. Interest has developed recently, however, in the use of certain synthetic colloidal silicas to improve the fluidity of particulate solids. For example, a pyrogenic silica and a silico-aluminate were examined for their ability to improve the flow properties of spray-dried egg yolk (5). In general, the colloidal silicas consist principally of silicon dioxide and are characterized by low bulk density and very fine particle size.

Although glidants are apparently used in pharmaceutical operations, a survey of the literature has revealed the lack of any definitive study demonstrating the usefulness of glidants in tableting. Furthermore, there are no reports of a critical evaluation of the newer, silica-type flow conditioners. Consequently, a study of glidants in tableting, with particular emphasis on these silica-type flow conditioners appeared to be justified. Since tablet production of the future may well depend to a large extent upon direct compression, this study was designed, in

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particular, to establish the usefulness of glidants in direct compression.

EXPERIMENTAL

Before an investigation of glidants could commence, it was necessary to establish an experimental procedure which would truly measure increases in fluidity and, preferably, have practical industrial significance. Although angle of repose measurements and flow rate through orifices give results relatable to fluidity, reproducibility is relatively poor. It was felt that the simple measurement of tablet weight, which is a reflection of the ability of a powder or granulation to flow into a die cavity, would not only give comparable fluidity values, but would also have practical importance from a production standpoint.

Intuitively, it may be predicted that the addition of a glidant will, by enhancing fluidity, result in the more efficient filling of a die cavity. This more efficient die fill should then be reflected in increased tablet weights and decreased weight variation for the same size die and fill volume. Preliminary experimentation proved this premise to be true and glidants were evaluated using tablet weight as the measured parameter.

Materials.—Relatively free-flowing powders, microcrystalline cellulose[1] and spray-dried lactose,[2] were used as fillers, thereby eliminating the need for granulation. Microcrystalline cellulose possesses many unique advantages which make it an ideal filler to be used for such a study (6). In the first place, microcrystalline cellulose is fluid enough so that it will flow sufficiently well by itself to allow for production of uniform tablets at relatively high tablet machine speeds. At the same time, measurable effects can still be attained when glidants are added. Furthermore, because it is an "anti-adherent," it can be directly compressed without the need for a lubricant to aid ejection of the tablets from the die. As will be seen later, this is an extremely important quality, as it eliminates the need for lubricants which will cloud the action of glidants, making it impossible to separate individual effects. Microcrystalline cellulose also gives a very hard tablet at low pressures, minimizing tablet press vibration and decreasing the chances of erosion or chipping of tablets causing weight variation not attributable to die fill.

The glidants studied and their suppliers appear in Table I.

Preparation of Powders.—Uniform treatment of the powders to be evaluated was extremely important since reproducibility and meaningful results can only be obtained from this type of experiment if the history of each batch is carefully duplicated.

All powders were weighed and placed in a Patterson Kelly V-mixer model LB331 with no agitation bar. Mixing time was carefully controlled at 15 min. In order to promote good blending and uniform dispersion, all glidants or lubricants (which were needed with high concentrations of spray-dried lactose) were passed through a 100-mesh screen before blending with fillers. The importance of screening such additives, particularly when experimental batches are small, was stressed by Marlowe (7). The powders were removed from the blender and compressed immediately whenever possible to eliminate possible aggregation effects, which may result on standing. All blending and compression, except for the runs on the Stokes machine, were carried out in semi air-conditioned laboratories where relative humidity remains constant.

Compression.—Tablets were compressed on either a Stokes model B-2 or a Colton model 216 rotary tablet machine. Once the experiment commenced, the die fill adjustment was locked so that it remained constant throughout the course of the investigation. Occasionally, it was necessary to adjust compression pressure as glidants and fillers were changed in order to allow for production of tablets which were hard enough to withstand handling. Feed frames were adjusted close to the die table during direct compression to minimize leakage of powders. The same $^{7}/_{16}$ in. concave punches and dies were used in all experiments. Hopper filling was accomplished by placing a polyethylene bag full of the powder or granulation into the hopper and gently pulling the bag out from around the particulate mass.

Sampling.—The tablet press was allowed to run for approximately 1 min. before collecting tablets. One hundred tablet samples were drawn from the collected tablets by a "grab sample" method without replacement. Each tablet in the sample was weighed on a Mettler balance accurate to 0.1 mg. The weight of each tablet was recorded to the nearest milligram. The statistics determined were mean tablet weight, standard deviation, and coefficient of variation. The percentage change in mean tablet weight between the test tablets and control tablets was also calculated.

Experiment 1.—This experiment was carried out using microcrystalline cellulose and a blend consisting of 70 parts spray-dried lactose and 30 parts microcrystalline cellulose on a weight to weight basis.

The object of the experiment was twofold: (*a*) to compare the relative efficiency of various selected glidants when added to the 2 different tablet fillers;

TABLE I.—PROPERTIES[a] AND SOURCES OF THE GLIDANTS TESTED

Glidant[b]	Chemical Type	Ultimate Particle Size, mμ	Bulk Density, lb./cu. ft.
A	Pyrogenic silica	15	2.2[c]
B	Hydrated sodium silico-aluminate	22[d]	3[f], 18–20[e]
C	Amorphous non-gelled precipitated silica	12[d]	10[e]
D	...	13	4[e]
E	Silica hydrogel	10×10^3	15–20[e]
F	Silica hydrogel exhibiting aerogel type structure	3.3×10^3	4–5[e]

[a] Adapted from *References 11, 13–16.* [b] *A*, Cab-O-Sil, Cabot Corp., Boston, Mass.; *B*, Zeolex, type 7, J. M. Huber Corp., New York, N. Y.; *C*, Quso, type F-20 and D, Quso, type G-32, Philadelphia Quartz Co., Philadelphia, Pa.; E, Syloid, type 63 and F, Syloid, type 244, Grace Davison Chemical Co., Baltimore, Md. [c] Pour density. [d] Effective glidant particle size increased due to densification prior to shipping. [e] As packaged. [f] Aerated bulk density.

[1] Marketed as Avicel by American Viscose Corp., Marcus Hook, Pa.
[2] Western Condensing Co., Appleton, Wis.

TABLE II.—THE EFFECT OF SILICA-TYPE GLIDANTS ON WEIGHT AND WEIGHT VARIATION OF MICROCRYSTALLINE CELLULOSE TABLETS COLTON MODEL 216[a]

Glidant, 1% Concn.	Av. Wt., mg.	Change in Wt., %	S.D., mg.	Coefficient of Variation, %
Control	256.5	...	4.55	1.77
A	313.2	+22.11	2.66	0.84
B	314.8	+22.73	2.85	0.91
C	305.3	+19.03	4.75	1.56
D	306.7	+19.57	4.04	1.32
E	273.8	+ 6.74	4.21	1.54
F	303.2	+18.21	4.55	1.50
Calcium acetate	256.5	...	8.49	3.31

[a] Press speed: 800 tablets/min.

TABLE III.—THE EFFECT OF SILICA-TYPE GLIDANTS ON WEIGHT AND WEIGHT VARIATION OF MICROCRYSTALLINE CELLULOSE TABLETS STOKES MODEL B-2[a]

Glidant, 1% Concn.	Av. Wt., mg.	Change in Wt., mg.	S.D., mg.	Coefficient of Variation, %
Control	260.3	...	5.81	2.23
A	320.2	+23.01	2.81	0.88
B	324.2	+24.55	2.75	0.85
C	320.8	+23.24	3.43	1.07
D	313.2	+20.32	2.77	0.88
E	273.3	+ 4.99	4.69	1.72
F	310.5	+19.29	2.57	0.83
Calcium acetate	262.8	+ 0.96	2.58	0.98

[a] Press speed: 800 tablets/min.

(*b*) to compare these effects on 2 different rotary tablet machines to determine whether there was any significant between-machine effects. The same tooling was used on both machines. For the purposes of comparison, an attempt was made to adjust the initial fill on both machines so that identical average weights of the control batches (plain filler) were obtained.

The glidants were tested at a concentration of 1% by weight. In addition, magnesium stearate, at a concentration of 0.5% by weight, was added as a lubricant to the filler containing spray-dried lactose. Initially, the additives were preblended with about one-third, by volume, of the respective fillers. After about 30 sec. of shaking in a polyethylene bag, the preblend was added to the remaining two-thirds of the batch in the V-blender. Each batch of 1500 Gm. was then divided into 2 series, one of which was run on the Colton press and the other on the Stokes press at an average speed of 800 tablets/min. (Tables II–V.)

Experiment 2.—In view of the suggestion in the literature that fines (8) or talc (9) concentration is critical for effective fluidity, this experiment was undertaken to determine the role that glidant concentration plays in over-all powder flow. Glidants *A* and *B* were studied since they represent glidant types. Batches of 500 Gm. each, containing 0.1, 0.25, 0.5, 0.75, 1.0, and 2.0% by weight of glidant were prepared by simple blending according to the previously described procedures. Tablets were compressed on the Colton press at its maximum output of 1200 tablets/min. (Table VI).

Experiment 3.—The object of this experiment was to investigate the effect of press speed upon glidant efficiency. It was hoped that this experiment would affirm the present method of evaluation as a measure of powder fluidity. Glidants *A* and *B* in concentrations of 0.5% by weight were used as the previous experiment had shown that 0.5% was an optimum glidant concentration. The tablets were prepared on the Colton press at outputs of 480, 800, and 1200 tablets/min. (Table VII.)

RESULTS

As anticipated, experiment *1* showed a general increase in tablet weight and decrease in coefficients of variation for tablets compressed from microcrystalline cellulose when glidants are added. Surprisingly enough, the microcrystalline cellulose/spray-dried lactose blend showed results apparently reverse from those obtained with the plain microcrystalline cellulose. While it would appear that the standard deviations of the Stokes series were less than that obtained from tablets prepared on the Colton press, statistical analysis showed that there was no significant difference between the 2 series at the 95% confidence level. Similarly, statistical

TABLE IV.—THE EFFECT OF SILICA-TYPE GLIDANTS ON WEIGHT AND WEIGHT VARIATION OF SPRAY-DRIED LACTOSE/MICROCRYSTALLINE CELLULOSE BLEND (70:30) TABLETS COLTON MODEL 216[a]

Glidant,[b] 1% Concn.	Av. Wt., mg.	Change in Wt., %	S.D., mg.	Coefficient of Variation, %
Control	540.4	...	1.68	0.31
A	468.3	−13.34	2.55	0.54
B	515.9	− 4.53	1.94	0.38
C	485.5	−10.16	2.21	0.46
D	491.2	− 9.10	2.37	0.48
E	521.3	− 3.53	3.43	0.66
F	482.5	−10.17	1.90	0.39
Calcium acetate	541.2	+ 0.15	1.48	0.27

[a] Press speed: 800 tablets/min. [b] 0.5% magnesium stearate added to each batch.

TABLE V.—THE EFFECT OF SILICA-TYPE GLIDANTS ON WEIGHT AND WEIGHT VARIATION OF SPRAY-DRIED LACTOSE/MICROCRYSTALLINE CELLULOSE BLEND (70:30) TABLETS STOKES MODEL B-2[a]

Glidant,[b] 1% Concn.	Av. Wt., mg.	Change in Wt., %	S.D., mg.	Coefficient of Variation, %
Control	553.3	...	3.39	0.61
A	486.3	−12.11	2.23	0.46
B	530.0	− 2.40	3.15	0.59
C	510.1	− 7.81	4.45	0.87
D	505.7	− 8.60	2.49	0.49
E	534.8	− 3.34	3.14	0.59
F	508.5	− 8.10	2.38	0.47
Calcium acetate	554.8	+ 0.27	2.63	0.47

[a] Press speed: 800 tablets/min. [b] 0.5% magnesium stearate added to each batch.

testing of the difference in variation between the spray-dried lactose blend tablets produced on the 2 machines revealed no significant differences at the 95% confidence level.

Experiment 2 showed that tablet weights increased, with little or no improvement in weight variation, up to a concentration of about 0.5%. As glidant concentration was increased beyond this point, tablet weights decreased, showing, for the most part, little or no change in weight variation.

Although the glidants were quite effective at each production rate in experiment *3*, it was apparent that as press speed increased, powder fluidity became more critical, as evidenced by decreased mean tablet weights and a tendency toward an increase in coefficient of variation.

DISCUSSION

The physical and chemical properties of the silica-type glidants used in these experiments are listed in Table I. Glidant *B* is unique in that it is a silicoaluminate while the other glidants are colloidal silicas consisting primarily of silicon dioxide. Although glidant *B* has an ultimate particle size on the same order of magnitude as glidants *A* and *D* (10–20 mμ), it exhibits a mean effective particle size of 0.5–1 μ as a result of the manufacturer's "densification treatment" for increasing the bulk density (5). Glidant *C* has also been "densified" and exhibits a larger mean agglomerate size than does glidant *D*.

Glidants *A* and *B* appeared to be the most effective of the glidants in terms of over-all performance. In general, the glidants with the smaller particle sizes tended to be the most effective ones, although glidant *B*, with its larger effective particle size, proved surprisingly effective.

The weight increases observed in these experiments cannot be explained by the filling of void spaces with glidant alone (10). The true density of microcrystalline cellulose is about 1.54 Gm./cm.^{3}, and its bulk density is about 0.29 Gm./cm.^{3}. From these data it can be shown that about 81% of the total volume occupied by the powder is void space. On the other hand, the true density and bulk density of glidant *A* is 2.2 Gm./cm.^{3} and 0.039 Gm./cm.^{3}, respectively (11). In a similar manner, it can be shown that about 98% of the volume occupied by this glidant is void space. Thus, if the void space of microcrystalline cellulose was filled by glidant *A*, there would be an increase in weight of only 11%. Since glidant *A* in concentration of 1% (by weight) was able to increase tablet weights by as much as 24%, it is quite obvious that some function other than the filling of void spaces must be involved. Presumably, this function is the coating of particles (5, 10), resulting in an increased fluidity which manifests itself by allowing the particles to fill the die cavity in a more efficient manner. Furthermore, this process of coating the microcrystalline cellulose particles, instead of merely filling in the voids, would actually increase the bulk volume of the powder blend, thereby making any increase in weight of the final tablet even more significant than was demonstrated, since any weight increase must in part be negated by an increase in bulk volume.

The results with the spray-dried lactose/microcrystalline cellulose blend were not anticipated. In this series, the glidants which were the most effective in causing weight increases with microcrystalline cellulose were the most effective in causing weight decreases in this test, but not necessarily in the same order. Rather, there was a general agreement with bulk density in that the glidants exhibiting the lowest bulk density, disregarding particle size, elicited the greatest loss in mean weight. This pattern suggests that the decrease in tablet weights is a result of an increase in the bulk volume of the total blend, due to the addition of the extremely high bulk volume silicas. This suggestion follows since the bulk of the spray-dried lactose blend, being of considerably larger particle size than the microcrystalline cellulose, presents a smaller total surface area to which the glidant particles can become attached. In addition, the spray-dried lactose blend offers a smaller void space and less chance for the glidant to simply fill in between the particles. It is also quite possible that this blend may already have possessed maximum fluidity, owing to the free flowing properties of spray-dried lactose itself, and the addition of glidant actually hindered flow by acting as excess fines. No doubt, some clouding of glidant activity could have also resulted from the inclusion of magnesium stearate, which was necessary to allow for tablet ejection from the dies.

Because of these many factors, it was impossible to determine whether any useful purpose had been accomplished by adding glidants to the spray-dried lactose blend. However, the fact that coefficients of variation tended to increase and not decrease would seem to indicate that the glidants actually hindered fluidity in this case.

The effects of glidant concentration of microcrystalline cellulose tablets proved quite interesting. Effectiveness was demonstrated with a concentration of only 0.1% by weight. The additions of glidant beyond the 0.5% level caused no further increases in tablet weight but rather resulted in a decrease. Coefficients of variation showed uniform improvement over the control at all concentrations of glidant *A* but tended to be higher than the control for batches containing glidant *B*. This difference between glidants as reflected in coefficients of variation can be accounted for on the basis of the smaller effective particle size of glidant *A*. The fact that, beyond a concentration of 0.5%, tablet weights decreased, may be due to an increase in bulk volume of the mixture. If the glidant does adhere to particle surfaces rather than fill in void spaces, as indicated, then the effect would be to increase the particle diameter and hence, to increase the bulk volume.

The effect of machine speed points out the critical nature of powder or granule fluidity in tableting. As press speed increases, dwell time decreases, and the need for fluidity becomes more apparent. This effect was reflected in a decrease in tablet weight for the same die-fill as machine speed increased. Along with this effect was a tendency toward higher coefficients of variation at higher press speeds. Although they were affected by speed, both glidant blends, *A* and *B*, showed significant glidant effects at each speed run.

The use of calcium acetate in experiment *1* was analogous to the use of acetic acid or a soluble acetate in the cement industry. According to the

TABLE VI.—THE EFFECT OF GLIDANT CONCENTRATION ON WEIGHT AND WEIGHT VARIATION OF MICROCRYSTALLINE CELLULOSE TABLETS[a]

Glidant, %	Glidant A			Glidant B		
	Av. Wt., mg.	S.D., mg.	Coefficient of Variation, %	Av. Wt., mg.	S.D., mg.	Coefficient of Variation, %
Control[b]		$\bar{x} = 253.5$	S.D. = 3.30	C.V. = 1.30		
0.1[c]	301.8	3.50	1.16	271.2	3.51	1.29
0.25	304.5	3.58	1.18	282.7	5.73	2.03
0.50	304.5	3.43	1.13	291.6	7.27	2.49
0.75	298.9	3.28	1.10	299.5	5.17	1.73
1.0	286.9	3.33	1.16	287.9	4.61	1.60
2.0	277.6	2.17	0.78	280.3	8.04	2.87

[a] Press speed: 1200 tablets/min. [b] Plain microcrystalline cellulose; mean of 4 controls. [c] Data for the 0.1% samples represent the means of replicates.

theory, fine particles of calcium acetate form on the surface of cement particles, rendering the cement free-flowing (12). These studies show that calcium acetate does not affect flow properties in the same way as the silicas. Indeed, in no case was a significant increase in mean tablet weight elicited. However, in most cases, there was a marked decrease in coefficient of variation, indicating that calcium acetate exerted some influence on flow properties. No explanation is presently obvious and more work is necessary before any conclusions can be drawn.

On the basis of the experimental results there is little question that glidants can play an important role in tablet compression, particularly in the direct compression of semifluid, nongranulated powders. The results obtained in these experiments are not only of theoretical importance, but are also of particular practical importance to tableting due to the method of evaluation employed. The experimental method employed proved to yield measures of particulate fluidity which may be more meaningful to the tableting industry than such methods as angle of repose and orifice flow since the measured parameter was actual tablet weights. However, since tablet sizes (or die-fill volume) only represent small parcels of the particulate mass, a large number of tablet weights were required in order to obtain a truly representative parameter. This aspect tended to make the method a tedious one.

On rare occasions unexplainable extreme weight variations were found, using this method of evaluation. This phenomenon occurred only twice in more than 60 runs and, in one case, involved a preliminary study. In the other cases which occurred during a regular run, the sample was repeated in replicate, and the average of the replicate was reported. This fact is noted in Table VII.

One of the problems associated with the use of glidants arises out of their very fine particle size and low bulk density. These qualitites make them very "dusty," thereby creating a problem in convenience of handling as well as posing an inhalation hazard. A second problem of more concern to industry arises out of the abrasiveness of silicas. This abrasiveness may result in wear of tooling in long-term use, necessitating replacement, and increasing production costs.

While glidants may play an important role in tableting, it may very well be that a quantitative evaluation of them will have even more significance in other pharmaceutical operations such as capsule filling or solid-solid blending.

SUMMARY AND CONCLUSIONS

1. A procedure is described, based on tablet weight and weight variation, which may be used effectively to measure the fluidity of semifluid powders and which gives results directly relatable to the tableting of such powders.

2. The technique is used to investigate the role of various silica-type glidants in direct compression on 2 different tableting machines using microcrystalline cellulose and a spray-dried lactose/microcrystalline cellulose blend as fillers.

3. All silica-type glidants were found to improve the flow properties of microcrystalline cellulose as reflected in increased tablet weights and decreased weight variations. The weight increases observed are attributed to the coating of filler particles with glidant, thereby allowing them to flow more readily and uniformly into the die cavities.

4. On the whole, glidants *A* and *B* were the most effective of the silica-type glidants when tested with microcrystalline cellulose as a filler. Glidant *E* was the least effective glidant. Glidant efficiency could generally be correlated with particle size, the smaller glidant particles effecting greater increases in tablet weight and decreases in weight variation.

TABLE VII.—THE EFFECT OF MACHINE SPEED AND GLIDANTS ON WEIGHT AND WEIGHT VARIATION OF MICROCRYSTALLINE CELLULOSE TABLETS

Glidant 0.5% Concn.	30 r.p.m. (480 Tablets/min.)			50 r.p.m. (800 Tablets/min.)			75 r.p.m. (1200 Tablets/min.)		
	Av. Wt., mg.	S.D., mg.	Coefficient of Variation, %	Av. Wt., mg.	S.D., mg.	Coefficient of Variation, %	Av. Wt., mg.	S.D., mg.	Coefficient of Variation, %
Control	262.1	2.80	1.07	259.2	3.32	1.28	253.5	3.30	1.30
A	311.1	1.55	0.50	305.7	3.19	1.04	304.5	3.43	1.13
B	306.3	2.77	0.90	302.2	3.10	1.03	291.6	7.28	2.50

5. All silica-type glidants were found to produce a decrease in mean tablet weight along with a tendency toward an increase in coefficient of variation for the same size die fill when used with the spray-dried lactose/microcrystalline cellulose blend. This phenomenon was attributed in large measure to the extremely high bulk volumes of the silica-type additives which have the effect of increasing the bulk volume of the entire powder blend, thereby decreasing effective tablet weights.

6. The statistical testing of coefficients of variation revealed no difference between the Stokes model B-2 tablet machine and the Colton model 216 tablet machine at the 95% confidence level.

7. Glidants *A* and *B* proved effective in concentrations as low as 0.1% by weight when added to plain microcrystalline cellulose. No increase in glidant activity was observed with concentrations beyond 0.5% by weight.

8. For the same die fill, tablet weights decreased and coefficients of variation tended to increase as tablet machine speeds increased when glidants *A* or *B* were added to microcrystalline cellulose. As would be expected, a powder fluidity becomes more critical when press speed is increased.

9. Although calcium acetate has proved to be an effective glidant in the cement industry, it did not increase tablet weights when added to microcrystalline cellulose or the spray-dried lactose/microcrystalline cellulose blend. It did appear to decrease coefficients of variation, but no explanation is offered.

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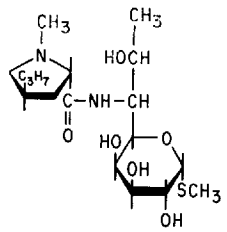
Automated Assay for the Antibiotic Lincomycin

By GEORGE C. PRESCOTT

A chemical assay for the antibiotic lincomycin has been developed which is based on the colorimetric determination of methanethiol generated from the acid hydrolysis of the methylthioglycosido group of the antibiotic. Fermentation beers and production samples as well as purified materials can be assayed by this procedure. Automation of this method increased the number of (completed) assays per man day from about 30 to about 150. Application of the assay to a series of standard solutions has given a mean recovery of 101 per cent and a standard deviation of 5.1 per cent. Analysis of a centrifuged beer containing added increments of lincomycin has given a mean recovery of 95.5 per cent and a standard deviation of 6 per cent.

LINCOMYCIN[1] is a medium spectrum antibiotic having the structure shown in I (1).

In 1962 the author developed an assay method which was based on the colorimetric determination of methanethiol generated from the acid hydrolysis of the methylthioglycosido group of the antibiotic (2). A particular advantage of this assay was that the thiol was separated from the interfering background material by distillation before it was reacted with the color reagent. Fermentation beers and production samples as well as purified products were assayed by this procedure. Early attempts to automate this assay with an automatic analyzer[2] were un-



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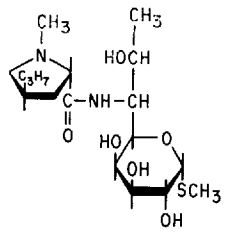

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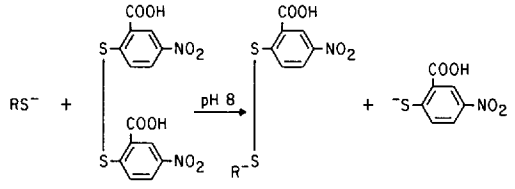
successful due to absorption of methanethiol gas by the Tygon tubing. This difficulty was eliminated by allowing the gas to contact only glass or polyethylene tubing. Other important improvements included the use of a water-soluble color reagent, better positioning of the mixing coils and gas separator, and the use of positive pressure on the system. Automation increased the number of (completed) assays per man day from about 30 to about 150.

The reactions involved in the assay are: acid cleavage of lincomycin to yield methanethiol, separation of the thiol, and reaction with a disulfide color reagent at pH 8.0 to release the highly colored anion. Full color development is almost instantaneous at room temperature.

The reaction of thiols with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) is shown in Scheme I.



Scheme I

EXPERIMENTAL

Instrumentation.—Technicon sampler No. 2, proportioning pump, colorimeter, and recorder.

Reagents.—*A.*—Sulfuric acid, approximately 12 *N*.

B.—Color reagent, 0.01% 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Dissolve 100 mg. of the reagent in 5 ml. of ethanol and dilute to 1 L. with water. The reagent appears to be stable for at least 1 month. In fact it is desirable to allow the reagent to stand for 24 hr. or longer as the slight haze which develops when the water is added disappears during this time.

C.—Tris buffer 0.1 *M*, pH 8.0. Dissolve 12.12 Gm. of 2-amino-2-hydroxymethyl-1,3-propanediol in 54.5 ml. of 1.0 *N* hydrochloric acid and dilute to 1 L. with water.

D.—Polysorbate 20.[3]

Procedure.—Set up the equipment as shown in the flow diagram (Fig. 1). Use a timing cam with the sampler 2 which allows 30 sec. for the sample and 90 sec. for wash or a rate of 30 samples/hr. Standardize the automatic analyzer as shown in the test manual.

The following points in technique have been found to be essential for a satisfactory assay.

(*a*) Tygon tubing is unsatisfactory for handling methanethiol gas. Glass and polyethylene tubing have been found to be satisfactory and *must* be used where indicated in the diagram.

(*b*) The gas separator must be connected in the manner indicated in the diagram which allows the aliquot for analysis to flow by gravity. The normal procedure of pumping this aliquot is unsatisfactory due to the Tygon manifold tubing.

(*c*) The mixing coils must be placed in a vertical position rather than in the usual horizontal position. The fittings must also be positioned in the approximate angle indicated.

(*d*) A positive air pressure of 3 lb./sq. in. greatly improves the bubble pattern and eliminates any back pressure in the system.

(*e*) As with most assays with this automatic analyzer the bubble pattern is greatly improved by using a small amount of surface-active agent in the reagents. The following amounts of polysorbate 20 seem to be about optimum under present conditions: 0.05 ml./L. in the acid, 0.1 ml./L. in the buffer, and 0.05 ml./L. in the water wash. The polysorbate 20 should be added to the acid and buffer on the same day that they are used.

Sample Preparation.—Prepare samples to contain from 25–150 mcg./ml. of lincomycin base per ml. of water. The pH of the solution is not critical since the sample will be hydrolyzed with strong acid. Highly colored samples cause no difficulty since the methanethiol gas is separated from the liquid before the colorimetry. However, solutions containing large amounts of carbonates or other materials which evolve gas on acidification must be degassed by acidifying at room temperature (lincomycin is not hydrolyzed at room temperature). Samples containing more than traces of volatile solvents should be evaporated and redissolved in water. Since the sample pick-up tubing is only 0.034 in. i.d., it is obvious that samples containing suspended solids must be clarified.

Standard Curves.—*Standard Curve for Purified Samples.*—Prepare standards to cover the assay range using lincomycin hydrochloride monohydrate primary standard. It has been found convenient to prepare standards containing 30, 60, 90, 120, and 150 mcg./ml. of lincomycin base equivalent in water. The concentration of lincomycin in each standard solution is calculated as lincomycin base, using a conversion factor based on the lincomycin base equivalent contained in the lincomycin hydrochloride monohydrate reference standard. These standards appear to be stable for several weeks at room temperature.

Standard Curve for Fermentation Beer Samples.—Prepare standards to cover the assay range in the same manner as described above, except use spent beer for the diluent in place of water. This spent beer must show no activity by either bioassay or chemical assay. It should be diluted with water to about the same dilution as the unknown samples.

Calculations.—Plot a transmission curve from the recorded peak averages of standards (Fig. 2). Draw a line connecting the transmission peaks of the standards and determine the concentration of each unknown from this curve. In most cases the curve is linear over the assay range, and it is equally satisfactory to determine the slope of the standard curve and calculate the concentration of the unknowns using this value. However, when the standard curve is not linear or does not pass through the origin the unknowns must be read directly from the curve. During the course of a long run it is necessary to correct for instrumental drift. This is the chief reason for running a standard for every 3

[3] Marketed as Tween 20 by the Atlas Powder Co., Wilmington, Del.

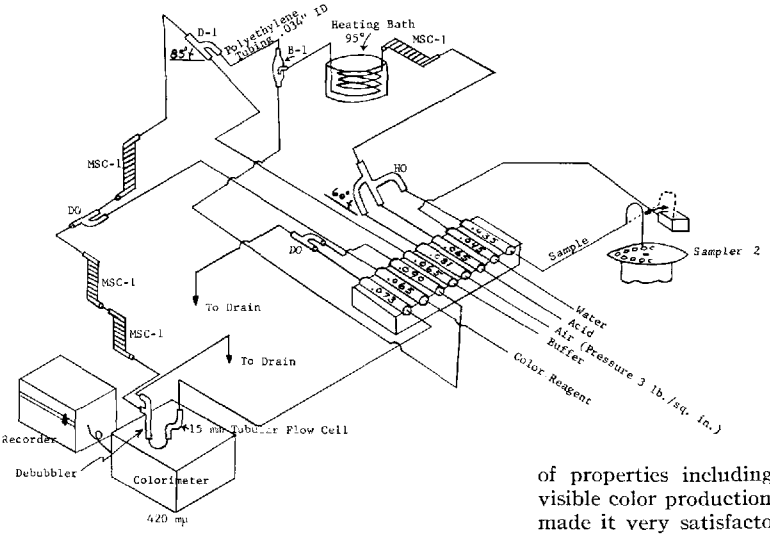


Fig. 1.—Automatic analyzer flow diagram for lincomycin using sampler 2. All glass fittings are designated by the Technicon AutoAnalyzer code.

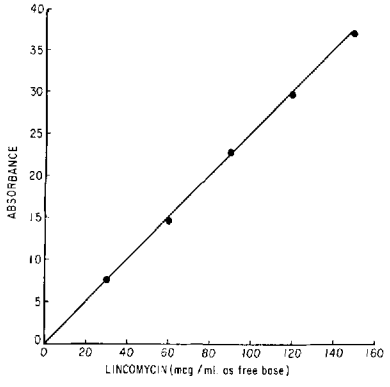


Fig. 2.—Standard curve for lincomycin assay.

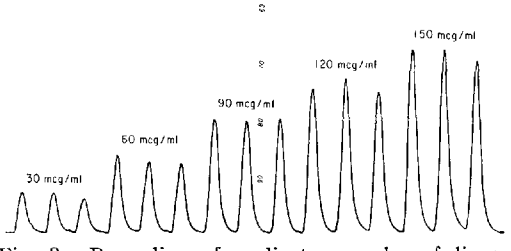


Fig. 3.—Recording of replicate samples of lincomycin standards.

samples throughout the entire run. This instrumental drift is inherent to the automatic analyzer and has been discussed by Thiers and Ogelsby (7).

RESULTS AND DISCUSSION

Choice of Reagent.—Several colorimetric reagents for thiol groups were investigated before the present reagent, 5,5′-dithiobis(2-nitrobenzoic acid) (3), was chosen. This reagent possessed a combination of properties including high sensitivity, stability, visible color production, and water solubility which made it very satisfactory for this assay. Some of the reagents investigated and rejected are: *N*-ethylmaleimide (4), bis(*p*-nitrophenyl)disulfide (5), 2,2-diphenyl-1-picrylhydrazyl (6), and sodium nitroprusside.

Specificity.—As would be expected, lincomycin analogs and metabolic products having a hydrolyzable thiol group give full molecular response in the assay. This applies to methylthiolincosaminide (1) as well as other lincomycin-related antibiotics (8). Most of the ingredients of the fermentation media do not show any response. These include methionine, ethionine, corn steep liquor, and Wilson's peptone No. 159. Blackstrap molasses, however, exerts a slight positive bias when it is added to standard samples. This may be the reason for the positive bias of fermentation samples when they are calculated from standards prepared in water. This bias is small and somewhat variable, averaging around 5%. Better agreement between the 2 assays is obtained by calculating the beer samples from a standard curve in which the crystalline standard is diluted with spent beer as described under *Standard Curves*.

Precision.—The plot of concentration *versus* absorbance is virtually linear at least over the concentration range of 25–150 mcg./ml. of lincomycin. At the described rate of assay there is no sample interaction as shown in the recording of replicate samples (Fig. 3). Application of the method to a series of standard solutions has given a mean recovery of 101% and a coefficient of variation of 5.1%. Analyses of a centrifuged beer containing added increments of lincomycin have given a mean recovery of 95.9% and a coefficient of variation of 6.0%.

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Enumeration of Primary Tissue Cells by an Electronic Device

By WILLIAM F. DALY, LEON P. PIASCIK, JR., and JAMES L. BITTLE

Cell yields from primary tissue suspensions of chicken embryos, rhesus, and cercopithecus monkey kidneys, were compared using the hemocytometer and Coulter counter, to determine the suitability of the electronic device for routine tissue culture practices. Plating efficiencies indicated that the Coulter counter is equal to or better than the hemocytometer for enumeration purposes. Results with the counter were more consistent and were reproducible, and there was a twofold saving in time, as well as elimination of the necessity of visual counts. The electronic counter has been used routinely in the laboratory for the past year, and in all instances, suitable monolayers for tissue culture production have been obtained.

A SIMPLE, quick, and reproducible method for determining cell yield from primary tissue has become a necessity for large scale tissue culture production. Many methods of estimating mammalian cell populations have been reported (1, 2) but the hemocytometer and the index-of-viability dyes, *e.g.*, trypan blue (3, 4) and eosin (5, 6), have been the most widely used means of determining viable cell concentrations. However, hemocytometer counts have proved time consuming and subject to serious error (7, 8), and the dye-exclusion tests are not always reliable indicators of cell cultivability (9).

This laboratory utilizes an electronic counter[1] which enumerates individual cells by a form of electronic "gating," for establishing cell inoculum. The instrument has been evaluated for blood cell counting (10, 11), and its application in growth studies has been demonstrated (12). The purpose of this paper is to compare the data obtained with both the hemocytometer and the electronic counter, and to determine the suitability of the instrument for routine tissue culture practices. The evaluation scheme closely paralleled that described by Brecher *et al.* (10).

EXPERIMENTAL

Culture Methods.—Primary cell suspensions were prepared from freshly harvested chicken embryos, rhesus, and cercopithecus monkey kidneys. Following trypsinization (1, 13, 14) the cells were resuspended in appropriate growth media, thoroughly mixed, and samples were removed for enumeration. The remaining cell suspension was diluted, inoculated into culture bottles, and incubated at 36°. Confluence monolayers developed in 16–18 hr. for chicken embryo cultures and 7 days for monkey kidney cultures.

Enumeration.—Enumeration with the electronic counter is accomplished by a form of electronic "gating." As a particle is drawn through a small aperture (100 μ), an equal amount of electrolyte is displaced causing a voltage drop due to an increase in aperture impedance. The resultant pulses are amplified, recorded by a decade counter, and visualized on an oscilloscope (10–12). For the authors' use, the instrument was calibrated with ragweed pollen according to the method described by Coulter Electronics.[2]

For monkey kidney cells the optimum threshold setting on model A was found to be 20 with aperture current setting of 3 and a gain of 2, whereas for chicken embryo cells settings were 5, 2, and 5, respectively. Cells were suspended in phosphate-buffered saline (15) and 4 successive counts were taken on each sample, representing 4 aliquots of the same cell population, or a total of 2 ml.

Hemocytometer.—Hemocytometer counts were made on samples composed of equal parts of cell suspension and 0.5% trypan blue. The viable cells in 2 squares (2 sq. mm.) were counted and multiplied by a dilution factor of 10,000 to obtain total cells per milliliter. This, therefore, represents a sampling of $^1/_{10,000}$ of 1 ml. Dead cells were also enumerated and a percentage of the total determined on each count.

Reproducibility Techniques.—Both systems were further evaluated by comparing multiple counts on the same primary cell suspension. Five different suspensions of rhesus monkey cells were enumerated with at least 20 counts obtained on each suspension with each method. In all instances, the cell suspensions were slowly mixed on a magnetic stirrer, and samples were removed and enumerated by both methods within a 2-hr. period (10). The mean cell counts obtained from the 5 monkey suspensions were compared, and the standard deviations and standard errors were determined.

Routinely, over a 6-month period, 3 types of primary cell suspensions—chicken embryo, rhesus, and cercopithecus monkey kidneys—were subjected to comparison counts with the hemocytometer and the electronic counter. Representative samples were taken of each suspension and single counts were obtained. The data for each tissue (Fig. 1) were plotted and fitted to a regression line (16) to determine the association existing between both procedures in evaluating a primary cell system.

A modified plating-efficiency technique was applied to both enumerating systems. A primary

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[1] Marketed by Coulter Industrial Sales Co., Division of Coulter Electronics, Inc., Chicago, Ill.

[2] Coulter counter for particle content and size distribution, Coulter Electronics, 1958.

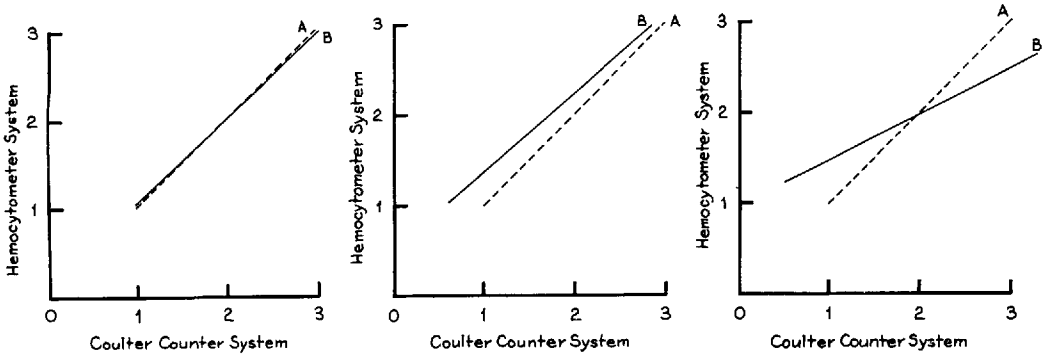

Fig. 1.—Relationship between hemocytometer and Coulter systems in evaluating primary tissue suspensions. Key: A, theoretical; B, Coulter counts plotted against hemocytometer counts. Counts are expressed in millions of cells per milliliter of suspension. Left: chicken embryo; middle: rhesus monkey kidney; right: cercopithecus monkey kidney.

suspension of rhesus monkey kidney cells was enumerated and, from this, two series of cultures were planted as determined from the counts of both systems. Each series consisted of two 2-oz. bottles at concentrations of 6,000, 12,000, 25,000, 50,000, 100,000, 200,000, and 300,000 cells/ml. Cell monolayer development was observed daily for 10 days.

RESULTS

The pooled standard deviation for the electronic method was 8.42×10^4 cells/ml. as compared with a standard deviation of 18.31×10^4 cells/ml. for the hemocytometer (Table I). The variability of each enumerating system on 5 separate monkey kidney suspensions is illustrated in Fig. 2 which represents that portion (95%) of the counted cells which lie in the interval of + or − 2 standard deviations. The standard errors for the electronic counter were all less than 2.4 while hemocytometer standard errors all exceeded 3.3 (Table I).

The data obtained from the routine comparison of primary cell suspensions provided correlation coefficients of 0.82 (chicken embryo), 0.76 (rhesus monkey kidney), and 0.70 (cercopithecus monkey kidney) which demonstrates the favorable relationship existing between both enumerating systems. The regression lines (Fig. 1) indicate the probability of a linear relationship.

Differentiation between counting systems was observed at a planting rate of 2.5×10^4 cells/ml. The cell cultures prepared from cells enumerated by the Coulter method had moderate growth in 10 days (40% confluency) as compared with 10–15% confluency in hemocytometer-enumerated cell cultures. No significant growth was noted at cell concentrations of 6000 and 12,000 cells/ml., whereas confluent monolayers were observed in 6 days at a concentration of 50,000 cells/ml. and in 4 days at concentrations of 100,000, 200,000, and 300,000 cells/ml.

DISCUSSION

The high degree of variation existing between mean hemocytometer and electronic counts has been demonstrated (Table I). It was shown from the 5 separate monkey kidney suspensions that the standard deviation for the electronic counter varied between 6.35–10.58 $\times 10^4$ cells/ml., whereas the standard deviation for the hemocytometer counts varied between 14.72 and 21.77 $\times 10^4$ cells/ml. The pooled standard deviation for the hemocytometer was more than twice that of the electronic counter. The standard errors agree well with those reported by Brecher (10), with the electronic counter errors all less than 2.4, while those of the hemocytometer all exceed 3.3.

The routine analysis of comparison counts provided correlation coefficients which demonstrate that both systems are fairly comparable. The coefficients are lower than those reported by Harris (12), probably because primary suspensions contain numerous cell types, blood, and debris. Correlation of hemocytometer and electronic counts was very good for chicken embryo tissue (Fig. 1, left), but to a lesser degree for simian tissue (Fig. 1, middle and right). In this study the cause of discrepancy was not determined, but the postulation of Mattern

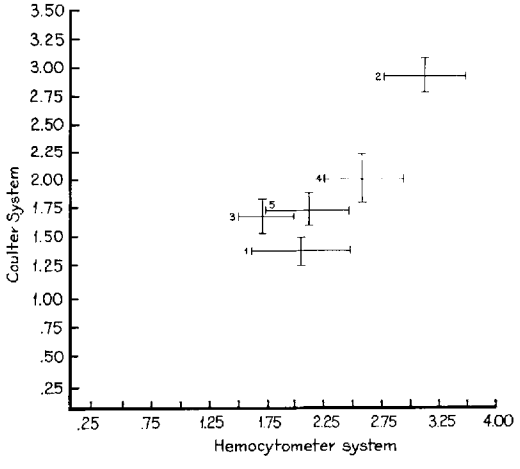

Fig. 2.—System reproducibility. Variation is expressed in terms of + or − two standard deviations from established mean cell count. Each kidney cell suspension is numerically indicated. Counts are expressed in millions of primary rhesus cells per milliliter.

TABLE I.—COMPARISON AND REPRODUCIBILITY OF ENUMERATING SYSTEMS (PRIMARY RHESUS KIDNEY CELLS)

Monkey Suspension	Method	Mean Count × 10^4/ml.	Dev.	Error
1	Hemocytometer	200.30	21.77	4.87
	Electronic[a] counter	136.55	6.35	1.41
2	Hemocytometer	310.20	17.90	4.02
	Electronic counter	290.95	8.02	1.79
3	Hemocytometer	165.55	14.72	3.29
	Electronic counter	169.00	9.29	2.08
4	Hemocytometer	256.00	17.76	3.97
	Electronic counter	203.35	10.58	2.37
5	Hemocytometer	207.88	18.63	3.73
	Electronic counter	174.44	7.51	1.50
Pooled	Hemocytometer		18.31	
	Electronic counter		8.42	

[a] Coulter counter.

et al. (11), that errors in hemocytometry must be involved, is accepted.

The plating efficiencies indicate that the electronic counter is equal to or better than the hemocytometer for determining cell inoculum. At a planting concentration of 2.5×10^4 cells/ml. a slightly higher per cent confluency was noted in the Coulter cultures.

From the data collected, it was noted that the number of nonviable cells from any one of the 3 cell suspensions never exceeded 10% of the total. Therefore, it must be assumed that the problem of variation in cell viability was overcome by the use of a standardized system of tissue culture preparation that consistently yielded a uniform population of viable cells.

This study indicates that the electronic counter is suitable for routine tissue culture practices, since it is more consistent than the hemocytometer and possesses excellent reproducibility. It is evident that the degree of variation existing in hemocytometry could eventually be responsible for the establishment of erroneous planting rates causing either a decrease or increase in the number of total cells available, which would directly affect the number of cultures obtained from a primary suspension. The utility of the electronic counter for routine operation is further supported by a twofold saving in time and the elimination of many stress factors that may contribute to erroneous visual counts. The electronic counter has been in operation in this laboratory for the past year as the standard enumerating method. During this period, 400 cercopithecus monkey kidneys, 220 rhesus monkey kidneys, and 61 lots of chicken embryo (approximately 206 embryos/lot) were trypsinized, planted, and in all instances gave rise to suitable monolayers for tissue culture production.

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Detection of *In Situ* Compound Instability by Two-Dimensional Thin-Layer Chromatography

By MELVIN H. PENNER, JOSEPH M. TALMAGE, and MILTON GELLER

Two-dimensional TLC, widely used for the resolution of chemically related compounds which cannot be resolved effectively by development in one solvent, has been employed for the detection of side reactions of the compound with the adsorbent or decomposition due to instability in the solvent system. The method involves the use of the same solvent system in both directions of development. Alteration of the compound is manifested by the appearance of secondary spots located off the theoretical diagonal line. Quingestrone and quingestanol acetate, both of which are steroidal 3-cyclopentyl enol ethers and show such instability, are presented as examples.

TWO-DIMENSIONAL chromatography using a different solvent for each development has shown particular value in the resolution of chemically related compounds which cannot be resolved effectively by development in one solvent. This technique has been used successfully by Brenner and Niederwieser (1, 2) for the resolution of 22 of the more common amino acids on Silica Gel G. Similar two-dimensional systems have been used for the resolution of 15 plasticizers (3), for 14 physiologically active indole derivatives (4), and for 9 tryptophane metabolites (5).

A substance is considered homogeneous in a chromatographic sense when the resulting chromatogram yields only 1 spot. In an extremely large number of cases, such homogeneity indicates genuine chemical purity. However, the appearance of multiple spots on a chromatoplate and the conclusion that the original substance is impure may be an erroneous one. In order to determine whether a compound undergoes a reaction with the adsorbent and/or decomposes during chromatography due to instability in the solvent system, the authors have employed two-dimensional thin-layer chromatography in which the same solvent is used in both directions. It is now recognized that a single pure substance may lead to the formation of more than 1 spot (6). Keller and Giddings (7) have reviewed in detail the problem of multiple spots in chromatography. Steroid 16β-esters have been shown to undergo reactions on alumina layers (8), and ethylene ketals have been hydrolyzed on silica gel plates (9). Alumina in columns has been reported by Mangold (10) to catalyze ester hydrolysis, isomerization of double bonds, and other reactions. Sterols are chemically altered when chromatographed on columns of dry silicic acid, and Linford (11) recommended that these compounds be separated by partition chromatography on silicic acid containing 35–40% water. Other citations of side reactions with the adsorbent have been reported by Stavely (12), Sarett (13), and Mattox and Mason (14). A comprehensive discussion of this problem has been presented by Neher (15). These alterations are by no means restricted to steroids and have been reported for sugars (16) and vitamins (17, 18).

Stahl (19) reported the use of a separation-reaction-separation (SRS) technique to study the inactivation of pyrethrines. This simple technique allowed the recognition of changes in the individual components of the mixture intentionally caused by irradiation of the chromatoplate with U.V. or sunlight following separation in the first direction. These alterations were quite evident after subsequent chromatographic development with the same solvent in the second dimension.

Two examples of steroidal 3-cyclopentyl enol ethers which show chromatographic instability are presented. It is now standard practice in this laboratory to chromatograph all substances by this technique which exhibit multiple spots by one-dimensional TLC.

EXPERIMENTAL

Procedure.—Commercially prepared standard silica gel plates, 20 × 20 cm., purchased from Analtech, Inc., were used in this study. These plates were activated for 30 min. at 105° just prior to use. Samples were applied as 0.2% solutions in hexane and approximately 10 mcg. of steroid spotted to 3 adjacent corners on the plate approximately 2.5 cm. from each edge. In this manner, the behavior of the compounds in each direction was apparent in the completed chromatogram.

Development.—The following solvent systems were used: (*a*) heptane–acetone, 2:1; (*b*) cyclohexane–ethyl acetate, 7:3; (*c*) cyclohexane–ether, 6:4; (*d*) heptane–propanol, 4:1; (*e*) toluene–methanol, 19:1.

The 3 points of application were marked and a finish line, 10 cm. from the starting points, was drawn in the layer with a needle. When the solvent front reached this line, the plate was removed from the development chamber, air dried, rotated 90°, and developed once more in the same solvent. Upon completion of this double development, the plates were air dried.

Detection.—For the detection, 50% methanolic sulfuric acid was found to be the most useful reagent. The entire surface of the plate was sprayed with the reagent and then heated in an oven at 105° for

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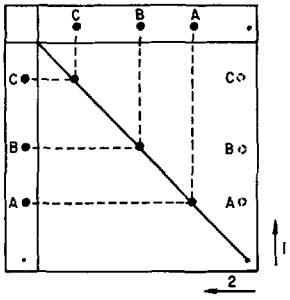

Fig. 1.—Theoretical distribution of a stable mixture in a two-dimensional chromatogram using the same solvent system in both directions.

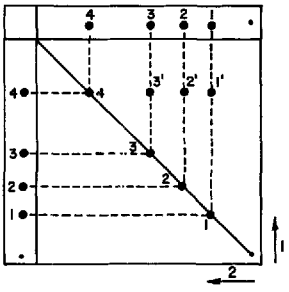

Fig. 2.—Two-dimensional chromatography of quingestanol acetate (17α-ethinyl, 19-nortestosterone acetate-3-cyclopentyl enol ether). Solvent system: heptane – acetone (2:1).

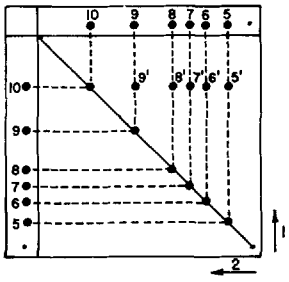

Fig. 3.—Two-dimensional chromatography of quingestrone (progesterone 3-cyclopentyl enol ether). Solvent system: toluene – methanol (19:1).

10–15 min. In addition to observing the colors formed upon heating, the plate was viewed under long wavelength U.V. light (360 mμ) to detect those spots which did not produce colored areas.

RESULTS AND DISCUSSION

In theory, if no alteration of the compound were to take place during the two-dimensional chromatography of a mixture using the same solvent system in both directions, all substances would be arranged in a diagonal line (Fig. 1). After the first development, the 3 substances comprising the mixture applied at the origin distribute themselves according to their R_f values as is indicated in Fig. 1 by the interrupted circles. After the second development with the same solvent system, all spots appearing above or below this diagonal correspond to substances which have been produced between the first and second chromatography (secondary spots). Both steroids when chromatographed by paper chromatography in heptane/methyl cellosolve (20) (capable of separating the decomposition products from the parent compound) represented 1 spot material.

Figure 2 represents the chromatographic pattern obtained for quingestanol acetate when heptane/acetone (2:1) was used as the solvent system in both directions. The following spots have been identified: (*1*) 6β-hydroxy-17α-ethinyl-19-nortestosterone acetate, (*2*) 6α-hydroxy-17α-ethinyl-19-nortestosterone acetate, (*3*) 17α-ethinyl-19-nortestosterone acetate, (*4*) 17α-ethinyl-19-nortestosterone acetate-3-cyclopentyl enol ether. Zones 1′, 2′, 3′ are indicative of decomposition of quingestanol acetate (4) during chromatography in the second dimension. Solvent systems B, C, and D gave evidence of similar decomposition patterns.

Figure 3 represents another example of the use of the two-dimensional technique for the detection of instability during TLC. Chromatographically pure quingestrone was chromatographed in both dimensions using toluene–methanol (19:1). Again from the completed chromatogram, it is evident that this steroid has undergone decomposition during the development as is evident from spots 5′–9′. Spots 5, 6, 9, and 10 have been identified as 6β-hydroxyprogesterone, 6α-hydroxyprogesterone, progesterone, and quingestrone, respectively. Spots 7 and 8 are minor and have not been identified. It has been postulated that quingestrone is unstable on silica gel due to an ether interchange reaction with the hydroxyl groups of silica gel. When comparatively large quantities of material are spotted, trails appear which furnish evidence of the decomposition pathway.

SUMMARY

Two-dimensional thin-layer chromatography using the same solvent system for development in both directions has been applied for determining compound instability during chromatography. Two examples of steroidal 3-cyclopentyl enol ethers which show such instability are presented. This technique has many advantages over elution and rechromatography of the parent compound, namely: (*a*) speed, (*b*) provides evidence of decomposition pathways, (*c*) for sensitive compounds, elution and concentration of the eluate may cause further alteration of the compound, (*d*) strongly adsorbed compounds may not be eluted in sufficient quantities, and (*e*) destructive spray reagents may be the only method of detection possible. The use of this method allows for the unequivocal determination of whether the cause of multiple spots by conventional TLC is indeed due to the presence of a mixture or an unstable compound.

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Some Observations on Nonlogarithmic Titration Curves for the Determination of Dissociation Constants and Purity

By LEWIS J. LEESON and MICHAEL BROWN

The method of Benet and Goyan for the determination of purity and ionization constant of a weak acid or base was investigated and found to work well. The general equation was modified in order to extend its applicability to titrations involving a volume change. The question of the correct type of activity coefficient to be used for calculating the data was considered, and some experimental evidence presented to indicate that the results are not markedly influenced by the one employed.

In recent publications (1, 2) Benet and Goyan have presented a technique for determining both the purity and the dissociation constant of a weak acid or base. This procedure is based on a nonlogarithmic linear plot of titration data. In these laboratories the authors use their method routinely, and have found it to be an excellent one. It is felt, however, that two points in the method warrant further discussion. The first is that the equation presented by Benet and Goyan assumes no volume change occurring during the titration. The second point is concerned with the correct activity coefficient to be used in the computations. Therefore, the basic equation has been rederived to make it generally applicable to all titrations, whether or not a volume change occurs. In addition, studies have been performed in an attempt to decide upon the correct type of activity coefficient to be used in the calculations.

EXPERIMENTAL

All pH measurements were made on the expanded scale of a Radiometer model 25 SE meter equipped with an A. H. Thomas 4855-B 10 glass electrode and a Corning fiber-type calomel electrode. Samples were prepared by adding to the titration vessel 100 ml. of nitrogen-sparged, triple distilled, pyrogen-free water; 2 ml. of 0.0953 *N* HCl solution (where necessary); sufficient 2 *M* KCl solution to obtain the desired ionic strength; and the sample to be titrated. Blank solutions were prepared identically. Titrations were performed under nitrogen in a water-jacketed vessel, the temperature of which was 25 ± 0.1°. A 5-ml. semimicro buret was employed; the titrant was 0.1008 *N* KOH solution standardized by titration with potassium acid phthalate, and the 1 *N* acetic acid was Fisher certified reagent. All linear relationships were calculated by the method of least squares.

DISCUSSION

Modification of the Equation.—The following equation was developed by Benet and Goyan (2) for the titration of an acid HA with a strong base MOH:

$$Z = A^0 - \frac{1}{K^c} Z\,[H^+] \qquad \text{(Eq. 1)}$$

where

Z = concentration of conjugate base formed during titration = $[H^+] + [M^+] - [OH^-]$,
$[M^+]$ = concentration of M^+ ion in solution from addition of MOH,
A^0 = concentration of all acid species in solution = $[HA] + [A^-]$, and
K^c = apparent ionization constant of acid HA.

Upon first examination, Eq. 1 appears to be a linear function. Therefore a plot of Z *versus* $Z[H^+]$ should be a straight line with a slope of $-1/K^c$, and an intercept of A^0, from which value the purity may be determined. On further consideration, however, it is apparent that this is true only when the total volume change, which occurs during a titration, is considered negligible. Since A^0 represents the sum of all forms of HA present in solution at any time, a volume increase, which results by addition of titrant, will cause A^0 to vary from the beginning to the end of the titration. Therefore in cases where the titration volume cannot be considered constant, Eq. 1 does not represent a linear function. Although it is recognized that Benet and Goyan were able to consider their volume changes negligible, in this work a volume increase of 2–5% is not uncommon. Therefore, the authors felt it desirable to alter Eq. 1 so as to circumvent any error that may be introduced by a volume change.

If one were to consider the system in terms of absolute amount of material (number of moles) in the titration vessel rather than concentration (moles/liter), the equation can be rederived to make it independent of volume change. For the titration of an acid HA with a strong base MOH, the equation of electroneutrality may be written in terms of absolute molar quantities rather than concentration units.

$$H^+ + M^+ = A^- + OH^- \qquad \text{(Eq. 2)}$$

where

H^+ = absolute numbers of moles of hydrogen ion present in solution,
M^+ = absolute number of moles of base added to the solution,
A^- = absolute number of moles of conjugate base present in solution, and
OH^- = absolute number of moles of hydroxyl ion present in solution.

Therefore

$$Z' = A^- = H^+ + M^+ - OH^- \qquad \text{(Eq. 3)}$$

The apparent ionization constant for HA may be written:

$$K^c = \frac{[A^-]\,[H^+]}{[HA]} = \frac{A^-\left(\frac{10^3}{V}\right)[H^+]}{HA\left(\frac{10^3}{V}\right)} = \frac{A^-\,[H^+]}{HA} = \frac{A^-\,[H^+]}{(A^0 - A^-)} \qquad \text{(Eq. 4)}$$

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TABLE I.—COMPARISON OF EXPERIMENTALLY DETERMINED ACTIVITY COEFFICIENTS WITH LITERATURE VALUES

	$\gamma\pm$		
μ	Determined	Lewis and Randall (4)	Kielland (3)
0.09	0.80	0.80	0.83
0.05	0.82	0.83	0.86
0.036	0.83	0.84	0.87
0.018	0.88	0.88	0.89

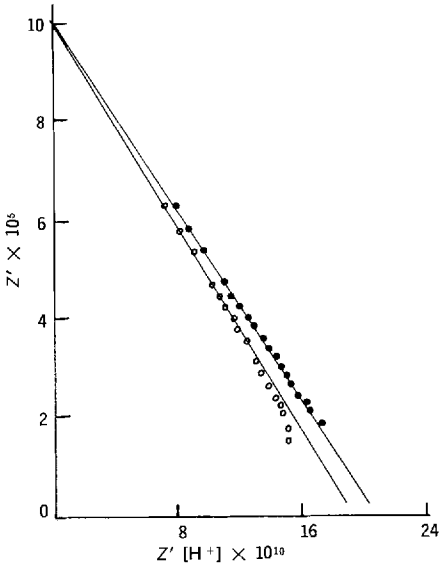

Fig. 1.—Plot of Z' *versus* Z' $[H^+]$ calculated at $\mu = 0.05$ with both the Kielland and $\gamma \pm$ activity coefficients. Key: ●, data using Kielland values; ○, data using $\gamma \pm$ values.

where

$[A^-]$ = concentration of conjugate base = $A^-(10^3/V)$,
A^- = absolute number of moles of conjugate base,
V = volume of solution in milliliters,
$[HA]$ = concentration of acid = $HA\ (10^3/V)$,
HA = absolute number of moles of acid,
$[H^+]$ = concentration of hydrogen ion,
A^0 = absolute number of moles of acid added initially = $HA + A^-$.

Substituting from Eq. 3 into Eq. 4 and rearranging,

$$Z' = A^0 - \frac{1}{K^c} Z'[H^+] \qquad \text{(Eq. 5)}^1$$

Although Eq. 5 is identical in appearance to Eq. 1, there are important differences which make it an exact linear function. The A^0 term in Eq. 5 represents *absolute* number of moles of acid added to the solution. All the terms used to calculate Z' (including H^+) are also *absolute* quantities. The $[H^+]$ in Eq. 5, however, is a *concentration* term.

The modified equation of Benet and Goyan permits the use of their method without any assumptions as to volume increase during titration. In addition, it also decreases the amount of calculations required, since the only adjustment necessary for volume change is made with the hydrogen ion. In practice, HCl is often added to the solution being titrated,[2] so that calculations in terms of concentration units would require an adjustment for both Cl^- and M^+ ions at each point in the titration. However, if calculations are made with absolute quantities, the amount of Cl^- is a constant and is equal to the absolute amount of HCl added, whereas M^+ is merely the volume of MOH added multiplied by its normality.

The authors have found that working in terms of absolute amounts of materials is a good general method for decreasing the computations required in titrimetric techniques. Its use in chelation studies, where numerous species are present (chelating metal, M^+, Cl^-, etc.), has again been found to simplify calculations because only one volume adjustment is necessary in most cases.

TABLE II.—COMPARISON OF RESULTS WITH ACETIC ACID USING BOTH TYPES OF ACTIVITY COEFFICIENTS

	Results		
	$\gamma\pm$	Kielland	Theory
% Purity	99.96	100.1	100.0
Thermodynamic pKa	4.76	4.74	4.75[a] 4.76[b,c]

[a] Determined from conductance measurements (5). [b] Data reported at an ionic strength of 0.0004 M (6). [c] Data reported at infinite dilution (7).

Activity Coefficient.—For evaluating Z', it is necessary to convert the hydrogen ion activity, obtained from the pH value, to a concentration term by use of an activity coefficient. In their work, Benet and Goyan used the single ion activity coefficients determined from theoretical considerations by Kielland (3). For the authors' calculations, the more familiar, experimentally obtained, $\gamma\pm$ values as listed in Lewis and Randall (4) were employed. These two types of activity coefficients differ somewhat.

In an attempt to determine which activity coefficient value is the better for use in these studies, blank solutions were prepared containing all constituents except the material being titrated. Therefore, since the ionic strength and amount of HCl present were known, the apparent activity coefficient could be evaluated by converting the pH of a solution to hydrogen ion activity, and dividing this by the known hydrogen ion concentration. The results of this study (Table I) indicate that the values obtained more closely approximate the $\gamma\pm$ values than they do the theoretically determined ones.

In addition, the purity and ionization constant of acetic acid were calculated using both the Kielland and $\gamma\pm$ values (Fig. 1). When $\gamma\pm$ is employed, the relationship is linear over the entire range studied, whereas with data at higher ionic strengths (0.09 and 0.05) the Kielland single ion coefficient

[1] For the titration of a base to which a fixed amount of HCl (W) has been added, $Z' = A^0 - K^c \frac{Z'}{[H^+]}$ where $Z' = W + OH^- - H^+ - M^+$.

[2] When an amount of HCl (W) is added to the solution, $Z' = H^+ + M^+ - W - OH^-$. These studies are often performed in a pH range where the hydroxyl ion is considered negligible, and $Z' = H^+ + M^+ - W$.

produces a curved segment at lower pH values.[3] In calculating the purity and ionization constant, only the linear portion of each plot was used. Nevertheless, it may be seen (Table II) that the intercept obtained (and therefore the purity evaluated) is not markedly influenced by the choice of activity coefficient. It is rather the ionization constant that reflects the difference, as may be seen by the two slopes in Fig. 1. A plot of pKa′ *versus* $\sqrt{\mu}$ demonstrated an essentially linear relationship for both sets of data. These were extrapolated to infinite dilution to evaluate a thermodynamic ionization constant. The results (Table II) indicate that the pKa obtained with both values are consistent with the literature (5–7). Therefore, since the two sets of data are the same, the type of activity coefficient employed for calculations appears to be a matter of personal choice.

[3] In this area of the titration, hydrogen ion makes its greatest contribution to Z' values, so that an error in the activity coefficient would be most evident at these higher acidities. This is comparable to Benet and Goyan's type A curve in Fig. 1 (2) for the case where high erroneous pH values are substituted. In all cases the Kielland values are higher than the $\gamma\pm$, and would result in a lower hydrogen ion concentration.

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Identification of Some Barbiturates by Paper and Thin-Layer Chromatography

By Z. F. AHMED, Z. I. EL-DARAWY, M. N. ABOUL-ENEIN, M. A. ABU EL-NAGA, and S. A. EL-LEITHY

Paper and thin-layer chromatographic procedures are described which can serve to separate a multiple mixture of 12 different barbiturates of toxicological interest.

THE APPLICATION of paper and thin-layer chromatography seems, so far, to be the most promising approach in the identification of barbiturates (1–9). The different procedures adopted leave much to be desired and a simple method for the separation and identification of a multiple mixture of barbiturates is of great value in medico-legal analysis.

A simple, rapid method of separation and identification of 12 barbiturates encountered either alone or in a mixture during the toxicological studies in the National Centre of Criminological Research is reported in this paper. The procedures adopted are based on the application of both paper and thin-layer chromatographic techniques to the following barbiturates: phenobarbital U.S.P., cyclobarbital,[1] barbital U.S.P., diallylbarbituric acid N.F., allylisopropylbarbituric acid,[2] butobarbital,[3] amobarbital U.S.P., secobarbital U.S.P., methylphenobarbital,[4] ethyl-*n*-hexylbarbituric acid,[5] pentobarbital U.S.P., and hexobarbital.[6]

EXPERIMENTAL

Paper Chromatography

The earlier attempt of Kybing (3) and Ledvina (4) for the chromatographic separation of barbiturates on formamide paper gave promising results. It was, therefore, decided to find out the most appropriate system of formamide and the developing solvent which fulfills speedy and efficient separation.

The following systems were investigated: (*A*) paper impregnated with formamide, (*B*) solvent containing formamide, and (*C*) formamide included in both the paper and the solvent.

Paper.—Sheets of Whatman No. 1 filter paper were impregnated with 20–30% formamide in acetone for about 10–15 min. The air-dried sheets were kept in a dark place away from dust. It is recommended that the paper be freshly impregnated.

Solvents.—Chloroform–benzene–ammonium hydroxide, concentration 13:3:6, was employed for system *A* (paper impregnated with formamide). Chloroform–*n*-butanol–formamide–5 *N* ammonium hydroxide, concentration 5:3:1:3, was employed for system *B* (solvent containing formamide). Chloroform–benzene–formamide–5 *N* ammonium hydroxide, concentration 12:2:1:5, was employed for system *C* (formamide included in both the paper and the solvent).

Reagent.—Silver reagent: (*a*) Silver nitrate, A. R., 0.5% methanolic solution. (*b*) Methanol–ammonium hydroxide, concentration 9:1. (*c*) Sodium hydroxide, A. R., 5% methanolic solution. The reagent is prepared by mixing solutions (*a*), (*b*), and (*c*) in the ratio 5:1:2. The reagent has to be freshly prepared.

Standard Solution of Barbiturates.—The above-mentioned barbiturates were used in a chloroform solution of a concentration of 1.5 mcg./μl.

Procedure.—The sheets were spotted in duplicate with 3–4 μl. of the chloroformic solution of the barbiturates and placed into a chamber previously saturated with the stationary phase. The solvent front de-

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[1] Marketed as Phanodorn by Winthrop Laboratories.
[2] Marketed as Alurate by Roche Laboratories.
[3] Marketed as Soneryl by Specia.
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TABLE I.—R_f VALUES OBTAINED USING STANDARD SOLUTIONS OF BARBITURATES[a]

Barbiturate Used	Solvents A	B	C
Barbital	0.06	0.65	0.09
Phenobarbital	0.07	0.58	0.06
Secobarbital	0.13	0.80	0.56
Diallylbarbituric acid	0.15	0.68	0.12
Cyclobarbital	0.18	0.70	0.23
Allylisopropylbarbituric acid	0.19	0.69	0.29
Butobarbital	0.24	0.73	0.35
Amobarbital	0.31	0.72	0.46
Pentobarbital	0.41	0.83	0.64
Ethyl-*n*-hexylbarbituric acid	0.58	0.79	0.78
Methylphenobarbital	0.63	0.71	0.90
Hexobarbital	0.77	0.76	0.85

[a] R_f values given here represent the average of 6 determinations.

TABLE II.—R_f VALUES OF BARBITURATES OBTAINED BY USING SILICA GEL G AND KIESELGUHR[a]

Barbiturate Used	Mobile Phase[b] (a)	(b)	(c)
Phenobarbital	0.20	—	—
Cyclobarbital	0.30	0.46	...
Barbital	0.31	0.13	...
Diallylbarbituric acid	0.34	0.24	...
Allylisopropylbarbituric acid	0.50		...
Butobarbital	0.53	...	...
Amobarbital	0.58	...	...
Secobarbital	0.63	...	0.43
Methylphenobarbital	0.64	...	0.90
Ethyl-*n*-hexylbarbituric acid	0.64	...	0.67
Pentobarbital	0.66	...	0.50
Hexobarbital	0.77	—	—

[a] R_f values given here represent the average of 6 determinations. [b] (*a*) for Silica Gel G; (*b*) and (*c*) for kieselguhr.

scended to the proper height (30 cm.) within 2–2.5 hr. After drying at room temperature in a stream of air for 10–15 min., the barbiturates were developed as white spots using the standard method of spraying with silver reagent (10). Table I shows the typical results obtained using the standard solution of barbiturates.

Thin-Layer Chromatography

Apparatus.—The apparatus used was essentially the one designed by Stahl (11) using 20 × 20 cm. glass plates.

Adsorbent.—Silica Gel G (Merck); kieselguhr (Merck) impregnated with formamide.

Mobile Phase.—(*a*) Ethyl acetate *n*-hexane–ammonium hydroxide, concentration 20:9:10, was employed for the Silica Gel G. (*b*) Carbon tetrachloride–chloroform, concentration 1:2, was employed for kieselguhr. (*c*) Carbon tetrachloride–chloroform, concentration 1:1, was employed for kieselguhr.

Reagent.—Silver reagent (10).

Standard Solution of Barbiturates.—The above-mentioned barbiturates were used in a chloroformic solution of a concentration of 1.5 mcg./μl.

Procedure.—Each plate was covered to a thickness of about 250 μ with a paste consisting of 4 Gm. of silica gel in 12 ml. of distilled water (or 4 Gm. kieselguhr in 16 ml. of 20% formamide in acetone). Precautions were taken to prevent air bubbles. The chromatoplates were dried in air for 15 min. at 105° (for silica gel) and 1 hr. at 60° (for kieselguhr).

The plates while still hot were spotted in duplicate with 3–4 μl. of the chloroformic solution of the barbiturates, and placed into a chamber containing the mobile phase. The solvent front ascended to the proper height (15 cm.) within 45 min. (in case of silica gel) and 20 min. (in case of kieselguhr). After drying at room temperature in a stream of air for 15 min., the barbiturates were developed as white spots against a grayish brown background using the standard method of spraying with silver reagent (10).

DISCUSSION

The 2 systems, *viz.*, paper impregnated with formamide and formamide included in both the paper and the solvent afforded satisfactory means of separation of the multiple mixture of barbiturates. The only 2 instances in which the separation failed when applying these 2 systems were phenobarbital–barbital and cyclobarbital–allylisopropyl barbituric acid mixtures as shown in Table I.

Regarding the thin-layer chromatography, silica gel gave 5 distinct ranges of R_f values, *viz.*, phenobarbital (R_f 0.2), cyclobarbital–barbital–diallylbarbituric acid (R_f 0.32), allylisopropylbarbituric acid–butobarbital–amobarbital (R_f 0.54), secobarbital – methylphenobarbital – ethyl - *n* - hexylbarbituric acid–pentobarbital (R_f 0.64), and hexobarbital (R_f 0.77). When applying kieselguhr as adsorbent, the cyclobarbital–barbital–diallylbarbituric acid mixture as well as secobarbital–methylphenobarbital–ethyl-*n*-hexylbarbituric acid–pentobarbital mixture could be effectively separated as shown in Table II.

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Physicochemical and Physiologic Factors Affecting the Absorption of Warfarin in Man

By ROBERT A. O'REILLY*, EINO NELSON†, and GERHARD LEVY‡

The relationship between the *in vitro* dissolution kinetics and the *in vivo* intestinal absorption characteristics of tablet preparations of the coumarin anticoagulant warfarin has been studied. The composition of the dissolution medium had a significant quantitative and qualitative effect on dissolution kinetics. Warfarin absorption in man varied with respect to lag time, absorption rate constants, and the type of kinetics observed. These variations may result from variable gastric emptying times and from differences in the pH of gastric fluids.

THE IMPORTANCE of pharmaceutical formulation in modifying the gastrointestinal absorption of drugs from tablets has been documented extensively (*References 1–3* and references cited therein.) To predict the *in vivo* absorption characteristics of various preparations, reliable *in vitro* testing procedures must be available. Recent studies (4, 5) have suggested that the development of such testing procedures may be feasible. Consistent absorption rate and complete physiologic availability are desired characteristics for all drugs, but these properties are particularly necessary for the coumarin anticoagulants. These agents have important therapeutic indications, *e.g.*, coronary heart disease, pulmonary embolism, and prevention of clotting after insertion of artificial heart valves. The magnitude and duration of pharmacologic activity of this class of drugs is a function of drug level in the body (6); excessively high coumarin levels can lead to hemorrhage with potentially disastrous consequences. The incidence of bleeding during anticoagulant therapy is related directly to excessive reduction of prothrombin activity (7).

The dosage form characteristics of coumarin anticoagulants can have a pronounced effect on their absorption. Lozinski (8) reported that a change in formulation of bishydroxycoumarin tablets caused such an augmentation of the therapeutic response that hemorrhage ensued in some instances. O'Reilly *et al.* (9) administered bishydroxycoumarin as tablets, powder, and solution and found appreciable differences in and correlations between the time of maximum blood levels and the onset of action. The drug was rapidly absorbed as a solution and slowly absorbed as tablets. From 10 to 30% of the dose was recovered in the feces after administration of tablets; no unchanged drug was found in the feces after intravenous administration. Warfarin is more readily and completely absorbed than bishydroxycoumarin (9). A comparison of warfarin blood levels after oral administration of 100 mg. in solution, in tablets containing the drug as the free acid, and in tablets containing sodium warfarin, showed that the most rapid absorption occurred from solution (9). Surprisingly, the free acid in tablets was as readily absorbed as the sodium salt in tablets (9).

The purpose of the present study was to compare the *in vitro* dissolution and *in vivo* absorption characteristics of different tablet preparations of warfarin and to examine the relationship between these two properties.

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† Deceased.

EXPERIMENTAL

Absorption Study.—Normal adult volunteers received 100 mg. of warfarin (as 25-mg. tablets) orally in the morning on an empty stomach. The tablets were swallowed whole together with 250 ml. of water. Food was withheld for at least 2 hr. after drug administration. Blood specimens were obtained at frequent intervals (3 to 7 samples) during the first 6 hr., and then at 12, 24, 48, and 72 hr. after drug administration. Warfarin concentrations in plasma were determined by the method of O'Reilly *et al.* (10). The amount absorbed as a function of time was calculated by the method of Wagner and Nelson (11).

Determination of Dissolution Rate.—*In vitro* dissolution tests were carried out by the beaker method of Levy and Hayes (12). For the initial screening tests 350 ml. water was used as the dissolution medium and a stirring rate of 60 r.p.m. was employed. In subsequent tests, the dissolution medium consisted of 300 ml. of 0.01 to 0.1 *N* hydrochloric acid for the first 30 min. Fifty milliliters of tris(hydroxymethyl)aminomethane solution of sufficient concentration to yield a final pH of 7.4 was then added. A stirring rate of 50 r.p.m. was employed in these experiments. From 3 to 5 tablets were used for each test. Warfarin was determined spectrophotometrically at 270 and 306 mμ in the organic phase obtained after chloroform (5 ml.) extraction of a 3-ml. aqueous sample acidified by addition of 1 ml. of concentrated HCl.

Tablet Preparations.—The several tablet preparations tested consisted of commercially available as well as experimental formulations obtained from a pharmaceutical manufacturer. Each of the tablets contained 25 mg. of warfarin as the free acid or the sodium salt.

RESULTS AND DISCUSSION

The *in vitro* dissolution rate in water of 7 different tablet preparations was evaluated. Markedly different rates were observed; dissolution half-lives ranged from 4 to about 1400 min. Data for 4 of the preparations are shown in Fig. 1. A and C are commercial products containing sodium warfarin, B is an experimental formulation containing sodium warfarin, and D is an experimental formulation containing warfarin as the acid. Preparations A and B dissolved rapidly, C dissolved slowly, and D very slowly. Accordingly, forms A, C, and D were chosen for clinical study.

The times for 50% *in vivo* absorption (including absorption lag time) of the 3 preparations in a total of 10 tests are listed in Table I. There were marked

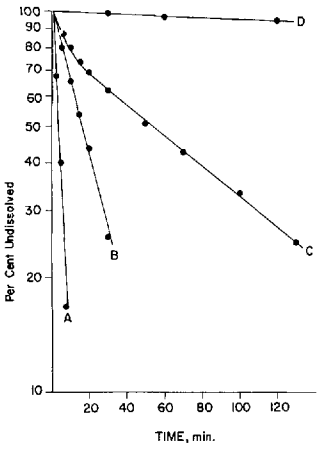

Fig. 1.—Semilogarithmic plot of per cent warfarin undissolved from several tablet formulations *vs.* time. The medium used was distilled water.

TABLE I.—TIME FOR 50% ABSORPTION[a] OF WARFARIN FROM DIFFERENT TABLET PREPARATIONS

Subject	Sex	Age	Prep. A	Prep. C	Prep. D
1	M	32	20	135	57
2[b]	F	36			48
3	M	24	21		
			84	230	
4	M	25	78	230	114

[a] In minutes. [b] This subject received 75 mg.; all others received 100-mg. doses.

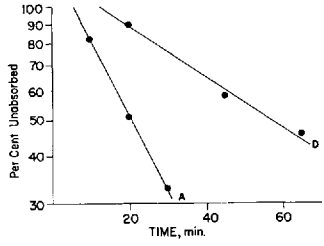

Fig. 2.—Apparent first-order absorption of warfarin from preparations A (subject *3*) and D (subject *1*).

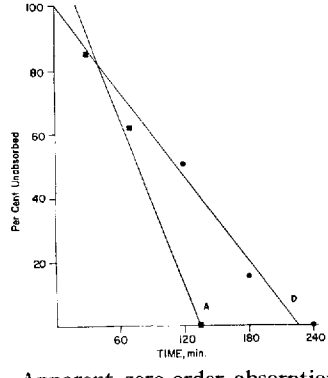

Fig. 3.—Apparent zero-order absorption of warfarin from preparations A (subject *3*) and D (subject *4*).

intra-product differences which overshadowed any product-to-product differences. Based on the dissolution rate experiments, product D was expected to be very slowly and incompletely absorbed. Actually, absorption was quite rapid and complete in each case, as determined from the area under the blood level *versus* time curves and the prothrombin times. The variability of absorption was apparently not due to individual differences between test subjects; subject *3* showed a fourfold variation in absorption half-time on repeated experiments.

Although the variability of absorption made it difficult to obtain blood samples at optimum intervals, the kinetics could be determined in most of the experiments. Preparations A and D, which differed so markedly in the *in vitro* dissolution test, were of particular interest. They were absorbed by both apparent first and zero-order kinetics. Data indicative of apparent first-order absorption are shown in Fig. 2, and examples of apparent zero-order absorption kinetics are depicted in Fig. 3.

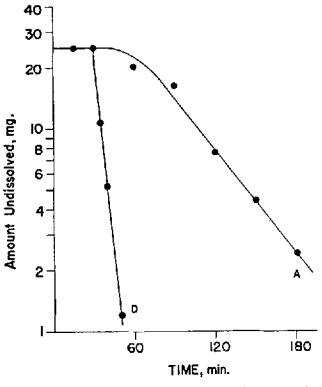

Fig. 4.—Apparent first-order dissolution of warfarin preparations A and D. Conditions: 30 min. dissolution in 0.1 *N* HCl, then dissolution in the medium brought to pH 7.4 by adding tris(hydroxymethyl)aminomethane.

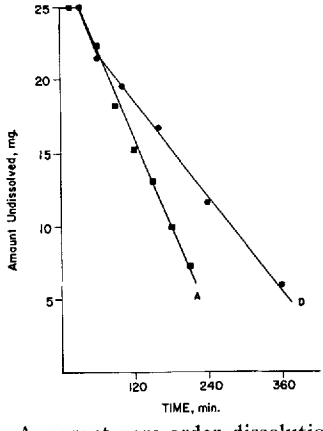

Fig. 5.—Apparent zero-order dissolution of warfarin preparations A and D. Conditions: 30 min. dissolution in 0.01 *N* HCl, then dissolution in the medium brought to pH 7.4 by adding tris(hydroxymethyl)aminomethane.

The marked contrast between the results of the clinical study and those of the dissolution experiments prompted an examination of the conditions of the dissolution procedure and their effect on the dissolution kinetics of warfarin in different tablet preparations. Small changes in stirring rate did not affect significantly the relative dissolution rates of the different preparations. The use of a buffer solution at pH 7.4 instead of water did not result in significant changes. Essentially no dissolution occurred in 0.01 to 0.1 *N* hydrochloric acid solutions because of the extremely low aqueous solubility of warfarin acid. However, tablets A and D disintegrated readily in 0.1 *N* hydrochloric acid but not in water or in 0.01 and 0.05 *N* hydrochloric acid. Based on this observation and the analogy to the passage of drug from the acidic environment of the stomach to the more neutral intestinal fluids, dissolution rates were determined under the following conditions. The tablets were placed in 0.01, 0.05, or 0.1 *N* hydrochloric acid for 30 min., and tris-(hydroxymethyl)aminomethane was then added to increase the pH of the medium to 7.4.

Under these conditions tablets A and D dissolved by apparent first-order kinetics after exposure to 0.1 *N* hydrochloric acid (Fig. 4), and by apparent zero-order kinetics after exposure to 0.05 or 0.01 *N* hydrochloric acid (Fig. 5). While tablets A dissolved about 350 times more rapidly than tablets D in water (Fig. 1), tablets D dissolved about 14 times faster than tablets A in pH 7.4 buffer after exposure to 0.1 *N* hydrochloric acid (Fig. 4).

The results of the dissolution experiments with hydrochloric acid solutions as the initial medium correlate with the clinical studies, and serve to explain the surprisingly rapid absorption of the warfarin acid tablets. The dissolution data, together with physiologic considerations, may also explain the qualitative and quantitative variability observed in the absorption study. Warfarin is almost insoluble in acidic gastric fluids and is therefore apparently not absorbed from the stomach. This has been demonstrated already in previously reported experiments (9) and may account for the lag times for absorption. For example, the longer time required by subject *1* to absorb warfarin from solution than from tablets A probably resulted from fortuitously rapid gastric emptying of the tablets in the latter instance. The variation in the type of absorption kinetics observed (*i.e.*, zero-order or first-order) can result from variations in the pH of gastric fluids within the physiologic range (13). Thus, absorption of warfarin from tablets A and D is determined in part by gastric emptying rate and gastric pH. Absorption from tablets C is probably influenced mainly by gastric emptying, since dissolution was not affected markedly by variations in the concentration of hydrochloric acid in the *in vitro* experiments.

The results of this study show the pronounced effect of solvent composition on dissolution kinetics of drugs in certain tablet formulations. The study illustrates also the difficulty of obtaining correlations with *in vitro* dissolution rates when absorption is influenced markedly by gastric emptying rate. Similar results were obtained recently with enteric-coated tablets (14). Because of the long half-life of warfarin (10), the blood levels of this drug are relatively insensitive to variations in absorption rate, unless absorption is so slow that the drug is absorbed incompletely. With more rapidly eliminated drugs, wide variations in absorption rates could limit seriously their usefulness for oral administration, even if complete absorption occurs in each case. The need for pretreatment with acid in order to obtain rapid dissolution, observed with tablets A and D, suggests that these tablets have a matrix which dissolves or disintegrates readily only in acid. It would be desirable that, in most instances, conventional compressed tablets be so formulated that release of the active ingredient is not a sensitive function of pH in the physiologic range.

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Analgesic and Local Anesthetic Activity of Dimethyl Sulfoxide

By RALPH W. MORRIS

To provide data with which to settle some of the current controversy over the clinical claims for dimethyl sulfoxide (DMSO) specific pharmacologic tests were performed. DMSO is "relatively nontoxic" to rodents but appears to be more toxic as the phylogenetic order is ascended. Considerable irritation results from the introduction of DMSO into the conjunctival sac of the rabbit. No topical or nerve block anesthetic activity was demonstrated. Infiltration anesthetic potency of DMSO was so low as to be of no therapeutic importance. No analgesia, free of sedation, was found. Topical application of trypan blue with DMSO produced considerable dermal staining but no distribution of the dye as contrasted with the rapid systemic absorption and distribution of the dye when given subcutaneously with DMSO. No evidence was obtained in these experiments which could support clinical claims made to date.

CONSIDERABLE controversy has been aroused by recent claims of striking clinical activity by dimethyl sulfoxide (DMSO) in certain musculoskeletal injuries and inflammations, including acute and chronic subacromial bursitis, acute musculoskeletal trauma, osteoarthritis, rheumatoid arthritis, gouty arthritis, scleroderma, and Dupuytren's contracture (1, 2). In an attempt to resolve some of the controversy specific pharmacologic tests were selected to determine whether the reported activity in man could be demonstrated in laboratory animals. Special consideration was given to testing for DMSO activities most nearly associated with the therapeutic claims of "local analgesia," "penetrant carrier," and "relatively nontoxic" (1).

EXPERIMENTAL

DMSO, certified reagent grade, Fisher Scientific Co., was kept in tightly sealed bottles since DMSO is very hygroscopic, rapidly picking up 70% of its own weight in water while evolving as its heat of solution 60 cal./Gm. at 20° (3). Analgesic activity was made relative to several standard analgesics (*i.e.*, morphine sulfate, meperidine hydrochloride, and codeine phosphate). Analgesic activity was determined in Carworth Farms (CF-1) mature female albino mice by the classical hot plate method, essentially that of Ohlsson (4), and by the Bianchi and Franceschini (5) modification of the Haffner tail clamp method (6). The DMSO was administered intraperitoneally, 0.01 ml./Gm. of mouse body weight, in at least 4 doses between 5.5 and 11.0 Gm./Kg., diluting with 0.9% saline whenever necessary. Design was such that the sample size was at least 10 and usually 20 mice per test dose of each drug. Three types of local anesthetic activity were investigated: topical anesthesia by the classical corneal response in rabbits, infiltration anesthesia by the guinea pig intracutaneous wheal technique of Bulbring and Wajda (7), and nerve block anesthesia by a modification of the frog sciatic nerve-gastrocnemius muscle preparation of Sinha (8). Three to 6 guinea pigs with 6 response sites per guinea pig were used for each dose of drug in the intracutaneous wheal test. Procaine and lidocaine hydrochlorides served as standards.

The effects of DMSO on membrane permeability were determined by noting the latent periods required for the adsorption, distribution, and subsequent discoloration of connective tissues from topically and subcutaneously administered trypan blue in mice. All median response doses (*i.e.*, ED_{50} and LD_{50}) and their 95% confidence limits were determined by the method of Litchfield and Wilcoxon (9), whereas all other statistical analyses were performed by the methods of Snedecor (10).

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RESULTS AND DISCUSSION

Toxicity.—A primary concern with the introduction of any new substance into human pharmacology is the precision with which projections of acute human toxicity can be made from tests on laboratory animals. Table I summarizes the available data on the acute lethality of DMSO as obtained from the literature (11, 12) and experiments recently completed in this investigator's laboratory. From Table I it would appear that a variably increasing lethality to DMSO occurs as the phylogenetic order is ascended.

Ventricular fibrillation and death occurred within 3 min. after the administration of 1.65 Gm./Kg. of 100% DMSO into the marginal ear vein of the rabbit. The LD_{50} for intravenous DMSO in female albino rabbit was 1.34 Gm./Kg. based on 3 doses between 1.10 and 1.65 Gm./Kg. given to a total of 28 rabbits. Female rabbits appear to be more sensitive to DMSO than are rodents, but are about equally sensitive as male dogs (Table I). DMSO

TABLE I.—ACUTE LETHALITY OF DIMETHYL SULFOXIDE

Animal	Rt. of Administration[a]	LD_{50} in Gm./Kg. (95% Confidence Limits)
Chicken	Oral	13.74[b]
Mouse		
	i.v.	5.75[c]
	i.p. (male)	10.10 (9.22–11.06)
	i.p. (female)	9.95 (9.35–10–59)
	Oral	21.40[c]
	Oral	21.98[b]
Rat		
	i.v.	5.36[c]
	Oral	28.30[c]
Rabbit	i.v. (female)	1.34 (0.96–1.88)
Dog		
	i.v. (male)	2.40[c]
	i.v. (male)	1.50[d]

[a] Routes of administration: i.v., intravenous; i.p., intraperitoneal. [b] From Brown *et al.* (11). [c] From Wilson *et al.* (12). [d] Preliminary value based on intravenous titration in 4 dogs.

might therefore be said to be "relatively nontoxic" (1) when relating chickens or rodents to rabbits and dogs. To date human toxicity following topical application consists of warmth, erythema, itching, local urticaria, increased skin pigmentation, dermatitis, fatigue, and lethargy (2). This observed topical toxicity plus the relative ease with which DMSO is said to be absorbed through the epidermis (1) should arouse considerable concern over the potential systemic toxicity of DMSO in man if it were applied *via* a route of administration (*e.g.*, parenteral) or to hyperpermeable tissue (*e.g.*, inflamed or open wounds) thereby facilitating an even more rapid and extensive absorption and distribution than by the topical route. There has been a recent report of a death possibly related to an acute allergic reaction to DMSO (13). Such reports must be thoroughly investigated, however.

Analgesia.—The unusual term "local analgesia" as used by Rosenbaum *et al.* (1) does not give recognition to the striking pharmacologic difference between analgesics and local anesthetics. Therefore, specific tests were made to determine if DMSO was a local anesthetic, an analgesic, or both. (Table II.) The analgesic ED_{50} for DMSO and morphine sulfate in mice are 6700 and 15 mg./Kg., respectively, using the Haffner tail clamp method 20 min. after the intraperitoneal administration of the test solutions. The other analgesics tested are all less potent than morphine which is in keeping with their known clinical potency. Attempts to determine DMSO analgesic activity in mice by the classical hot plate method were unsuccessful because DMSO produced extensive sedation thereby making the mouse incapable of performing the test response (*i.e.*, jumping to the lip of a 10-in. high glass cylinder sitting on a thermostatically controlled hot plate maintained at 60 ± 0.5°). On the basis of this observation a sedative rather than analgesic ED_{50} for subcutaneously administered DMSO was determined as being 6.92 Gm./Kg. Criteria for the sedative end point was the presence of a thoroughly depressed, immobile, but not ataxic mouse which was still capable of righting itself from the dorsal to the ventral positions within a 30 sec. test period (*i.e.*, neither asleep nor anesthetized). Such similarity in the DMSO sedative and analgesia ED_{50} values seriously question whether the failure of the mice to respond to the Haffner tail clamp stimulus might not be equally as well explained on the basis of sedation as analgesia. This possibility is presently being explored.

TABLE II.—CENTRAL NERVOUS SYSTEM ACTIVITY OF DIMETHYL SULFOXIDE IN THE MATURE FEMALE ALBINO MOUSE

Pharmacologic Activity	ED_{50}, mg./Kg. (95% Confidence Limits)	No. Doses	Total Sample Size
Sedation, s.c.			
DMSO	6920 (3980–12040)	2	20
Analgesia, i.p. Haffner tail clamp			
DMSO	6700 (6200–7240)	5	110
Morphine sulfate	11.6 (9.47–14.21)	5	110
Meperidine HCl	38.3 (31.39–46.73)	3	90
Codeine phosphate	34.5 (28.51–41.75)	4	100
Dextropropoxyphene HCl	36.7 (31.75–42.43)	3	60

TABLE III.—LOCAL ANESTHETIC ACTIVITY OF DIMETHYL SULFOXIDE

	ED_{50} in % Soln. (95% Confidence Limits)
Topical	
Rabbit (female):	
DMSO	Inactive[a]
Infiltration	
Guinea pig (female):	
DMSO	51.0 (38.5–67.6)
Lidocaine HCl	1.17 (0.90–1.52)
Procaine HCl	0.84 (0.62–1.13)
Sciatic nerve block	
frog: DMSO	Inactive[a]

[a] No anesthesia produced by maximum dosage used, a 100% solution.

Local Anesthesia.—No alterations in the pupillary response to penlight illumination or to touch with a glass probe were noted during 2 hr. test periods following the instillation into the conjunctival sac of the rabbit of 0.25 ml. of DMSO solutions, varying from 30 to 100%. In contrast to the nonirritant report of Brown (11) all 24 female albino rabbits that received DMSO, irrespective of the per cent concentration, experienced considerable conjunctival inflammation and limbic corneal vasodilatation. The irritation and vasodilatation cannot be solely the consequence of the heat of solution (*i.e.*, counter-irritant activity) since even 30% solutions (well below the 62% equillibrium point of DMSO–water mixture) produced irritation and vasodilatation. The duration of the severe irritation (30.8 ± 20.7 min.) does not appear to be dose dependent; however, the experiments were not designed to provide statistical evaluation of such a regression function. A positive corneal reflex to touch with a glass probe (*i.e.*, an eyelid movement) was present before and throughout the 90 min. period following the introduction of all per cent solutions of DMSO into the conjunctival sac. In all 24 rabbits tested with DMSO, blockade of the corneal reflex was produced after 90 min., the blockade persisting for the remainder of the test period (*i.e.*, 120 min. in 19 rabbits and 165 min. in 5 rabbits). No explanation of this delayed blockade of the corneal reflex is possible, since the experiments were not designed with this question in mind.

The ED_{50} of DMSO as an infiltration anesthetic was 51.0% solution compared to the ED_{50} of 1.17 and 0.84% solutions for lidocaine and procaine hydrochlorides, respectively. (Table III.) No evidence of nerve block anesthesia was obtained when 100% DMSO was tested on each of four sciatic nerve-gastrocnemius muscle preparations; therefore, further nerve block testing was discontinued.

Membrane Permeability.—Topical application of 1% solutions of trypan blue in either distilled water or DMSO solutions varying from 50–100% to the shaved scapular area of the mouse failed to induce systemic absorption and distribution of trypan blue, although extensive staining at the site of application occurred with the solutions containing

DMSO. However, the administration of 1% trypan blue in solution with 50–100% DMSO facilitated the speed with which subcutaneous injections of 1% trypan blue were absorbed and distributed to the connective tissues in the ears, tail, and paws of mice. Trypan blue in DMSO solutions were significantly more rapidly distributed than 1% trypan blue in distilled water (*i.e.*, 6.9% more rapidly in 50% DMSO and 3.9% faster in 100% DMSO: each with $p < 0.001$). After 24 hr. all mice that had received topical administration of 1% trypan blue, with or without DMSO, showed no signs of the typical bluish discoloration of connective tissues. On the other hand, the previously noted statistically significant effect of subcutaneously administered DMSO on the absorption and distribution of trypan blue solutions were even more evident after 24 hr., *i.e.*, all visible connective tissues were dark blue after trypan blue in 100% DMSO, moderately blue in 50% DMSO, and pale blue when in distilled water.

All animals receiving DMSO, regardless of dosage, very rapidly (*i.e.*, usually within 2 or 3 min.) gave strong evidence in their expired air of extremely rapid DMSO metabolism, *i.e.*, to dimethyl sulfone in mice, guinea pigs, rabbits, and dogs (11, 14); to dimethyl sulfide in cats (15); and to unspecified metabolites in man (1, 2, 14). Shortly thereafter similar evidence could also be found in the urine, feces, and saliva.

The rapidity with which DMSO is metabolized jeopardizes precise determinations of chronic toxicities and confounds acute toxicities, especially when the DMSO is administered by any route other than the intravenous. Even DMSO data by the intravenous route of administration would have to be considered misleading if one accepts the literature reports that DMSO induces perivascular inflammation (12) and extensive protein denaturation as evidenced by the rapid development of hemolytic anemia (12, 15). No evidence of perivascular inflammation was seen in any of the 13 rabbits that survived the 1.10 Gm./Kg. dose of DMSO during the acute toxicity tests or during the 6 hr. of intravenous administration of DMSO in each of 4 dog carotid artery blood pressure preparations (16).

Unequivocal evaluation of the toxicologic and pharmacologic data for DMSO is therefore rather difficult. First, the physical properties, especially the heat of solution (3, 11), confounds attempts to attach chemical and/or biological mechanisms to observed pharmacologic responses. Second, the biochemical instability of DMSO in the systemic circulation is so great that an uncertain number of potentially and variably toxic metabolities are very rapidly produced. Third, determining precise values for such an impotent agent as DMSO makes for rather inaccurate estimates of its very subtle activities. Fourth, the public and scientific controversy attendant to the plethora of DMSO lay articles published to date[1] creates an atmosphere in which strictly objective evaluations by all parties is difficult. Recent statements by the Food and Drug Administration, U. S. Department of Health, Education, and Welfare (17–19), and in *Medical Letter* (2, 13) have introduced some stability into the very controversial therapeutic merits of DMSO.

On the basis of the experiments reported here, no evidence was obtained in support of the therapeutic claims of "local analgesia," "penetrant carrier," or "relatively nontoxic" in humans (1).

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dl-2,6-Diamino-4-hexynoic Acid, a Growth Inhibitor

By JOHN L. NEUMEYER and WILLIAM FIRSHEIN*

***dl*-2,6-Diamino-4-hexynoic acid, a lysine analog, was synthesized by the interaction of *N*-(4-chloro-2-butynyl)phthalimide and the sodium salt of ethyl acetamidomalonate. A two-step hydrolysis yielded the desired acetylenic diamino acid. *dl*-Lysine, identical with the known amino acid, was obtained by the reduction of the triple bond in I. This acetylenic analog of lysine inhibited the growth of DNA, RNA, and protein synthesis in resting cell suspensions of pneumococci at a concentration of 100 mcg./ml.**

THE UNSATURATED amino acids, methallylglycine and crotylglycine, were first prepared by Albertson and co-workers, (1) and were found to be potent inhibitors of microorganisms. On the basis that olefinic unsaturation produces powerful inhibition of microorganisms, Gershon and co-workers (2) prepared two amino acids containing the acetylenic linkage, propargylglycine and 2-amino-3-methyl-4-pentynoic acid, and found that these acetylenic analogs were more potent growth inhibitors than their ethylenic analogs.

Similarly, several cyclopentenyl, cyclopentyl, and cyclohexenyl amino acids have been demonstrated (3, 4) to be competitive inhibitors of certain naturally occurring amino acids in various microorganisms. Unsaturation within the cycloalkyl moiety in all cases resulted in compounds acting as amino acid antagonists. The importance of steric requirements was further exemplified by Shive and co-workers (4) who synthesized *cis* and *trans* dehydrolysine and found the *trans* compounds to be the only isomer active as a competitive lysine antagonist.

Davis *et al.* (5) have recently reported that the introduction of a carbon-carbon triple bond into the carbon chain of lysine (I) forces the lysine into a fixed conformation and yields a competitive growth inhibitor of *Leuconostoc dextranicum* 8086. The authors were thus prompted to report their findings on the synthesis and biological properties of *dl*-2,6-diamino-4-hexynoic acid (I), their investigations having been carried out independently.

$$NH_2CH_2C{\equiv}C{-}CH_2CHNH_2COOH$$

"Acetylenic" Lysine

I

The authors' biological results (Table I) show that at a concentration of 100 mcg./ml., "acetylenic" lysine (I) is an effective inhibitor of DNA, RNA, and protein synthesis. Higher concentrations than 100 mcg./ml. do not increase the inhibition, while lower concentrations than 100 mcg./ml. are less effective. The control levels are depressed equally for all three components (about 80% inhibition). Since these results are obtained with "resting" cells (with respect to multiplication), it cannot be stated definitely that similar results would be obtained in growing (multiplying) cultures. However, since the synthesis of three essential components (DNA, RNA, and protein) required for *any* multiplication are inhibited by the modified lysine, it is likely that multiplication in growing cultures would also be inhibited. As for the mechanism of action of "acetylenic" lysine, at least two possibilities exist. One is that modified lysine may be a general growth inhibitor rather than a specific antagonist of DNA, RNA, and protein synthesis, since it is unlikely that the drug would affect reactions involved in the synthesis of all three components. Rather, it is possible that "acetylenic" lysine acts on some basic cell process that indirectly affects DNA, RNA, and protein synthesis, such as respiration. Two is that since the modified lysine was reported (5) to be a competitive antagonist of lysine, it is possible that the drug inhibits protein synthesis specifically, which then results in an inhibition of DNA and RNA synthesis.

TABLE I.—EFFECTS OF *dl*-2,6-DIAMINO-4-HEXYNOIC ACID HYDROCHLORIDE ON NUCLEIC ACID AND PROTEIN SYNTHESES OF PNEUMOCOCCI

dl-2,6-Diamino-4-hexynoic Acid Hydrochloride (mcg./ml.)	% Increase Over 0 Time after 70 min.		
	DNA	RNA	Protein
None	20	18	30
25	18	18	25
50	10	9	14
100	4	3	6
200	4	None	3

EXPERIMENTAL[1]

N-(4-Chloro-2-butynyl)phthalimide.—To a solution of 74 Gm. (0.6 mole) of 1,4-dichloro-2-butyne (General Aniline and Film Corp.) in 1000 ml. of dimethylformamide at 90° in a 2-L. 3-necked flask equipped with a stirrer, thermometer, and a funnel was slowly added with constant stirring 55 Gm. (0.3 mole) of potassium phthalimide. Stirring with heating (90°) was continued for 4 hr. after which the solution was poured into an ice-water mixture.

The precipitate which formed was filtered (suction) and washed with 200 ml. of 5% sodium hydroxide followed by two washings with 200 ml. of water. The white crystals were dried (vacuum) and recrystallized from ethanol to yield 28 Gm. (40%), m.p. 121–122°. [Lit. (5) m.p. 120–121°.]

Anal.—Calcd. for $C_{12}H_8ClNO_2$: C, 61.75; H, 3.41; Cl, 15.21. Found: C, 61.69; H, 3.61; Cl, 15.15.

Ethyl 2-Acetamido-2-carbethoxy-6-phthalimido-4-hexynoate.—To a 500-ml. 3-necked flask fitted with a condenser and mechanical stirrer was added a solution of 21.8 Gm. (0.1 mole) of ethyl acetamidomalonate (Winthrop Laboratories) in 200 ml. of absolute ethanol. After the malonate had completely dissolved in the ethanol, 2.3 Gm. of sodium was added and the mixture was rapidly taken up to reflux temperature. At this point 30.4 Gm. of

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[1] All melting points are uncorrected and were obtained in a Thomas-Hoover silicone-filled capillary melting point apparatus.

N-(4-chloro-2-butynyl)phthalimide was added in 1 part. The reaction mixture was then heated at reflux for 8 hr., allowed to cool, and the solid precipitate was filtered off. The precipitate was then extracted with hot absolute ethanol and the extract was allowed to cool. White crystals of the hexynoate separated. These crystals were again recrystallized from absolute ethanol to yield 37% of product, m.p. 149–150°. [Lit. (5) m.p. 139–142°.]

Anal.—Calcd. for $C_{21}H_{22}N_2O_7$: C, 60.86; H, 5.35; N, 6.76. Found: C, 61.24; H, 5.62; N, 7.08.

Ethyl 2-Acetamido-6-phthalimido-4-hexynoate.—A small amount could be isolated from the above reaction by evaporation of the filtrate from the reaction mixture. The resulting red residue was triturated with water, the water decanted off, and the remaining solid recrystallized from absolute ethanol, m.p. 224–226°.

Anal.—Calcd. for $C_{18}H_{18}N_2O_5$: C, 63.15; H, 5.30. Found: C, 63.09; H, 5.55.

dl-2,6-Diamino-4-hexynoic Acid Hydrochloride.—Ethyl 2-acetamido-2-carbethoxy-6-phthalimido-4-hexynoate (41.5 Gm., 0.1 mole) was hydrolyzed with 5.5 Gm. of 85% aqueous hydrazine in 200 ml. of ethanol by the method of Sheehan (6). As the solution was taken up to reflux temperature, the hexynoate slowly went into solution and the reaction became clear, but shortly thereafter, phthalhydrazide began to precipitate as a white solid. After 1 hr. of stirring at reflux, the reaction mixture was cooled to room temperature, and the phthalhydrazide was filtered off. The reaction liquor was then reduced under vacuum to yield a viscous semi-clear yellow residue. Without further isolation the crude hydrazine hydrolysate was dissolved in a small amount of water and then was added to an excess of concentrated hydrochloric acid. This mixture was then stirred at reflux for 1.5 hr. The reaction mixture was cooled to room temperature and the solvents partially removed under vacuum, cooled in an ice bath, and filtered. This filtration was carried out in order to remove any residual phthalhydrazide.

The filtrate, containing the product, was then completely evaporated under vacuum, the residue triturated with acetone to yield a crystalline product. The product thus formed consisted of a mixture of the monohydrochloride and the dihydrochloride of "acetylenic" lysine (I). Pure monohydrochloride (11.1 Gm., 62% yield) was obtained by recrystallization of the mixture of the mono- and dihydrochlorides from an ethanol–water–pyridine solution. This compound melted at 236° when a sample was placed in the oil bath at 220° and heated at approximately 2°/min.[2]

Anal.—Calcd. for $C_6H_{10}N_2O_2 \cdot HCl$: C, 40.35; H, 6.16; Cl, 19.85; N, 15.69. Found: C, 40.56; H, 6.21; Cl, 19.3; N, 15.71.

Catalytic Hydrogenation of 2,6-Diamino-4-hexynoic Acid Monohydrochloride.—A solution of the monohydrochloride (0.1816 Gm.) of the acetylenic amino-acid (I) in water (10 ml.) was catalytically hydrogenated over platinum oxide (0.053 Gm.) at room temperature and atmospheric pressure. When 2 *M* equivalents of hydrogen had been absorbed, the catalyst was removed, the filtrate was reduced to dryness under reduced pressure, and the residue was crystallized from 5 ml. of 95% ethanol containing 2 drops of pyridine. The crystals which formed were filtered off and dried, m.p. 260–262°. [Lit. (7) m.p. 262–264°.] The melting point of a mixture of an authentic sample of *dl*-lysine monohydrochloride with a sample of the product isolated above was not depressed (m.p. 261–262°). The infrared spectra of this product was identical with that of an authentic sample of *dl*-lysine monohydrochloride.

Biological Test Methods.—Virulent pneumococci derived from type III (strain A66) were grown in 3 L. of a casitone, tryptone (both Difco), albumin (fraction V from bovine serum albumin, Armour) medium supplemented with glucose and K_2HPO_4 (8) for 24 hr. at 37°. After this time, additional glucose and phosphate were added and the cells were incubated for 3 hr. The viable cell count after this 3-hr. period was approximately $4–6 \times 10^9$ cells/ml. The culture was centrifuged in a refrigerated centrifuge (International) at 30,000 × g for 20 min., washed 3 times with cold Na-K-phosphate buffer (0.02 *M*, pH 7.5), and inoculated into 3 ml. of a suspending medium consisting of this same buffer, glucose, casitone (both 1.0%), and catalase (0.005%, Nutritional Biochemicals). The suspending medium was prepared double strength so that additions could be made without affecting the over-all concentration of the basic constituents. *dl*-2,6-Diamino-4-hexynoic acid hydrochloride was added in 4 concentrations: 25, 50, 100, and 200 mcg./ml. (in the suspending medium). All samples were prepared in duplicate and the inoculated suspensions contained $1–2 \times 10^9$ viable cells/ml. The suspensions were incubated at 37° for 70 min. in a gyrotory water bath shaker (New Brunswick Scientific) at 200 r.p.m. DNA, RNA, and protein were then extracted as described previously (9) and measured by the procedures of Brody (10), Drury (11), and Lowry *et al.* (12), respectively.

[2] Davis *et al.* (5) reported their 2,6-diamino-4-hexynoic acid dihydrochloride as having a m.p. 178–183° dec.

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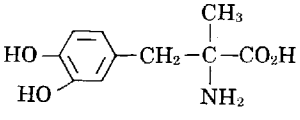
Cyclic Methyldopa Analogs as Potential Antihypertensive and Antineoplastic Agents

By J. H. BURCKHALTER and GÜNTER SCHMIED*

Three cyclic methyldopa analogs (III*a*, *b*, and *c*) were synthesized by hydrolysis of the appropriate spirohydantoins (V), which were obtained through the Bucherer synthesis. Amino acids (III*a* and *b*) and hydantoins (V*a* and *c*) were inactive against experimental tumors. The 5 hydantoins tested were devoid of anticonvulsant effect.

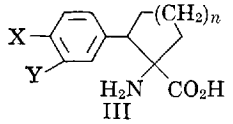
THE AMINO acid, methyldopa (I), is a recognized drug for hypertension (1), while another amino acid, 1-aminocyclopentane-1-carboxylic acid (NSC-1026, II), possesses inhibitory activity against



I

II

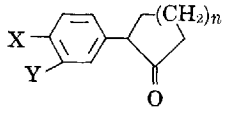
tumors (2). The structures of these substances, which are aliphatic α-amino acids fully substituted at the α position, suggested the synthesis of hybrids of I and II which are represented by structure III.



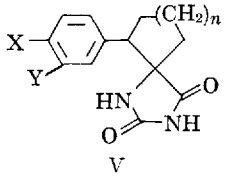
III

a, ($n = 1$, X = Y = H)
b, ($n = 2$, X = Y = H)
c, ($n = 2$, X = Y = OH)

The approach to compounds of type III was through appropriate ketones IV which were converted by



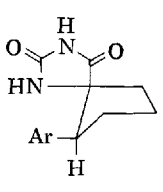
IV



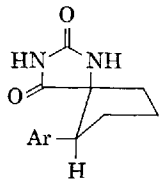
V

means of potassium cyanide and ammonium carbonate in a Bucherer-Bergs synthesis (3) to the spirohydantoins (V*a–e*) of Table I. Demethylation of hydantoins V*b*, *d*, and *e* to phenolic spirohydantoins (V*f*, *g*, and *h*) of Table I was effected by means of refluxing overnight with 47% hydriodic acid and glacial acetic acid. All the hydantoins of Table I exhibited characteristic infrared absorption at 3.1 μ and a doublet in the carbonyl region between 5.6 and 5.9 μ.

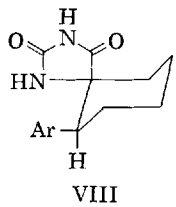
Hydantoins of structure V are representable by the following structures as *cis-trans* pairs (VI and VII; VIII and IX). However, separation of any



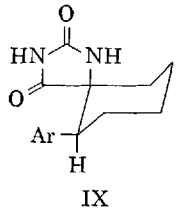
VI



VII



VIII



IX

isomers was not possible by means of thin-layer chromatography. In each case only a single spot was observable. This observation agrees with the recent conclusion (4) that the Bucherer-Bergs synthesis is stereoselective and only a small percentage yield of a second isomer is sometimes isolable.

Methods employing barium hydroxide or hydrogen chloride in glacial acetic acid failed to hydrolyze the spirohydantoins (V). Despite poor solubility, hydantoins (V*a* and V*c*) were hydrolyzed by heating at 140–150° with 60% sulfuric acid (5). After neutralization of the mixture with barium carbonate and desalting by ion-exchange resin, the yield of amino acid was 33% of III*a* and 51% of III*b*. The *N*-(2,4-dinitrophenyl) derivative of each amino acid was prepared. Amino acid (III*c*) was obtained by hydrolysis of hydantoin (V*e*) and demethylation of the unisolated intermediate by means of hydriodic acid. The crude hydriodide was converted to the hydrochloride of III*c* by ion exchange. Over-all yield from the hydantoin was 12%.

PHARMACOLOGICAL RESULTS

The following summary of results available to date was prepared from reports submitted by Dr. Joseph Leiter, Cancer Chemotherapy National Service Center, Bethesda, Md. Detailed information concerning test procedures may be found in publications from that office (6).

Amino acids (III*a* and *b*) and spirohydantoins V*a* and *c* were screened by the Southern Research Institute, Birmingham, Ala., and were all found to be nontoxic and inactive against sarcoma 180, solid Friend virus leukemia, and lymphoid leukemia L-1210 in mice and also inactive in cell culture.

Professor Ewart Swinyard, College of Pharmacy, University of Utah, found that spirohydantoins V*a*, *c*, *f*, *g*, and *h* were devoid of appreciable anti-

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TABLE I.—6-ARYL-1,3-DIAZASPIRO[4.4]NONANE-2,4-DIONES (V, n = 1) AND 6-ARYL-1,3-DIAZASPIRO[4.5] DECANE-2,4-DIONES (V, n = 2)

Compd.	Aryl	n	M.p., °C.	% Yield	Molecular Formula	Anal., % Calcd.	Found
V*a*	Phenyl	1	215–217 dec.	61	$C_{13}H_{14}N_2O_2$	C, 67.81	67.72
						H, 6.13	6.16
						N, 12.17	12.28
V*b*	4-Methoxyphenyl	1	226–229 dec.	46	$C_{14}H_{16}N_2O_3$	C, 64.60	64.66
						H, 6.20	6.26
						N, 10.76	10.79
V*c*	Phenyl	2	250–252 dec.	60	$C_{14}H_{16}N_2O_3$[a]	C, 68.61	68.61
						H, 6.61	6.46
						N, 11.47	11.30
V*d*	4-Methoxyphenyl	2	235–240 dec.	65	$C_{15}H_{18}N_2O_3$	C, 65.67	65.66
						H, 6.61	6.45
						N, 10.21	10.10
V*e*	3,4-Dimethoxyphenyl	2	221–224 dec.	58	$C_{16}H_{20}N_2O_4$	C, 63.14	62.92
						H, 6.62	6.57
						N, 9.21	9.35
V*f*	4-Hydroxyphenyl	1	345–350 dec.	62	$C_{13}H_{14}N_2O_3$	C, 63.40	63.14
						H, 5.73	5.94
						N, 11.38	11.56
V*g*	4-Hydroxyphenyl	2	389–392 dec.	67	$C_{14}H_{16}N_2O_3$	C, 64.60	64.64
						H, 6.20	6.27
						N, 10.76	10.74
V*h*	3,4-Dihydroxyphenyl	2	317–328 dec.	64	$C_{14}H_{16}N_2O_3$	C, 60.86	60.99
						H, 5.84	5.84
						N, 10.14	10.02

[a] Product analyzed correctly for monohydrate until dried at 150° (0.3 mm.). Previously reported by Tiffeneau, M., Tchoubar, B., Saiaslambert, M., and LeTellier-Dupré, M., *Bull. Soc. Chim. France*, **1957**, 445; m.p. 255°, yield 25%.

convulsant activity in mice as measured by maximal electroshock seizure test.

Owing to the difficulty of obtaining screening of compounds obtained under a grant from the National Institutes of Health, no data are available pertaining to possible antihypertensive activity of the amino acid (III).

EXPERIMENTAL

2 - Arylcycloalkanones.—2 - Phenylcyclopentanone (7), 2-phenylcyclohexanone (8), and 2-(4-methoxyphenyl)-cyclohexanone (9) were obtained as described in the literature. 2-(4-Methoxyphenyl)-cyclopentanone had not been prepared before. The method described in *Reference 9* was used, but difficulty was encountered in its isolation. Its identity was established by formation of the hydantoin (V*b*). 2-(3,4-Dimethoxyphenyl)-cyclohexanone, a new compound, was obtained from 3,4-dimethoxybenzaldehyde by a general method (10), but similarly the hydantoin (V*e*) was made from the crude ketone.

Spirohydantoins (Table I).—The general procedure of preparation was based upon that of Henze and Speer (11). The appropriate ketone was mixed with 2 equivalents of potassium cyanide and 4 equivalents of ammonium carbonate in about 300 ml. of 50% alcohol per 0.1 mole of ketone. In the case of V*c*, the starting ketone was first dissolved in 50 ml. of alcohol. The mixture was stirred for about 4 to 5 hr. at 58–60°. Cooling the mixture to room temperature yields a pure first crop. Furthermore, less pure product was obtained by evaporation of the filtrate to about two-thirds of its *volume and* then acidifying. (Hood!) The hydantoins were recrystallized from 50% alcohol.

Thin-layer chromatography of hydantions, using silica gel as adsorbent and with a developing time of 50 min., gave the following R_f values of approximately the same magnitude, for solvent system chloroform/acetone (1:1) and chloroform/acetone (2:1), respectively: V*a*, 0.83 and 0.59; V*c*, 0.79 and 0.54; and V*e*, 0.77 and 0.49. Sulfuric acid spray and heat were used for development of spots.

Demethylation of Hydantoins Vb, d, and e.—A mixture of 0.01 mole of the methoxyphenylspirohydantoin, 20 ml. of 47% hydriodic acid, and 20 ml. of glacial acetic acid was heated at reflux overnight. After the mixture was cooled and diluted with water, crystallization was induced. Evaporation of the mother liquors gave less pure product.

1 - Amino - 2 - phenylcyclopentane - 1 - carboxylic Acid (IIIa).—A mixture of 23 Gm. (0.1 mole) of crude hydantoin (V*a*) and 400 ml. of 60% sulfuric acid was heated with stirring for 6 hr. at 140–150°. The cooled solution was neutralized with barium carbonate, the precipitated barium sulfate was removed by filtration and washed several times with hot water. From the hot water 2.3 Gm. of unchanged V*a* separated. The solution was concentrated and desalted by absorption of the amino acid in a strongly acidic ion-exchange resin (Dowex 50), washing out the anions and replacing the amino acid with 2 *N* ammonia. When, upon concentrating, the amino acid began to crystallize, an equal volume of hot alcohol was added. Cooling gave 6.9 Gm. (34% yield) of III*a*, m.p. 290° dec. With paper chromatography, only 1 spot was obtained. Whatman's No. 1 paper was employed in ascending flow of solvent at room temperature for the following R_f values: 0.86 (120 butanol/30 glacial acetic acid/50 water); 0.89 (160 phenol/40 water); 0.83 (150 phenol/40 ethanol/10 water); 0.70 (60 butanol/60 pyridine/60 water). Ninhydrin spray was used for development.

Anal.—Calcd. for $C_{12}H_{15}NO_2$: C, 70.22; H, 7.37; N, 6.82. Found: C, 70.18; H, 7.41; N, 6.85.

N - (2,4 - Dinitrophenyl) - 1 - amino - 2 - phenylcyclopentane - 1 - carboxylic Acid.—This was made from III*a* and 2,4-dinitrophenyl-1-fluorobenzene (12). Two recrystallizations from dilute alcohol gave a product, m.p. 220–230° dec. Paper chromatography produced tailing. Thin-layer chromatography using Silica Gel G as adsorbent and solvent system 95 chloroform/5 methanol/1 glacial acetic acid gave after 50 min. R_f value 0.70 (single spot).

Anal.—Calcd. for $C_{18}H_{17}N_3O_6$: C, 58.45; H, 4.09; N, 11.38. Found: C, 58.68; H, 4.39; N, 11.20.

1 - Amino - 2 - phenylcyclohexane - 1 - carboxylic Acid (IIIb).—As in the preparation of III*a*, 26.2 Gm. (0.1 mole) of crude hydantoin V*c* was hydrolyzed. After 6 hr., 38% of V*c* was recovered, 28% after 24 hr., and none remained after 2 days of heating but carbonization resulted. In the last case, the yield of III*b* was 51%, m.p. 286–290° dec. Paper chromatography gave only 1 spot, and R_f values for the same solvent system used for III*a* were, respectively, 0.87, 0.85, 0.82, and 0.73.

Anal.—Calcd. for $C_{13}H_{17}NO_2$: C, 71.20; H, 7.82; N, 6.39. Found: C, 71.02; H, 8.02; N, 6.36.

N - (2,4 - Dinitrophenyl) - 1 - amino - 2 - phenylcyclohexane-1-carboxylic Acid.—This was made (12) as a derivative of III*b*, m.p. 204–206° dec., after 2 crystallizations from 50% alcohol. R_f 0.68 (thin-layer, adsorbent Silica Gel G, 95 chloroform/5 methanol/1 glacial acetic acid).

Anal.—Calcd. for $C_{19}H_{19}N_3O_6$: C, 59.22; H, 4.97; N, 11.16. Found: C, 59.34; H, 5.05; N, 11.10.

1 - Amino - 2 - (3,4 - dihydroxyphenyl) - cyclohexane-1-carboxylic Acid (IIIc) Hydrochloride.—A mixture of 20 Gm. (0.062 mole) of crude hydantoin (V*e*) and 400 ml. of 60% sulfuric acid was stirred and heated at 135–140° for 2 days. The dark solution was neutralized with barium carbonate. The precipitated barium sulfate was removed by filtration, washed with hot water, and the combined solutions evaporated to dryness to give 10 Gm. of dark brown material. It was dissolved in 100 ml. of 47% HI and heated at reflux for 24 hr. Diluted with water and filtered, the brown solution was extracted with ether. The aqueous layer was evaporated to dryness. The product was dried under reduced pressure to remove excess hydrogen iodide, after which it was dissolved in water and adsorbed on an ion-exchange column (Dowex 50). The iodide ions were removed with water and the amino acid eluted with 4 *N* hydrochloric acid. The solution was evaporated to dryness to give 3 Gm. of a slightly gray solid. It was purified by several reprecipitations from glacial acetic acid by ether. A yield of 1.8 Gm. (10% from V*e*) of slightly tan solid III*c* hydrochloride was obtained, m.p. > 300°.

The product is acidic and gives a dark green color with ferric chloride. I.R. spectrum shows a broad band at 2.8 to 4.55 μ, suggesting phenolic and carboxylic hydroxyls, C—H and RNH_3^+ absorption. A carbonyl peak appears at 5.8 μ.

Anal.—Calcd. for $C_{13}H_{18}ClNO_4$: C, 54.20; H, 6.31; N, 4.87. Found: C, 54.34; H, 7.00; N, 4.65.

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Diisopropylammonium Dichloroacetate I. Assay, Distribution Coefficient, and Solubility Studies

By GEORGE E. CREVAR and ROBERT W. GOETTSCH*

Solubility and distribution coefficient values were determined for the drug, diisopropylammonium dichloroacetate, using a nonaqueous titration method. A second assay procedure is presented for the drug utilizing the modified Fujiwara colorimetric method.

IN RECENT YEARS, several papers have described the pharmacological properties of diisopropylammonium dichloroacetate.[1] The hypotensive properties of the drug have been reported (1–3). The antitoxic action of diisopropylammonium dichloroacetate has been demonstrated against potassium cyanide and quinine hydrochloride (4). Biological studies with diisopropylammonium dichloroacetate have indicated its effect on the blood levels of galactose, glycine, and cholesterol (5), on the utilization of amino acids by the liver and other tissues (6), and on the interference with oxidation-reduction processes (7). Villari, Mazzacca, and Coraggio (8) observed the activation effect of diisopropylammonium dichloroacetate on mice infected with MHV-3 (mouse hepatitis virus).

During preliminary biopharmaceutical investigations of diisopropylammonium dichloroacetate, it was necessary to find suitable assay procedures for solubility and distribution coefficient studies. A colorimetric assay for diisopropylammonium dichloroacetate has been reported by Maruyama and Hasegawa (9). Their procedure is based on the color reaction involving naphthorescorcinal and glyoxalic acid, an alkaline degradation product of diisopropylammonium dichloroacetate. More recently, a colorimetric method for the determination of polyhalogenated organic compounds was reported by Leibman and Hindman (10). The method used by these authors is a modification of the Fujiwara alkaline pyridine reaction (11).

The purpose of this paper is to report on the application of two analytical procedures for the assay of diisopropylammonium dichloroacetate: a colorimetric method based on the modified Fujiwara technique (10) and a nonaqueous titration procedure (12). These assays have proven applicable for the determination of the solubility and the distribution coefficient of this agent.

EXPERIMENTAL

Nonaqueous Titrimetry

Analysis of Diisopropylammonium Dichloroacetate.—The nonaqueous titration procedure described by Fritz (12) was utilized for the analysis of diisopropylammonium dichloroacetate. Each milliliter of 0.1 *N* potassium methoxide solution is equivalent to 23.014 mg. of the drug.

Analysis of Solutions of Diisopropylammonium Dichloroacetate.—Aliquots of diisopropylammonium dichloroacetate solutions (aqueous and nonaqueous) were pipeted into 100-ml. beakers and the solution allowed to evaporate to dryness. The residue was dissolved in 20 ml. of neutralized DMF and the solution titrated with potassium methoxide solution as previously described.

Solubility Studies.—An amount of diisopropylammonium dichloroacetate known to be in excess of that required for a saturated solution was weighed accurately and placed in a glass-stoppered bottle containing the solvent. The bottle was placed in a rotating constant-temperature bath and equilibrated for 2 hr. at 26 ± 0.1°. After equilibration, aliquot samples of the supernatant liquid were removed and assayed for the drug by nonaqueous titration.

Distribution Coefficient Studies.—The distribution coefficients of diisopropylammonium dichloroacetate between benzene–water and chloroform–water systems were determined by nonaqueous procedures. Accurately weighed samples of the compounds were added to glass-stoppered bottles containing 5 ml. of distilled water and 5 ml. of the organic solvent. The bottles were placed on a rotating constant-temperature bath and equilibrated for 2 hr. at 26 ± 0.1°. After equilibration, the 2 layers were separated and analyzed for diisopropylammonium dichloroacetate by nonaqueous titration.

Colorimetric Procedure.—Aqueous solutions of diisopropylammonium dichloroacetate were analyzed colorimetrically by the modified Fujiwara reaction (10). The proposed procedure differs from that reported by Leibman and Hindman (10) in that it is recommended that suitable standard solutions of this drug be run simultaneously. This general procedure was extended to the determination of the solubility of diisopropylammonium dichloroacetate in several solvents.

RESULTS

Table I shows the results obtained from the analysis of diisopropylammonium dichloroacetate by a nonaqueous titration procedure. The method proved to be suitable for the crystalline salt as well as for aqueous and nonaqueous solutions of the drug. The nonaqueous procedure is accurate, rapid, and has many potential applications.

TABLE I.—ANALYTICAL DATA FROM NONAQUEOUS TITRATION STUDIES

Diisopropylammonium Dichloroacetate	mg. Taken	mg. Found	% Recovery[a]
Powder	118.35	118.24	99.9 ± 0.17
Aqueous soln.	100.00	100.3	100.3 ± 0.4
Chloroform soln.	99.87	100.87	101.0 ± 0.26

[a] Average of 5 or more determinations ± standard deviation.

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[1] Marketed as Diedi by the Italsebar Co., Milan, Italy.

TABLE II.—SOLUBILITY OF DIISOPROPYLAMMONIUM DICHLOROACETATE[a]

Solvent	Solubility in Gm./100 ml. Soln.[b]
Distilled water	58.0 ± 0.32
Ethanol, 95%	46.3 ± 0.31
Chloroform	50.6 ± 0.21
Benzene	15.6 ± 0.22

[a] Average of 5 or more determinations ± standard deviation. [b] All data represent studies conducted at 26 ± 0.1°.

TABLE III.—DISTRIBUTION COEFFICIENTS OF DIISOPROPYLAMMONIUM DICHLOROACETATE[a]

Concn. of Diisopropylammonium Dichloroacetate, Gm.[b]	Benzene/Water	Chloroform/Water $K = \frac{C_{CHCl_3}}{C_{H_2O}}$	Chloroform/Water $K = \frac{C_{CHCl_3}}{(C_{H_2O})^n}$[c]
0.5029	0.017	...	...
1.0059	0.017	...	...
1.0019	...	0.22	0.345
1.5110	...	0.34	0.345
2.0034	...	0.44	0.348

[a] All data represent studies conducted at 26 ± 0.1°. Leaders indicate data were not determined. [b] Concentration of the drug partitioned between 5 ml. of organic phase and 5 ml. of aqueous phase. [c] The value of n was found to be 2.3.

TABLE IV.—COMPARATIVE SOLUBILITY STUDIES BY NONAQUEOUS AND COLORIMETRIC PROCEDURES

Solvent	Solubility in Gm./100 ml. Soln. Nonaqueous	Solubility in Gm./100 ml. Soln. Colorimetric[a]
Distilled water	58.0 ± 0.33	60
Ethanol, 95%	46.3 ± 0.31	48.5
Chloroform	50.6 ± 0.21	[b]
Benzene	15.6 ± 0.22	15

[a] Standard deviations were not determined for the colorimetric assay. [b] The colorimetric procedure is not applicable with chloroform as the solvent (10).

Tables II and III, respectively, include data demonstrating the use of this method for the determination of solubilities and distribution coefficients.

In the solubility studies, solvents were selected to cover a broad range of dielectric constants (2.28–80). It was observed that, with the exception of chloroform, the solubility of diisopropylammonium dichloroacetate increased as the polarity of the solvent increased. The solubility in chloroform was much greater than anticipated from the polarity of the solvent (Table II). It is proposed that the increased solubility of this drug in chloroform may be attributed to an association between solute and solvent molecules.

An examination of the distribution studies of diisopropylammonium dichloroacetate (Table III) reveals that in the partitioning between benzene and water, the expression of $K = C_{org.}/C_{H_2O}$ is a constant value. This type of expression was not adequate for calculating the distribution coefficient between chloroform and water. However, a constant value was obtained from the use of the following expression: $K = C_{org.}/(C_{H_2O})^n$ where the value of n is obtained from the slope of the line by plotting log $C_{org.}$ against log C_{H_2O}. The data obtained suggest an association of the drug molecule in the organic phase.

The modified Fujiwara colorimetric procedure proved to be suitable as an assay method for this drug. However, reproducible results were obtained only if appropriate standards were run simultaneously with the test solutions. In analyzing 4 samples of the same concentration (0.4 mg./ml.) in 2 successive runs, the average deviations of the absorbance measurements were found to be ±0.003 and ±0.004. The utilization of a standard curve gave variable results. It was also found necessary to use a freshly prepared benzidine–formic acid reagent. Additional studies on the solubility of diisopropylammonium dichloroacetate in several solvents were conducted utilizing the modified Fujiwara colorimetric procedure. The data obtained from these studies are in agreement with those obtained in the nonaqueous titration experiments (Table IV).

This colorimetric procedure may prove valuable in studying the degradation of this drug and related molecules possessing the dichloroacetic acid moiety.

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Estimation of Thiamine by Inverse Isotope Dilution II

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In a continuation of a previous study, the cleavage of thiamine hydrochloride by sodium sulfite was proved to be a quantitative reaction. An inverse isotope dilution procedure based on this cleavage was shown to be both accurate and precise in the analysis of pure thiamine hydrochloride in quantities of 10, 50, and 100 mg. It was also used in the analysis of thiamine hydrochloride in 4 pharmaceutical products.

A previous paper (1) reported a preliminary study of a thiamine assay by inverse isotope dilution. Thiamine hydrochloride was cleaved with sodium sulfite-^{35}S to form ^{35}S labeled 4-amino-2-methylpyrimidyl-5-methanesulfonic acid as the derivative. Quantitative assays of thiamine in the presence of other vitamins were performed.

This assay procedure was studied further. The cleavage reaction was proved to be quantitative by direct isotope dilution. Samples containing 10, 50, and 100 mg. of thiamine hydrochloride and 4 commercial products were assayed. The results are reported in this paper.

EXPERIMENTAL

Preparation of Carrier.—The carrier was prepared in the manner reported previously. Purity was proved by paper chromatography with 2 solvent systems, propanol–ammonia (2:1) and butanol–acetic acid–water (4:2:1), and by potentiometric titration. The latter method gave an average purity of 100.3% for 3 determinations.

Measurement of Radioactivity.—The radioactivity of all samples was measured in the manner previously reported. Samples were counted for a period of time sufficient to maintain a counting error of 1.0% or less.

Proof That Cleavage Reaction Is Quantitative.—Williams *et al.* (2) reported that the cleavage reaction is quantitative. This report was based on the weight of product recovered in a cleavage reaction. The recovery was less than 100%.

In order to prove that the reaction is quantitative, direct isotope dilution was used. Labeled derivative was prepared by cleaving 5 Gm. of thiamine hydrochloride with sodium sulfite-^{35}S in the manner used for the preparation of the carrier. The purity of the product was confirmed by paper chromatography with the 2 solvent systems used for the carrier.

The direct isotope dilution procedure was performed in duplicate. Approximately 400 mg. of thiamine hydrochloride (U.S.P. reference standard), previously dried to constant weight over phosphorus pentoxide, was weighed accurately into a weighing bottle. The thiamine was dissolved in 3 ml. of water, 3.00 ml. of a 1 *M* sodium sulfite solution was added, the pH was adjusted to 4.9–5.0 with 2.6 *N* hydrochloric acid, and the reaction mixture was set aside for 12 hr.

A solution of the labeled derivative was prepared by dissolving 100.5 mg. in sufficient ammonia T.S. to make 25.00 ml. To the reaction mixture was added 5.00 ml. of this solution, the pH was again adjusted to 4.9–5.0, and the mixture was set aside for 12 hr. The crystals were collected with suction, washed with about 25 ml. of ice cold water, transferred to a 50-ml. beaker, and dissolved in the least amount of ammonia T.S. The pH was adjusted to 4.9–5.0 with glacial acetic acid, and the mixture was allowed to stand at room temperature for 12 hr. The crystals were collected, washed, and dissolved in about 5 ml. of water by boiling. The mixture was set aside at room temperature for 12 hr. The crystals were then collected, washed, and dried in an oven at 105° for 2 hr.

Approximately 50 mg. of the product was accurately weighed and dissolved in sufficient ammonia T.S. to make 50.00 ml. A sample was prepared for counting by adding 1.00 ml. of this solution to 15 ml. of scintillator. In a similar manner, a sample of the labeled derivative was prepared for counting in order to determine its initial specific activity.

The results are shown in Table I.

Table I.—Yield of Cleavage Reaction

Thiamine HCl Cleaved, mg.	Labeled Derivative Added, mg.[a]	Final Specific Activity, c.p.m./mg.	Thiamine HCl Recovered, mg.	Recovery, %
501.4	20.1	4378	499.7	99.7
342.2	20.1	6207	342.6	100.1

[a] Initial specific activity was 69,982 c.p.m./mg.

Table II.—Analysis of Thiamine Hydrochloride

Thiamine HCl Analyzed, mg.	Initial Sp. Act. of Derivative, c.p.m./mg.	Thiamine HCl Recovered, mg.
100.0	79,926[a]	100.3 ± 1.03[d]
50.0	81,654[b]	49.74 ± 0.53[e]
10.0	118,534[c]	10.07 ± 0.22[d]

[a] Average of 3 replicates. [b] Average of 4 replicates. [c] Average of 6 replicates. [d] Average of 12 replicates. [e] Average of 11 replicates.

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TABLE III.—ANALYSIS OF THIAMINE HYDROCHLORIDE IN PHARMACEUTICAL PRODUCTS

Product	Thiamine HCl Content Labeled, mg.	Thiamine HCl Recovered, mg.		Internal Standard Recovered, %
		Isotope Dilution	Thiochrome Method	
A[a]	5	5.32 ± 0.04[c]		99.4[c]
B[b]	10	8.35 ± 0.16	11.4[c]	100.6
C[a]	50	50.25 ± 1.20		100.1
D[a]	100	105.8 ± 0.20	102	100.1

[a] Tablet. [b] Vitamin B complex capsule. [c] Average of 3 replicates.

Analyses of Thiamine Hydrochloride at 10, 50, and 100-mg. Levels.—These analyses were performed in the manner presented in the previous paper with the following exceptions. The thiamine hydrochloride was a manufacturing grade (Roche). The quantity and specific activity of the labeled sodium sulfite were adjusted to the amount of thiamine hydrochloride cleaved. For the 50 and 100-mg. levels, 6 mg. of carrier was used for each milligram of thiamine hydrochloride cleaved; for the 10-mg. level, the ratio was 30 to 1. In addition to recrystallization with glacial acetic acid, the carrier diluted derivative was recrystallized from water. The initial specific activity of the labeled derivative at each level was determined by performing the cleavage reaction on U.S.P. thiamine hydrochloride reference standard. The results are shown in Table II.

Analyses of Thiamine Hydrochloride in Pharmaceutical Products.—Four pharmaceutical products (Table III) with thiamine hydrochloride contents ranging from 5 to 100 mg. were selected for analysis. These analyses were performed in the manner used for pure thiamine hydrochloride with the following exceptions. For product *A*, the ratio of carrier added to thiamine hydrochloride cleaved was 60 to 1. The binders, diluents, and other insoluble materials present in the products were removed after the carrier was added by filtration with a Büchner funnel and a double thickness of Whatman No. 1 filter paper. Due to the large amounts of insoluble materials present, it was thought that constant stirring of the reaction mixture would be necessary for quantitative cleavage. It was shown that this was not necessary. For each product, 6 samples were cleaved. To 3 of these samples was added thiamine hydrochloride (Roche, for ampul use) as an internal standard in an amount equal to the labeled thiamine hydrochloride content of the product.

The average tablet weight and the average net capsule weight were determined for each product. For products *C* and *D*, 12 tablets were powdered in a mortar and an amount of the powder equivalent to 10 tablets was dissolved in water in a volumetric flask. An aliquot of this solution was used in the analysis. For product *A*, single tablets were used directly in the analysis. For product *B*, capsule fill equivalent to the average was used.

Products *B* and *D* were assayed by the thiochrome method by the supplier.

The results are shown in Table III.

DISCUSSION

The direct isotope dilution analysis proved that the cleavage reaction is quantitative. Thiamine hydrochloride, U.S.P. reference standard, was used to assure high purity.

The accuracy and precision of the inverse isotope dilution analysis were good for both pure thiamine hydrochloride and pharmaceutical products. For the pure thiamine hydrochloride the accuracy and precision were within 1 and 2.2%, respectively, for all 3 levels. For the pharmaceutical products, the precision and accuracy indicated by recovery of internal standard were comparable.

The reason for the poor agreement between the 2 methods of analysis for product *B* is not apparent. The recovery of internal standard was complete, and the analysis was run twice with consistent results.

SUMMARY

The inverse isotope dilution method was shown to be applicable for the analysis of pure thiamine hydrochloride in quantities of 10 mg. or greater. It was used for the analysis of thiamine hydrochloride in pharmaceutical products and was successful in three out of four.

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Modified Computer Programs for Determining Eutectic Temperatures

Sir:

In a recent publication (1), the errors associated with both an expression for calculating eutectic temperatures and the computer program for solving this expression were elucidated. The general form of the equation which was derived from freezing point depression and solubility curves (2) was as follows:

$$\log\left(\frac{1}{T_e} - \frac{1}{T_0}\right) = -\frac{Lf'}{2.303R}\left(\frac{1}{T_e} - \frac{1}{T_0'}\right) - \log\frac{Lf}{R} \quad \text{(Eq. 1)}$$

where Lf and Lf' = the heat of fusion of the solvent and differential heat of solution, respectively; T_0 and T_0' = melting point of the solvent and of the solid, respectively; and T_e = the eutectic temperature.

Because of an approximation introduced in the freezing point equation during the derivation of the eutectic expression in which $-x$ was substituted for ln $(1 - x)$, deviation occurs for highly soluble salts where the mole fraction, x, exceeds a value of 0.1. However, a new program has been written in which the approximation has been eliminated in accordance with the following expression:

$$1 = \exp\left[-\frac{Lf'}{R}\left(\frac{1}{T_e} - \frac{1}{T_0'}\right)\right] + \exp\left[-\frac{Lf}{R}\left(\frac{1}{T_e} - \frac{1}{T_0}\right)\right] \quad \text{(Eq. 2)}$$

To simplify the expression, the following definitions are introduced:

$$A = Lf/R \qquad A' = Lf'/R$$
$$B = Lf/RT_0 = A/T_0 \qquad B' = Lf'/RT_0' = A'/T_0'$$

Then

$$1 = \exp\left[-A'/T_e + B'\right] + \exp\left[-A/T_e + B\right] \quad \text{(Eq. 3)}$$

TABLE I.[a]—EUTECTIC TEMPERATURE

Diff. Heat of Soln., cal.	M.p. °C. (T_0') 20	40	60	100	140	180	220	260	300
100	116.59	115.00	113.62	111.35	109.57	108.13	106.95	105.95	105.10
300	90.65	88.05	85.80	82.14	79.27	76.97	75.09	73.52	72.19
500	77.69	74.46	71.68	67.17	63.68	60.90	58.65	56.78	55.21
700	69.05	65.35	62.18	57.08	53.17	50.09	47.61	45.58	43.88
900	62.63	58.55	55.09	49.55	45.34	42.07	39.46	37.33	35.37
1100	57.55	53.17	49.47	43.59	39.18	35.79	33.11	30.95	29.18
1300	53.38	48.75	44.85	38.71	34.17	30.71	28.01	25.86	24.12
1500	49.87	45.02	40.95	34.62	29.99	26.51	23.83	21.72	20.02
1700	46.85	41.81	37.61	31.12	26.45	22.98	20.35	18.30	16.68
1900	44.21	39.00	34.69	28.09	23.41	19.99	17.43	15.46	13.91
2100	41.88	36.53	32.11	25.44	20.77	17.42	14.95	13.07	11.62
2300	39.79	34.31	29.82	23.10	18.47	15.21	12.83	11.06	9.71
2500	37.91	32.32	27.76	21.02	16.46	13.29	11.03	9.36	8.11
2700	36.21	30.51	25.90	19.15	14.67	11.62	9.48	7.93	6.77
2900	34.65	28.86	24.21	17.48	13.10	10.16	8.14	6.71	5.65
3100	33.22	27.35	22.66	15.97	11.69	8.89	7.00	5.67	4.71
3300	31.90	25.95	21.24	14.60	10.44	7.78	6.01	4.79	3.93
3500	30.67	24.67	19.94	13.36	9.33	6.80	5.16	4.05	3.27
3700	29.54	23.47	18.73	12.23	8.34	5.94	4.42	3.41	2.72
3900	28.47	22.36	17.62	11.20	7.45	5.19	3.79	2.88	2.26
4100	27.48	21.32	16.58	10.26	6.65	4.54	3.25	2.43	1.88
4300	26.55	20.35	15.62	9.40	5.94	3.96	2.78	2.04	1.56
4500	25.67	19.43	14.71	8.62	5.30	3.45	2.38	1.72	1.29
4700	24.84	18.58	13.87	7.89	4.73	3.01	2.03	1.44	1.07
4900	24.06	17.77	13.09	7.23	4.22	2.62	1.74	1.21	0.89
5100	23.32	17.00	12.35	6.63	3.76	2.28	1.48	1.02	0.73
5300	22.61	16.28	11.66	6.07	3.35	1.99	1.26	0.85	0.61
5500	21.94	15.60	11.01	5.56	2.98	1.73	1.08	0.72	0.50
5700	21.31	14.95	10.39	5.09	2.65	1.50	0.92	0.60	0.42
5900	20.70	14.34	9.82	4.66	2.36	1.30	0.78	0.50	0.34

[a] Eutectic values are negative.

TABLE II.—EUTECTIC TEMPERATURE FOR SOME PHARMACEUTICALS AND INORGANIC ELECTROLYTES

Compd.	Exptl.	Eutectic Temp., °C. Calcd. (no approx.)	Calcd. (with approx.)	Lit. Value
Methylphenidate hydrochloride	−11.7	−11.91	−11.39	...
methanesulfonate	−10.1	− 9.07	− 8.77	...
phosphate	− 4.29	− 3.97	− 3.92	...
Phentolamine hydrochloride	− 1.3	− 2.35	− 2.34	...
methanesulfonate	−11.0	− 8.54	− 8.28	...
phosphate	− 0.75	− 0.88	− 0.88	...
Sodium chloride	−21.6	−26.74	−24.01	−21.6
Sodium bromide	−27.7	−33.88	−29.76	−28.0
Sodium iodide	−29.9	−37.89	−33.09	−31.5
Potassium chloride	−11.1	−12.66	−12.03	−10.7
Potassium bromide	−12.9	−13.26	−12.72	−12.6
Potassium iodide	−22.8	−30.07	−26.67	−23.0
Lithium chloride	below −40	−23.70	−21.57	...
Cesium chloride	−24.1	−27.89	−24.98	...

An initial trial value of T_e equal to 273.1°K. is taken resulting in a value, Y, for the right hand side of Eq. 3, and Y' is calculated from $T_e' = T_e + \Delta T$, where $\Delta T = 0.01\ T_e$.

Then

$$\frac{\Delta Y}{\Delta T} = \frac{Y' - Y}{T_e' - T_e} \qquad \text{(Eq. 4)}$$

The error in Y is $1 - Y$, which can be corrected by incrementing T_e by an amount ϕ, where

$$\frac{\Delta Y}{\Delta T} = \frac{1 - Y}{\phi} \quad \text{or} \quad \phi = (1 - Y)\frac{\Delta T}{\Delta Y} \qquad \text{(Eq. 5)}$$

The next trial value will be $T_e + \phi$ and this procedure is repeated until the error in Y is arbitrarily small. In this procedure, the correction ϕ to be applied to the trial value of the eutectic can be calculated immediately, not requiring two previous iterations as in the former program. The procedure converges rapidly, usually after three iterations. Furthermore, since the correction does not depend on division by previous improvements, no difficulty is encountered when these values approach zero, as occurred, for example, in the former program for large values of Lf' and T_0'.

A program was written in Fortran II, and an IBM 1401 (8K) computer was used to print a revised table of eutectic temperatures in a condensed form to include values of Lf' from 100–5900 cal. in increments of 200 cal., and melting points T_0' from 20–60° in increments of 20° and from 60–300° in increments of 40° (Table I). The maximum error involved in interpolating values from the condensed table is 0.5°.

Independently, a somewhat different program for solving Eq. 2 was written in Manchester Autocode, and an ICT 1301 computer was used to obtain a similar table of eutectic temperatures as a function of Lf' and T_0'. The results of the two methods agree within 0.01°.

Since most pharmaceuticals do not exceed a 0.1 mole fraction concentration in water, the difference in the values obtained using Eq. 2 as compared to those using the approximation given by Eq. 1 is not of practical significance as shown in Table II. The major discrepancies as would be expected occur for highly soluble salts above 0.1 mole fraction in water.

Some previously published values (2, 3) have been recalculated with the new programs and they are listed in Table II.

The deviation which occurs in calculated and experimental values was discussed in the previous papers and is presently undergoing investigation.

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Identity of Viminalol Acetate

Sir:

In their work on substances occurring in *Sarcostemma viminale*, Torrance and Marais (1) showed that this plant contained, in addition to β-amyrin and friedelin, a compound which they called viminalol acetate, and which they stated appeared to belong to the lupeol series. In the present investigation it is shown unequivocally that the compounds described by Torrance and Marais (1) as viminalol and viminalol acetate were in all respects identical with α-amyrin and α-amyrin acetate.

In the previous communication (1) the best melting point reported after chromatography on alumina for viminalol acetate, referred to as *B*, was 160–162°. Thin-layer chromatography, however, showed that this material was not pure. After repeated chromatography of this substance on aluminum oxide using benzene as solvent, a product was finally obtained with a melting point of 200–215°, which gave a single spot on thin-layer chromatography. A pure sample of *B* was eventually obtained by hydrolysis of *B*, in ethanolic potassium hydroxide, to the alcohol. The alcohol was crystallized from 95% ethanol until a constant melting point of 180–182° was obtained. Thin-layer chromatography of this alcohol gave a single spot. This pure alcohol was then acetylated, using pyridine and acetic anhydride in the cold, to give a product which, after crystallization from alcohol, gave a chromatographically pure substance of melting point 216–219°. This melting point remained constant after recrystallization.

A comparison of these melting points with that of α-amyrin 186° (2, 3) and of α-amyrin acetate 226° (2, 3) suggested the possibility of viminalol acetate being identical with α-amyrin acetate.

A number of derivatives of viminalol acetate were prepared and their melting points compared with the corresponding derivatives of α-amyrin and α-amyrin acetate described in the literature. From the physical constants given in Table I for viminalol acetate, α-amyrin acetate, and their derivatives it can be seen that viminalol acetate was in fact α-amyrin acetate.

TABLE I.—SUBSTANCES AND MELTING POINTS

Substance	M.p., °C.
α-Amyrin acetate	216–219°; lit., 226° (2, 3)
Viminalol acetate	216–219°
α-Amyrin	182°; lit., 186° (2, 3)
Alkaline hydrolysis product of viminalol acetate	180–182°
α-Amyrone	126° (2, 3)
Chromic acid oxidation of hydrolysis product of viminalol acetate	124–127°
α-Amyrenonal acetate	278° (2, 3)
Chromic acid oxidation product of viminalol acetate	278–280°
α-Amyrene	115° (2, 3)
Clemmensen reduction product of viminalol	108–111°

Furthermore, no depression in the melting points of α-amyrin and α-amyrin acetate were obtained on admixture with viminalol and viminalol acetate, respectively. (Table I.)

Thin-layer chromatography at room temperature (22°) using silica gel (G nach Stahl) and a solvent system of ethyl acetate–cyclohexane (2:8) on α-amyrin, viminalol, α-amyrin acetate, and viminalol acetate gave R_f values of 0.53 for α-amyrin and viminalol and 0.94 for α-amyrin acetate and viminalol acetate.

Finally, comparison of the infrared spectra of viminalol and viminalol acetate with authentic samples of α-amyrin and α-amyrin acetate showed that the substance previously described (1) as an unknown triterpene acetate, *viz.*, viminalol acetate is identical with α-amyrin acetate.

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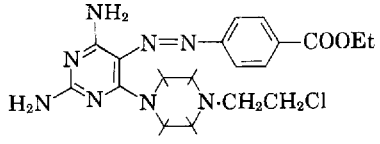
2,4-Diamino-6-substituted-5-(4-carbethoxyphenylazo)pyrimidines as Irreversible Inhibitors of Folic Acid Reductase

Sir:

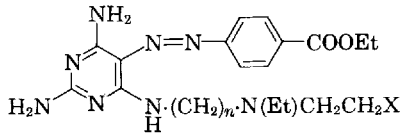
The theory of competitive, or active-site-directed, irreversible inhibition of enzymes has been discussed by Baker (1) and others (2–5). To achieve this type of inhibition, the inhibitor must form an initially reversible enzyme–inhibitor complex and, while in this form, be able to form a covalent bond between reactive groups located on the enzyme and on the inhibitor to yield a covalently bound enzyme–inhibitor complex.

Previous studies (6–9) have shown that 5-arylazopyrimidines possess antifolic properties. Evidence has been presented which suggests that a series of 2,4-diamino-6-substituted-5-arylazopyrimidines are competitive antagonists of rat liver folic acid reductase (10). Thus, it seemed possible that irreversible antagonists might be designed by the incorporation of appropriately located alkylating groups into the 5-arylazopyrimidine molecule.

Some irreversible inhibition was noted (10) with *N*-2-chloroethyl-*N'*-[2,4-diamino-5(4-carbethoxyphenylazo)-6-pyrimidyl]piperazine (I), but this was not particularly marked since lengthy preincubation (8 hr.) of the inhibitor and enzyme was necessary to produce a significant level of irreversible inhibition (10). It seemed probable that the ineffectiveness of I was due to the conformational rigidity of the alkylating side chain which would preclude, save in the most favorable circumstances, effective interaction between the alkylating group and a corresponding nucleophilic site on the enzyme. Accordingly, we have prepared (Table I) a series of 2,4-diamino-5-(4-carbethoxyphenylazo)pyrimidines (II) bearing alkylating 6-substituents of varying chain length and with increased flexibility relative to I. (The synthesis of these compounds and a more detailed analysis of their behavior will be published together in a later paper.)



I



II

The alkylating inhibitors (VIII–XII, Table I) produced irreversible inactivation at rates which were dependent on the length of the 6-alkyl chain despite the similar chemical reactivities of the alkylating groups (11). In order to exclude the possibility that these compounds inactivate folic reductase by random alkylation processes, we have studied the activity of *N*-(2-chloroethyl)-*N*-ethyl-*n*-butylamine (XIII), a compound that represents the alkylating moiety of X. When incubated with folic reductase at the same concentration as X, XIII required 90 min. to produce 50% inactivation and is clearly much less efficient than X which required only 6 min. to produce the same degree of inactivation. Furthermore, incubation of the enzyme with XIII and 2,4,6-triamino-5-phenylazopyrimidine [a competitive reversible antagonist, $K_I = 1.4 \times 10^{-8} M$, (10)] did not alter the time for 50% inactivation, suggesting (*a*) that any conformational change induced by the enzyme–inhibitor interaction does not expose a nucleophilic group that can be alkylated by XIII and (*b*) that XIII produces enzyme inactivation by a nonspecific process.

The data in Table I show that the alkylating

TABLE I.—INHIBITION OF FOLIC ACID REDUCTASE[a] BY 2,4-DIAMINO-6-SUBSTITUTED-5-(4-CARBETHOXYPHENYLAZO)PYRIMIDINES (II)

Compd.	*n*	X	$[I/S]_{50}$[b]	Time for 50% Inactivation, min.[c]
III	2	OH	2.9	>200
IV	3	OH	5.0	>200
V	4	OH	5.6	>200
VI	5	OH	4.8	>200
VII	6	OH	3.6	>200
VIII	2	Cl	2.0	50
IX	3	Cl	3.0	12
X	4	Cl	2.0	6
XI	5	Cl	2.75	10.5
XII	6	Cl	1.9	10
XIII	$CH_3(CH_2)_3N(Et)CH_2CH_2Cl$			90

[a] Folic acid reductase activity was determined at 37° in the presence of 20 μM NADPH, 10 μM sodium citrate, 10 μM $MgCl_2$, 100 μM dimethyl glutarate buffer (pH 6.1), 80 μM folic acid, rat liver homogenate supernatant and inhibitor in water to a final volume of 0.5 ml. (12). [b] $[I/S]_{50}$ is the ratio of the concentrations of inhibitor and substrate required to produce 50% inhibition of enzyme activity. Since $K_I/K_M \approx [I/S]_{50}$ these figures provide an approximate guide to the relative affinities of substrate and inhibitor. Because of the possibility of irreversible inactivation by VIII–XII during the determination of enzyme activity, the $[I/S]_{50}$ values for these compounds are only approximate. [c] Time for 50% inactivation of the enzyme in the absence of inhibitor was 240 min. Inactivation studies were done with an inhibitor concentration of $2 \times 10^{-4} M$.

and nonalkylating inhibitors bind less efficiently to the enzyme than folic acid ($K_M = 6.2 \times 10^{-6}$ M). Variation of the chain length of the 6-substituent has a relatively small effect on the $[I/S]_{50}$ ratios for both the alcohols (III–VII, $[I/S]_{50}$ range, 2.9–5.6) and the chloro compounds (VIII–XII, $[I/S]_{50}$ range, 1.9–3.0). However, incubation of the enzyme with the inhibitor before addition of the substrate resulted in varying degrees of irreversible inactivation of the enzyme. Enzyme inactivation in the presence of the reversible inhibitors (III–VII) was identical to that occurring in the absence of these inhibitors.

The data presented in this communication seem to establish the existence of a nucleophilic site on the enzyme folic acid reductase that can be specifically alkylated by appropriately designed antagonists. Further work is in progress, using analogs of these antagonists, that may further establish the position of this nucleophilic site relative to other binding sites of folic acid reductase.

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The title of the book has been changed with this 14th edition from its original title *Practical Physiological Chemistry* to *Hawk's Physiological Chemistry*. This timely volume is the result of the collaborative efforts of scientists and researchers outstanding in the many aspects of the biochemical field. *Hawk's Physiological Chemistry* is a comprehensive text which includes modern theory and practice without losing sight of the fundamental concepts and methodology which have historically constituted the structure of the science of biochemistry.

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Reviewed by Carolyn Damon
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The monographs provide unbiased information on the drugs and the Council emphasizes that these are not approved drugs; in some cases unfavorable evaluations are given. It is pointed out also in certain instances that a drug may be used for a particular condition, but further evaluation is necessary to substantiate its value. A total of 271 single entity drugs are covered in this edition. The information included in the monograph includes generic and chemical name, actions and uses, adverse reactions and contraindications, pharmacology, dosage, preparations available, trade name(s), and manufacturer(s).

New Drugs will be revised and published annually. Monographs for drugs over ten years old will be dropped and newer agents will be added, and as more information is gained about drugs still included, these monographs will be revised to incorporate this information.

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The title of the book has been changed with this 14th edition from its original title *Practical Physiological Chemistry* to *Hawk's Physiological Chemistry*. This timely volume is the result of the collaborative efforts of scientists and researchers outstanding in the many aspects of the biochemical field. *Hawk's Physiological Chemistry* is a comprehensive text which includes modern theory and practice without losing sight of the fundamental concepts and methodology which have historically constituted the structure of the science of biochemistry.

This 14th edition contains several innovations in nomenclature and abbreviations. Some of the older terminology has been modified to conform with recommendations of the International Union of Biochemistry. For example, readers will notice that NAD or nicotinamide adenine dinucleotide is used in place of DPN, Coenzyme I, or diphosphopyridine nucleotide and that its reduced form is abbreviated $NADH_2$. In keeping with this, NADP or nicotinamide adenine dinucleotide phosphate replaces TPN, Coenzyme II, or triphosphopyridine nucleotide; its reduced form is $NADPH_2$.

Some of the subjects receiving special emphasis in this new edition are the composition, configuration, and role of the nucleic acids and related compounds; the enzymes and cofactors involved in metabolic pathways; the biotransformations of nutrients and their metabolites and their estimation in blood and tissues; the use of radioisotopes for the study of metabolic processes; current concepts of the biochemistry of muscular contraction, blood coagulation, respiratory metabolism, detoxification, and so forth. The initial chapters of this book deal with basic physiochemical principles and with the chemistry of the major components of physiological tissues. This sophisticated discussion of the chemical principles important to biochemistry is clearly expressed in surprising detail. The information in all chapters is well documented for further study. Each chapter or section contains analytical procedures and instrumental techniques which may be used as laboratory experiments or aids to the researcher. Some of these procedures and techniques are new, replacing those no longer in wide use. However, many examples of the older, perhaps less precise, procedures have been retained because of their continued usage or historical importance. The reader will find numerous diagrams, illustrations, and tables throughout the text. Particularly noteworthy is the color print of a model of the molecular structure of deoxyribose nucleic acid in the frontispiece which is symbolic of the recent epoch-making developments in the elucidation of the structural configuration and biochemical and genetic significance of the ribonucleotides. This comprehensive textbook in physiological chemistry should prove indispensable to those in the medical and allied professions.

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Review Article

Microsomal Dealkylation of Drugs

Substrate Specificity and Mechanism

By ROBERT E. MCMAHON

ABOUT TWENTY-FIVE years ago Butler and Bush (1) reported the *in vivo* demethylation of dimethylbarbital in the dog. This observation represented the first example of the *N*-demethylation of a synthetic drug. In the intervening years the dealkylation reaction has become recognized as a major pathway of drug metabolism. However, studies at the *in vitro* level have been undertaken only in recent years. The first such study was that of Mueller and Miller at Wisconsin. They found (2) that the demethylation of the carcinogenic dye, *p*-dimethylaminoazobenzene could be effected by an *in vitro* system composed of the liver microsomal fraction, an enzyme in the soluble fraction together with oxygen, NADP, NAD, and hexose phosphate. In the 2 years that followed this discovery, the field developed rapidly and the general characteristics of the reaction became well established.

Among the early studies were those of LaDu *et al.* (3) and of Axelrod (4, 5). These workers demonstrated that the oxidative *N*-dealkylation of amines is catalyzed by the microsomal fraction (derived from the endoplasmic reticulum) from mammalian liver and requires molecular oxygen and NADPH. The requirement for a soluble enzyme, NADP, and hexose phosphate experienced by the earlier workers proved to be a requirement for a NADPH generating system. It was also demonstrated that alkyl groups other than methyl were oxidatively removed by the *N*-dealkylase system.

The over-all oxidative *N*-dealkylation reaction has the following stoichiometry:

$$R_2NCH_2R \xrightarrow{[O]} R_2NH + RCHO$$

A key discovery of the Brodie (6) group was that the *N*-demethylase activity was a member of a family of oxidative enzymes involved in the metabolism of drugs. All of these enzymes are located in the microsomal fraction of mammalian liver and require NADPH and molecular oxygen. In addition to dealkylation these enzymes catalyze aromatic hydroxylation, side chain oxidation, *S*-oxidation of thioethers, *N*-oxidation of tertiary amines, and the oxidation of phosphorothionates. A key property of these enzymes is their remarkable nonspecificity.

A number of excellent reviews of the general field of microsomal enzyme systems are available. Among these, the recent reviews by Gillette (7), Siekevitz (8), and Shuster (9) are particularly informative. In this review we plan to consider recent developments in the field of microsomal dealkylation from the viewpoint of the medicinal chemist interested in structure–activity studies and in the mechanism of the reaction. The review has been divided into three sections:

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TABLE I.—*In Vitro* DEMETHYLATION RATES

Substrate	H/W	μmole HCHO/Gm. Liver/hr. Guinea Pig	Rat	Mouse
I	4970	5.25	5.32	7.45
II	4060	6.31	7.87	7.90
III	406	4.44	4.47	5.80
IV	107	4.35	4.22	3.85
V	47	3.89	2.87	3.40
VI	35	1.75	2.89	3.60

structure–activity studies, the mechanism of the reaction, and a tabulation of important pharmacological agents which undergo *in vivo* dealkylation.

STRUCTURE ACTIVITY CONSIDERATIONS

Known substrates of the microsomal dealkylase system include tertiary amines, secondary amines, *N*-alkyl amides, *N*-alkyl carbamates, *N*-methyl barbituric acids, *N*-methyl sulfonamides, *N*-methyl pyrophosphoramides, *N*-methyl imides, *N*-methyl purine bases, arylalkyl ethers, and aryl methyl thioethers. The characteristics of the dealkylation of each of these classes of compounds are summarized below. A discussion of the chemical nature of reaction intermediates and of the possible mechanism of the reaction is presented in the concluding paragraphs.

Tertiary Amines.—The number of tertiary amines which are known to be dealkylated by the microsomal system is very large. Substrates of such widely varying structures have been found to be active that it is difficult to imagine any single chemical or physical property which could be used to correlate the experimental observations. However, Gaudette and Brodie (10) presented experimental evidence that an important requirement for the substrate is that it be lipid soluble. This proposal was particularly attractive since the endoplasmic reticulum is the principal lipid-bearing structure of the cell. Further, it provided an explanation of why foreign amines were readily acted upon by this system while naturally occurring polar amines, such as methylated amino acids, were not dealkylated.

The question of lipid solubility has been explored in detail by McMahon (11) who studied the relationship between lipid solubility (as measured by distribution between *n*-heptane and pH 7.4 buffer) and the rate of demethylation in both an *in vitro* system and in the intact animal. The compounds investigated were I, *dl*-propoxyphene; II, *dl*-propoxyphene carbinol; III, *p*-chlorophenylpropyldimethylamine; IV, phenylpropyldimethylamine; V, phenylethyldimethylamine; and VI, benzyldimethylamine.

The relative rates of demethylation of these six substrates by microsomes from three species are summarized in Table I. H/W represents the ratio of the equilibrium concentration in heptane to that in pH 7.4 buffer. It is clear from these data that there is a direct correlation of increasing rate with increasing lipid solubility. The two most lipid soluble amines, I and II, however, present an exception. In this case the esterification of the hydroxyl group of II results in a small increase in lipid solubility as expected, but it also results in a somewhat lowered demethylation rate. The introduction of the polar and reactive hydroxyl group produced an effect which overrides simple lipid solubility considerations. Apparently it is important in studies of this sort to select series of compounds which differ from each other by the presence or absence of such relatively inert groups as methylene, phenyl, halogen, etc. The results do suggest that a study of structure–activity relationships in a series of compounds in which the lipid solubility is high and not limiting might be rewarding.

In order for the *in vitro* work described above to be valuable to the medicinal chemist, it was important to correlate it with *in vivo* data in the same species. Radiocarbon labeling served for these studies. The results presented in Table II show an excellent correlation with the *in vitro* results.

With one minor exception the *in vivo* results correlate directly with *in vitro* rates. These studies then demonstrated a relationship between lipid solubility and both *in vitro* and *in vivo* demethylation rates. Also, although the *in vivo* rates of demethylation varied from species to species, the relative order of substrate activity was approximately the same in each species. It is likely that systematic studies of species variation will only be successful when a narrowly defined series of compounds such as these are used.

The structure–activity studies on the effect of lipid solubility were next extended to series of substrates which were totally aliphatic in nature (12). From this study, which will not be reviewed in detail here, it was abundantly clear

TABLE II.—*In Vivo* DEMETHYLATION RATES

Compd.	% Demethylation in 200 min. in— Guinea Pig	Rat	Mouse
I	61	50	47
II	84	55	83
III	58	21	20
IV	55	11	12
V	46	12	11
VI	18	16	9

that there is a direct correlation between lipid solubility and substrate activity. Recently Hansch, Steward, and Iwasa (13) have described an interesting mathematical treatment of the results of the demethylation studies cited above (11, 12) and have concluded that in these series the rate of demethylation could be correlated with lipid solubility and with the pKa of the amine.

There seems little doubt that lipid solubility is an important factor (perhaps the major factor) in controlling the rate of demethylation of tertiary amines. Other factors, probably chemical in nature, are also most certainly involved. To study these factors, however, will require careful and rigorous structure–activity studies. Thus, in a group of randomly selected compounds which are not chemically related, the effect of any single parameter will be obscured [*cf.* Mazel and Henderson (14)].

Secondary Amines.—Although secondary amines were among the earliest substrates to be studied (3, 4, 15), no systematic structure–activity studies have been reported. For example, lipid solubility is undoubtedly an important factor influencing the rate of demethylation of secondary amines, but no data are available at present which bear on this question. However, there are data available which allow comparisons of tertiary and secondary amines as substrates.

For example, a comparison (16) has been made of the kinetics of the demethylation of certain tertiary dimethylamines as compared to the corresponding secondary methylamine. In general it was found that the maximum rate (V_m) of demethylation is greater for tertiary amines. It is believed that the kinetic factor arising from the fact that the tertiary amine has two methyl groups while the secondary amine has but one is at least partly responsible for this observation. Secondary methylamines appear from k_m data to have a greater affinity for enzyme than does the corresponding tertiary dimethylamine. In the secondary amine there is less steric hindrance about the nitrogen which may facilitate binding. In addition, in each of the pairs of amines studied, the electron pair on nitrogen was more readily available for binding (*i.e.*, the secondary amine is the stronger base). Secondary amines are also considerably less responsive to the action of the potent demethylation inhibitor DPEA (17, 18) than are tertiary amines.

The difference in rate of demethylation of tertiary and secondary amines can have interesting pharmacological consequences. For example, McMahon *et al.* (19) found that in both the *in vitro* and *in vivo* systems acetylmethadol was demethylated at a rate considerably greater than was noracetylmethadol, the secondary amine analog. The result of this rate differential was that administration of acetylmethadol led to the accumulation of noracetylmethadol in tissues. Since noracetylmethadol itself is an active analgesic, these data serve to explain the clinical observation that the administration of acetylmethadol to patients leads to accumulated drug effects (20). Another example is that of the antidepressant imipramine. Gillette *et al.* (21) have presented experimental evidence that the delayed onset of action and the antidepressant properties are due to the *in vivo* formation of des-*N*-methyl imipramine.

One important systematic structure–activity study involving the dealkylation of both secondary and tertiary amines has yet to be carried out, *i.e.*, the *N*-dealkylation of amines in which the alkyl group removed is larger than methyl and ethyl. A few examples are known, however. LaDu *et al.* (3) have demonstrated the removal of the *N*-butyl group while Axelrod and Cochin (22) and Leadbeater and Davies (23) have studied the deallylation of nalorphine. The *in vivo* opening of the pyrrolidine ring of nicotine (24, 25), the debutylation of ethoxybutamoxane (26) and chlorethoxybutamoxane (27), and the *N*-deallylation of levallorphan (28) are further examples. The *in vitro* dealkylation of *N*-cyclopropyl-4-phenyl-4-carbethoxypiperidine has also been observed (McMahon, unpublished data).

O-Dealkylation.—In 1948 Huggens, Jensen, and Cleveland (29) reported the *O*-dealkylation of *p*-nitroanisole and *p*-nitrophenetole in both the intact rat and in liver homogenates. In 1955 Axelrod (30) demonstrated that the *O*-demethylation of codeine was closely related to *N*-demethylation by showing the demethylation to be catalyzed by liver microsomes and to require NADPH and oxygen. The products were morphine and formaldehyde. A later study from the same laboratory (31) concerned the effect of ring substitution upon the rate of demethylation of anisole derivatives. It was found that the *p*-substituted analogs were more readily demethylated than the *o*- or *m*-derivatives. The decreasing order of the rate of metabolism for *p*-substituted anisoles was CN, CHO, $NHCOCH_3$, COOH, $CH_2—NH_2$, allyl, NH_2, H. It is difficult at present to correlate these data with the electronic effects produced by the substituents. It is probable that both k_m and $V_{max.}$ are affected by aromatic substitution so

that it would probably be necessary to do a complete kinetic study of a series of substrates.

McMahon *et al.* (32) have studied the rate of dealkylation of a series of *p*-nitrophenylalkoxyethers in order to determine the effect of changing alkyl groups. In general it was found that with increasing chain length of normal alkyl groups, the rate of dealkylation decreases. However, when electron donating groups were introduced adjacent to the methylene, increased rates were observed. For example, allyl, cyanomethyl, and benzyl were more active substrates than *n*-propyl or chloroethyl. The dialkylaminoethoxy grouping which occurs frequently in drugs does not undergo *O*-dealkylation. It is likely that in this case *N*-dealkylation occurs as an alternative pathway. In studying the effect of aromatic substitution on the rate of dealkylation of substituted *p*-nitrophenyl benzyl ethers, a very clear steric effect emerged when groups were placed *ortho* to the methylene group.

In experiments in intact animals, it was found that an excellent correlation exists between the *in vitro* rates and the *in vivo* rates (32).

It is likely that *O*-dealkylation may not be confined to phenolic ethers but that totally aliphatic ethers will be found to be cleaved. In this connection the recent report (33) of the cleavage of glycerol ethers of long chain fatty alcohols is of considerable interest. This reaction requires molecular oxygen and liver microsomes but showed a requirement for a tetrahydropteridine as a cofactor as well as the usual NADPH. These observations suggest a possible relationship between this activity and the phenylalanine hydroxylation which has been the subject of the elegant researches of Kaufman (34).

For information on detailed experimental conditions which govern microsomal *O*-demethylation, the report by Nilsson (35) on the demethylation of the estrogenic isoflavone, biochanin A, should be consulted.

N-Alkylamides and Related Compounds.—The extensive researches by Butler and his associates have shown many *N*-methyl barbiturates and related *N*-methyl heterocyclics to be demethylated in the intact animal. [See Fishman (36) for a review and bibliography of the Butler studies.] Other examples include the demethylation of the herbicide, diphenamid (37), the demethylation of *N*-methyl-α-phenyl-α-ethylglutarimide (38), and the demethylation of diazepam (39, 40) and of chlordiazepoxide (41).

An early report of the *in vitro* demethylation of an amide was that of Hodgson and Casida (42) who investigated the demethylation of *N*-methyl carbamates and demonstrated the reaction to be a typical microsomal oxidation requiring oxygen and NADPH. More recent *in vitro* work (43, 44) has shown the demethylation of *N*-methyl barbiturates, *N'*-methylsulfonylureas, and simple amides to be microsomal *N*-demethylations as well.

Miscellaneous Substrates.—The most interesting recent development has been the demonstration by Mazel and co-workers that the microsomal dealkylases will readily *S*-demethylate various *S*-methyl ethers (45). This discovery serves to emphasize again the remarkable nonspecificity of the microsomal oxygenase systems.

Another interesting report is that of Schreiber and co-workers (46) who found that *N*-methyl sulfonamides are demethylated by the microsomal system, thus confirming earlier (47) *in vivo* observations. Smith, Keasling, and Forist (48) have also described the dealkylation of *N*-alkyl sulfonamides. They observed the *in vivo* removal of methyl, ethyl, allyl, isopropyl, butyl, and pentyl groups from the corresponding *N*-alkyl-*p*-bromobenzenesulfonamide in mice. The dealkylation of sulfonamides is reminiscent of microsomal oxidation of the cholinesterase inhibitor, octamethyl pyrophosphoramide (49). In this case the reaction does not go to completion spontaneously since the intermediate *N*-hydroxymethyl intermediate is reasonably stable (see below).

The observations of Henderson and Mazel (50) on the demethylation of various methylated purine bases should also be mentioned. In view of the role of methylated purines as minor constituents of RNA and DNA, these observations may have special significance (51).

Other Enzymes Which Catalyze Oxidative Dealkylation.—In addition to the liver microsomal system, other enzyme systems occur in nature which can oxidize *N*-methyl groups. There are, for example, several instances in alkaloid biogenesis in plants in which the oxidation of an *N*-methyl group appears to occur. In the Tilden lecture (1963), Battersby (52) speculated that the berberine bridge carbon may have its origin in the *N*-methyl group of a suitably substituted 1-benzyl-*N*-methyl-isoquinoline. It was proposed that in this conversion the initial oxidative step might be *N*-oxidation as had been proposed earlier by Wenkert (53) in connection with other alkaloid biotransformations. Direct attack on the methyl group, either by a heterolytic or a free radical mechanism, was suggested as an alternative mechanism (52). Although it has now been

demonstrated by radiocarbon studies that the *N*-methyl group is indeed the source of the berberine bridge, the oxidation mechanisms involved remain obscure (54, 55).

The discovery of the alkaloid leurocristine (56) among the alkaloids of *Vinca rosea* is also of interest. This alkaloid is identical with vincaleucoblastine except that the *N*-methyl has been replaced by an N—CHO group. This conversion could well have taken place in a two-step process through a hydroxymethyl intermediate in exactly the same way that nicotine is converted to cotinine (25) or tremorine is converted to oxytremorine (57) in the liver. *O*-Demethylation also occurs in plants. The elegant research of Rapoport (58) on the biogenesis of morphine leaves no doubt that thebaine is a precursor of morphine which means that two successive *O*-demethylations must occur on the pathway to the latter alkaloid. The demethylation of nicotine to nornicotine in the intact *N. glutinosa* plant has recently been established by Alworth and Rapoport (59).

Another class of enzymes which catalyze oxidative *N*-dealkylation and for which *N*-oxide intermediates have been suggested are found in liver mitochondria. These enzymes are responsible for the *N*-demethylation of *N*-methyl amino acids, such as *N*,*N*-dimethylglycine, as well as the oxidation of certain polar amines, such as dimethyl tryptamine, and appear to be dehydrogenases which require NAD as cofactor.

Horning and co-workers (60) have suggested that the dealkylations catalyzed by these mitochondrial dehydrogenases proceed *via* *N*-oxide intermediates. This suggestion was based on the observation of these workers and of others that *N*-oxides of certain tertiary amines and of dimethylamino acids rearrange in the presence of iron complexes to form carbinol amines which in turn dissociate to aldehyde and dealkylated amine or amino acid (61–67). Although subsequent studies (68, 69) have shown that the oxidation of dimethyltryptamine does not proceed through an *N*-oxide intermediate, the question of whether *N*-oxides are involved in the demethylation of dimethylamino acids has not been resolved.

MECHANISM OF MICROSOMAL DEALKYLATION

In their early work on the *in vitro* demethylation of dimethylaminoazobenzene, Mueller and Miller (2) presented indirect evidence to support the view that the reaction proceeded through an *N*-hydroxymethyl intermediate:

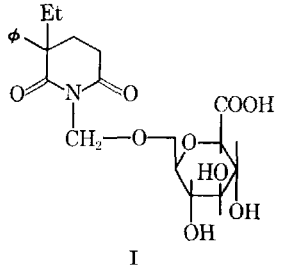
$$R_2NCH_3 \rightarrow [R_2NCH_2OH] \rightarrow R_2NH + HCHO$$

It is now well established that such an intermediate is involved and in certain cases is stable enough to be detected. For example, the active intermediate formed by microsomal oxidation of octamethyl pyrophosphoramide was demonstrated to be *N*-hydroxymethylheptamethyl pyrophosphoramide (49, 70–72).

A particularly relevant observation was that of Keberle *et al.* (38) who found among the metabolites of *N* - methyl -α- phenyl -α- ethylglutarimide the glucuronide of the *N*-hydroxymethyl intermediate (I). Recently McMahon and Sullivan (37) have found an analogous metabolite of the herbicide diphenamid (II). In these cases the hydroxymethyl intermediate has been "trapped" by conversion to a stable derivative, the glucuronide.

The most direct evidence, however, comes from the work of Dorough and Casida (73) who found that the unconjugated *N*-hydroxymethyl derivatives were relatively stable metabolites of α-naphthyl *N*-methylcarbamate and *o*-isopropoxyphenyl *N*-methylcarbamate.

There seems to be little doubt then that the hydroxymethyl compound is indeed an intermediate of *N*-dealkylation. It also seems reasonable to assume that *O*- and *S*- dealkylations proceed through analogous intermediates. There remains, however, the question of how the hydroxymethyl intermediate is formed in the microsomal dealkylation reaction. As a consequence of the work on alkaloid biosynthesis and on the mitochondrial amino acid demethylases discussed, it has been proposed that the *N*-hydroxymethyl intermediate arises from the rearrangement of a tertiary amine *N*-oxide intermediate.



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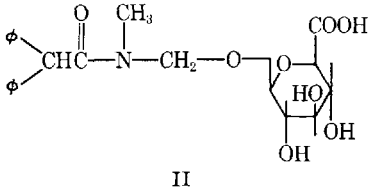

II

TABLE III.—COMPOUND DEALKYLATED IN THE INTACT ANIMAL

Compd.	Classification	Dealkylation Reaction	Species	Ref.
N-Methylbarbital	Sedative	*N*-demethylation	Dog	(1)
N-Methylphenobarbital	Sedative	*N*-demethylation	Dog, human	(106)
Hexobarbital	Sedative	*N*-demethylation	Dog	(107)
Methyl-5-ethyl-5-phenyl hydantoin	Anticonvulsant	*N*-demethylation	Dog, human	(108)
Trimethadione	Anticonvulsant	*N*-demethylation	Dog, human	(109)
Paramethadione	Anticonvulsant	*N*-demethylation	Dog, human	(110)
Caffeine	Stimulant	*N*-demethylation	Rat, dog, human	(111–113)
3-Methyl uric acid	...	*N*-demethylation	Rat, dog	(112)
Morphine	Analgesic	*N*-demethylation	Rat	(114)[a]
Methadone	Analgesic	*N*-demethylation	Dog	[b]
Pethidine	Analgesic	*N*-demethylation	Dog, man	[c]
α-*dl*-Acetylmethadol	Analgesic	*N*-demethylation	Rat	(19)
α-*dl*-nor-Acetylmethadol	Analgesic	*N*-demethylation	Rat	(19)
d-Propoxyphene	Analgesic	*N*-demethylation	Rat, mouse, human	(11, 115)
Ethoheptazine	Analgesic	*N*-demethylation	Rat	(116)
Y-535	Analgesic	*N*-demethylation	Rat	(117)
Dextromethorphan	Antitussive	*N*-demethylation and *O*-demethylation	Dog	(118)
Chlorcyclizine	Antihistamine	*N*-demethylation	Rat	(119)
Cyclizine	Antihistamine	*N*-demethylation	Rat	(119)
Deptropine	Antihistamine	*N*-demethylation	Rat	(120)
d-Methamphetamine	Sympathomimetic	*N*-demethylation	Dog	(121)
Ephedrine	Sympathomimetic	*N*-demethylation	Dog	(122)
Butynamine	Hypertensive	*N*-demethylation	Rat	(123)
Diphenamid	Herbicide	*N*-demethylation	Rat	(37)
Erythromycin	Antibiotic	*N*-demethylation	Rat	(83, 124)
Mepivacaine	Local anesthetic	*N*-demethylation	Rat	(125)
Methixene	Anticholinergic	*N*-demethylation	Rat	(126)
Imipramine	Antidepressant	*N*-demethylation	Rat, rabbit, dog, human, mouse	(31, 127, 128, 129)
Amitriptyline	Antidepressant	*N*-demethylation	Rat, mouse	(130, 131)
Nortriptyline	Antidepressant	*N*-demethylation	Rat	(98)
Thioridazine	Tranquilizer	*N*-demethylation	Rat	(132)
Promazine	Tranquilizer	*N*-demethylation	Dog	(133)
Chlorpromazine	Tranquilizer	*N*-demethylation	Rat, human	(134, 135)
Diazepam	Tranquilizer	*N*-demethylation	Dog, human	(39, 40)
N-Methyl-polythiazide	...	*N*-demethylation	Rat	(47)
Cotinine	...	*N*-demethylation	Dog	(136)

Continued on next page.

Several facts support this point of view. For example, Hanaki and Ishidate (74, 75) have developed a chemical system consisting of ascorbic acid and chelated iron which will demethylate dimethylaminoazobenzene through an intermediate *N*-oxide. Furthermore, studies with the microsomal system have shown that it can indeed form *N*-oxides from certain tertiary amines (76–78). Finally, it has been reported (79) that dimethylaniline *N*-oxide is readily converted to methylaniline and formaldehyde in the presence of liver microsomes. [This report, however, conflicts with an earlier report of Gillette (80) who found dimethylaniline *N*-oxide to be a poor substrate for these systems.]

There are, however, a number of observations which cast doubt on the *N*-oxide mechanism. Although this mechanism would be satisfactory for the dealkylations of tertiary amines, other mechanisms must be evoked to explain the dealkylation of the many other types of compounds which are dealkylated by microsomes. Another difficulty arises from the extensive work of Kiese on the oxidation of aniline derivatives by liver microsomes. He has shown (81) that the k_m value for oxygen in the *N*-oxidation reaction is some 40 times greater than it is for the *N*-dealkylation reaction. This would seem to rule out *N*-oxidation as an intermediate step in *N*-dealkylation. Recently McMahon and Sullivan (82) found that the microsomal demethylation of radiocarbon labeled *l*-propoxyphene in the presence of a pool of cold *l*-propoxyphene *N*-oxide did not result in the incorporation of radioactivity into the oxide pool. This observation also argues against the involvement of an *N*-oxide intermediate in dealkylation. An analogous study with erythromycin and erythromycin-*N*-oxide led Mao and Tardrew (83) to the conclusion that the *N*-oxide was not a reaction intermediate.

Also if *N*-oxide formation occurred as the rate-determining step, an isotope effect such as that seen in the demethylation of trideuteriomorphine (84) would not be expected. In addition the observation (12, 13) that steric interference about the nitrogen atom does not affect the re-

TABLE III.—(*Continued*)

Compd.	Classification	Dealkylation Reaction	Species	Ref.
Dimethylaminoazobenzene	Carcinogen	*N*-demethylation	Rat	(137)
Aminopyrine	Antipyretic	*N*-demethylation	Rat	(138)
N-Ethylbarbital	Sedative	*N*-deethylation	Dog	(139)
Benzquinamide	Neurosedative	*N*-deethylation and *O*-demethylation	Dog, man	(140, 141)
3,5-Diethyl-5-phenylhydantoin	Anticonvulsant	*N*-deethylation	Dog	(142)
Chloroquine	Antimalarial	*N*-deethylation	Man	(143)
Diethylpropion	Ovulation stimulant	*N*-deethylation	Human	(144)
Trifluralin	Herbicide	*N*-depropylation	Rat	(145)
Chlorpropamide	Antidiabetic	*N*-depropylation	Dog	(146)
N-Isopropyl-4-bromobenzene-sulfonamide	Anticonvulsant	*N*-deisopropylation	Mouse	(48)
Diallylmelamine-*N*-oxide	Vasodilator	*N*-deallylation	Dog	(147)
N-Allyl-4-bromobenzene-sulfonamide	Anticonvulsant	*N*-deallylation	Mouse	(48)
N-Dibenzyl-*β*-chloroethylamine	*α*-Adrenergic blocking agent	*N*-dechloroethylation	Dog	(148)
Butamoxane	*α*-Adrenergic blocking agent	*N*-debutylation	Dog	(149)
Ethoxybutamoxane	*α*-Adrenergic blocking agent	*N*-debutylation and *O*-deethylation	Rat, dog	(26)
Chlorethoxybutamoxane	*α*-Adrenergic blocking agent	*N*-debutylation and *O*-decthylation	Rabbit, dog, rat, mouse, guinea pig	(27)
6-Methylthiopurine	Antimetabolite	*S*-demethylation	Man, rat	(150, 151)
Mescaline	Hallucinogen	*O*-demethylation	Man	(152)
Brucine	Convulsant	*O*-demethylation	Rabbit	(153)
Codeine	Analgesic	*O*-demethylation	Rats	(154)
Biochanin A	Estrogen	*O*-demethylation	Rat	(155)
p-Nitroanisole	...	*O*-demethylation	Rat	(156)[d]
Phenacetin	Antipyretic	*O*-deethylation	Rabbit, man	(157, 158)
O-(2-Ethoxy)benzamide	Antipyretic	*O*-deethylation	Man	(159)
Benzyl-*p*-nitrophenyl ether	...	*O*-debenzylation	Rat	(32)[e]

[a] Although the metabolism of morphine has been studied very extensively, there is at present little evidence that *N*-demethylation is a significant pathway. For example, Rapoport (personal communication) has investigated the metabolism of radiomorphine in humans with some care and has found no normorphine among the metabolites. For a complete discussion of the metabolism of morphine and of other analgesics, see Way and Adler (160). [b] Pohland, Sullivan, and Lee, personal communication. [c] For complete bibliography see *Reference 160*. [d] For a discussion of the demethylation of substituted anisoles, see Williams (161). [e] These workers (32) have studied the *in vivo* cleavage of a wide variety of alkyl and arylalkyl-*p*-nitrophenyl ethers.

action rate argues against the *N*-oxide mechanism.

On the basis of data available at present it seems likely that *N*-oxidation and *N*-dealkylation represent alternative pathways for the metabolism of tertiary amines. In this connection the recent reports by Ziegler and co-workers (77, 78) are of interest. They have shown that cholate treatment of microsomes does not destroy the *N*-oxidizing capacity of the microsomes, although such a treatment frequently results in the loss of dealkylase and hydroxylase activity. Thus, the *N*-oxidizing capacity of microsomes may well be a separate system from that required for dealkylation.

Mention should be made of the suggestion of Oae (85, 86) that the demethylation of methionine might proceed through a sulfoxide intermediate (Pummerer reaction). Since methionine is metabolized by methyl transfer rather than methyl oxidation, such a mechanism would not be applicable for its demethylation, but the Oae work does nevertheless raise the question of possible involvement of sulfoxides in microsomal *S*-demethylation.

At the present time the microsomal dealkylation reaction is best understood as a hydroxylation reaction:

$$RXCH_2R' \xrightarrow[\text{oxygen''}]{\text{''Active}} RXCHOHR \rightarrow RXH + O{=}\overset{H}{C}{-}R'$$

X = *O*, *S*, or *N*.

This type of mechanism is attractive for several reasons. For example, it recognizes the close relationship between dealkylation and the microsomal hydroxylation reaction which it so closely resembles. It is also a general mechanism applicable regardless of the nature of X or R. In addition there exists ample analogy in both organic chemistry and biochemistry for the oxidative attack upon a saturated carbon atom.

Although considerable progress has been made in recent years in understanding the oxygen activating system of microsomes, the nature of

the "active oxygen" species that is formed is still obscure. Early investigators, including Udenfriend (87), Mason (88), Hayano (89), and Corey (90) discussed microsomal hydroxylation in terms of heterolytic mechanisms, but more recently free radical intermediates have received more attention. From results with nonenzymatic hydroxylation systems, Breslow and Lukens (91) have suggested hydroxyl radicals as the active hydroxylating species. On the basis of both enzymatic and nonenzymatic hydroxylation studies, Staudinger (92) has suggested the active intermediate to be either the OH or the OOH radical. Evidence for a free radical mechanism has also been presented by Nilsson, Orrenius, and Ernster (93). Finally Gillette (7) reviewed the literature on microsomal oxidation of drugs and found a free radical mechanism to be most consistent with the facts as they are now known.

The interaction of free radicals with *N*-methyl groups has ample analogy in chemical studies (94–96). In fact, Needles and Whitfield (96) have recently shown that dimethylamides are readily demethylated by a free radical mechanism in aqueous solution containing persulfate ion. Further work with systems of this sort would be of considerable value since they appear to be suitable models for the enzymic reaction.

The observation that *N*-dealkylation occurs even more rapidly than does *N*-demethylation (22, 23) is readily understandable in terms of a free radical mechanism, since the intermediate, resonance stabilized, allyl radical, $-\mathrm{N}(\dot{\mathrm{C}}\mathrm{H})-\mathrm{CH}=\mathrm{CH}_2$ would be expected to form readily. For the same reason the ready *O*-dealkylation of *O*-allyl, *O*-benzyl, and *O*-cyanomethyl aryl ethers (32) can be rationalized in terms of a free radical mechanism. It is interesting to note that the radical formed in *S*-demethylation would be substantially stabilized by electron-sharing resonance which is possible in the case of sulfur since it can expand its valence shell to nine electrons by utilizing a vacant 3d orbital (97).

Additional evidence for the free radical mechanism can be found in studies on side chain hydroxylation by microsomes. It is well known that (ω-1)-hydroxylation occurs to a much greater extent than does ω-hydroxylation. This may be due to the greater stability of the $-\dot{\mathrm{C}}\mathrm{H}\mathrm{CH}_3$ radical as compared to $-\mathrm{CH}_2\dot{\mathrm{C}}\mathrm{H}_2$. Also, there are many examples known in which hydroxylation occurs on a benzylic carbon instead of in the aromatic ring itself as might be expected (98–101). The known stability of benzyl radicals serves to explain these observations. In a number of studies of side chain hydroxylation in which a careful analysis of products has been made, it has been found that in addition to the major product smaller amounts of most other possible isomeric alcohols are also observed (102–104). Such nonspecificity is frequently observed in free radical reactions.

Additional support for a free radical mechanism has been recently presented by Hayaishi (105). He has proposed a generalized hypothesis for the action of oxygenases which involves a ternary complex of oxygen, ferrous iron, and substrate which reacts by a concerted one electron transfer to yield oxygenated substrate.

At present then it appears that microsomal dealkylation is best understood as a free radical hydroxylation reaction.

DEALKYLATION IN THE INTACT ANIMAL

In Table III is presented a listing of a variety of compounds that have been demonstrated to undergo dealkylation when administered to the intact animal. The listing is not comprehensive, but an effort has been made to include compounds of various chemical and physiological types.

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Research Articles

Pharmacological Activity of Thalicarpine

By R. A. HAHN*, J. W. NELSON, A. TYE, and J. L. BEAL

The effect of the alkaloid thalicarpine on the cardiovascular system of the anesthetized dog and on several smooth muscle preparations has been studied. In the dog, 2 mg./Kg. produced moderate pressor activity of rather long duration which was sometimes accompanied by a mild tachycardia. This effect does not appear to involve a neural pathway but may be due to a direct action either on the heart or on vascular smooth muscle. Intense, long lasting, noncholinergic hypotension was observed with doses of 10 mg./Kg. Direct depressant effects were seen on several smooth muscle preparations as well as reduction of spasmogenic effects induced by various drugs.

KUPCHAN *et al.* were the first to isolate the alkaloid thalicarpine and to describe its effect on mean arterial blood pressure of the cat (1). They reported that doses up to 5 mg./Kg. caused a transient lowering of blood pressure, while a dose of 10 mg./Kg. caused death. The hypotensive activity, in their opinion, was due to bradycardia, respiratory depression, and a weak adrenergic blocking action.

As part of a continuing study of the genus *Thalictrum* we have observed moderate pressor activity after the administration of 2 mg./Kg. of thalicarpine in the anesthetized dog. The pressor activity was sometimes accompanied by a mild tachycardia. A dose of 10 mg./Kg. was observed to produce an intense depressor response and bradycardia, with some degree of hypotension being observed for 1–3 hr. During this time of prolonged hypotension, no evidence of toxic symptoms was observed. The administration of thalicarpine to the anesthetized cat always produced a transient depressor response.

Reported here are the results of a study of the action of thalicarpine on mean arterial blood pressure of the anesthetized dog and on several smooth muscle preparations.

EXPERIMENTAL

Adult mongrel dogs of either sex were anesthetized with sodium pentobarbital (35 mg./Kg., i.p.). After surgical anesthesia was achieved the trachea was cannulated and bilateral cervical vagotomy performed. The right carotid artery was cannulated and blood pressure recorded *via* a mercury manometer on a kymograph. The right femoral vein was then cannulated with a 3-in. length of polyethylene tubing for the injection of drug solutions. When infusions were administered the left femoral vein was cannulated and the infusion given by means of a Harvard infusion pump. Heart rate was recorded by means of a Sanborn Twin Viso-

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recorder. In those dogs which were pretreated with reserpine (1 mg./Kg., i.p., 24 hr. prior to experimentation) the dose of sodium pentobarbital was reduced to 20 mg./Kg. (2).

Adult cats of either sex were anesthetized with sodium pentobarbital and prepared in a similar manner.

Helically cut 3-cm. strips of descending thoracic rabbit aorta were prepared according to the method of Furchgott (3). The tissue was bathed with Krebs bicarbonate solution and gassed with a mixture of 95% oxygen and 5% carbon dioxide in a 10-ml. tissue bath at a constant temperature of 37°. Movements were recorded isotonically on a slow moving kymograph by means of a frontal lever, with a magnification of 1:10 and a tension of 2 Gm. After mounting of the tissue, 1.5 hr. were allowed for equilibration before the addition of drugs. Two-centimeter lengths of adult rabbit ileum and uteri of adult rats were set up in a similar apparatus. These tissues were bathed with Tyrode's solution and 0.5 hr. was allowed for equilibration before the addition of drugs. A resting tension of 2 Gm. was used with the rat uterus, while light tension was used in the case of rabbit ileum.

Solutions of thalicarpine were prepared in physiological saline with the aid of dilute hydrochloric acid, those of dibenamine in polyethylene glycol, while all other drug solutions were prepared in physiological saline. Injection volumes administered to *in vivo* preparations never exceeded 1 ml. and were followed by a 1-ml. flush of saline, while drug solutions added to *in vitro* preparations never exceeded 0.2 ml. All drug solutions were prepared fresh on the day of the experiment. All doses refer to mg. or mcg. of the parent compound. Thalicarpine[1] was isolated and purified according to the method of Tomimatsu *et al.* (4).

Each observation represents a minimum of 3 experiments.

The following drugs were used: thalicarpine, dibenamine, reserpine phosphate, norepinephrine bitartrate, epinephrine bitartrate, dichloroisoproterenol hydrochloride, isoproterenol hydrochloride, dimethylphenylpiperazinium iodide, atropine sulfate, histamine phosphate, tripelennamine hydrochloride, methacholine chloride, and vasopressin.

RESULTS

Figure 1 shows the effect of administration of 2 mg./Kg. of thalicarpine to the anesthetized, vagotomized dog. A pressor response with gradual onset occurred with a peak mean value of +29.2 ± 2.0 mm. of Hg (mean ± standard error) with some degree of hypertension lasting for 10–35 min. Occasionally the pressor response was accompanied by a mild tachycardia. Some animals responded to this dose of thalicarpine in a biphasic manner; a slight, short-lived fall in blood pressure followed by the sustained pressor response. Further administration of this dose of thalicarpine was ineffective in producing a pressor response, and only transient depressor activity was seen. Doses of thalicarpine less than 2 mg./Kg. did not produce any demonstrable effect on arterial blood pressure.

[1] Its identity as thalicarpine was established by infrared comparison and mixed melting point determination with an authentic sample kindly supplied by Dr. S. M. Kupchan.

Upon the injection of 10 mg./Kg. of thalicarpine to the anesthetized vagotomized dog, a prompt intense depressor response occurred (Fig. 2). The maximal depressor response had a mean value of −112.5 ± 8.5 mm. of Hg and some degree of hypotension was observed for 1–3 hr. Bradycardia (30–40 beats/min.) accompanied this depressor activity. Despite this precipitous fall in blood pressure, no evidence of overt toxic effects was observed, the EKG and respiratory patterns being the same as observed before the injection. Doses of thalicarpine between 5 and 10 mg./Kg. produced increasing depressor activity.

Doses of thalicarpine up to 5 mg./Kg., when given to the anesthetized cat, produced only transient depressor activity. Pressor activity was never seen upon either the initial or subsequent injections.

In an attempt to determine the mechanism by which thalicarpine brought about the initial pressor response in the dog at the 2 mg./Kg. dose level, several experiments were conducted.

To determine if the release of endogenous catecholamines was necessary for the response, dogs were pretreated with reserpine (1 mg./Kg., i.p., 24 hr. prior to experimentation). A typical response of the reserpinized dog to 2 mg./Kg. of thalicarpine is shown in Fig. 3. Reserpine pretreatment did not prevent the initial pressor activity. Furthermore, when the initial pressor activity of thalicarpine had been demonstrated and further injections produced only a fall in blood pressure, the infusion of norepinephrine (1 mg. in 30 min.) did not return the ability of thalicarpine to increase arterial blood pressure. Dibenamine pretreatment (10 mg./Kg.), which abolished the pressor activity of injected epinephrine, had no effect on the pressor activity of thalicarpine.

To ascertain if augmentation of cardiac activity played a part in the pressor effect of thalicarpine, dogs were pretreated with dichloroisoproterenol (DCI). Although this pretreatment effectively abolished control responses to isoproterenol, thalicarpine still produced a pressor response (Fig. 4). DCI was also ineffective in preventing the depressor response to subsequent injections of thalicarpine.

A ganglionic stimulant action on the part of thalicarpine would not appear to account for the pressor action in view of the results obtained with dibenamine or with DCI. These drugs are known to be α- and β-adrenergic receptor blocking agents, respectively, and since neither agent blocked the response, a neural pathway does not seem to be

Fig. 1.—The response of the anesthetized, vagotomized dog to 2 mg./Kg. of thalicarpine. The initial injection produced a pressor response, while a subsequent injection caused only a fall in blood pressure. Key: blood pressure in mm. of Hg; heart rate indicated by numbers above blood pressure tracing (beats/min.); T, thalicarpine.

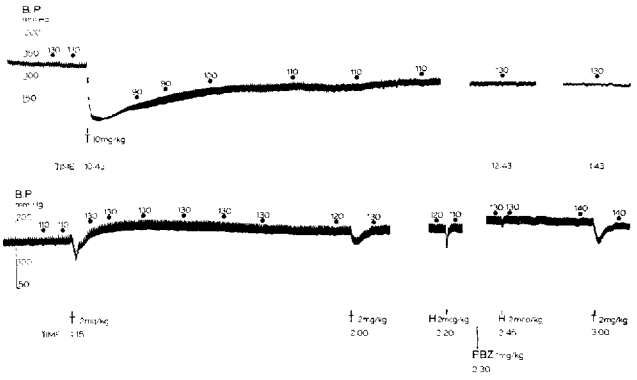

Fig. 2.—The response of the anesthetized, vagotomized dog to 10 mg./Kg. of thalicarpine. Key: blood pressure in mm. of Hg; heart rate indicated by numbers above blood pressure tracing (beats/min.); T, thalicarpine.

Fig. 3.—The response of the anesthetized, reserpinized dog to 2 mg./Kg. thalicarpine. Key: blood pressure in mm. of Hg; heart rate indicated by numbers above blood pressure tracing (beats/min.); T, thalicarpine; H, histamine; PBZ, tripelennamine.

involved. In addition, no augmentation of dimethylphenylpiperazinium (DMPP)-induced pressor activity was observed when DMPP (25 mcg./Kg.) was injected during a typical pressor response to thalicarpine.

The depressor action of repeated injections of thalicarpine at the 2 mg./Kg. dose was not blocked by atropine pretreatment, although the depressor effect of injected acetylcholine was completely abolished (Fig. 4). Vagotomy also did not alter the response. The possibility of histamine release was ruled out when pretreatment with tripelennamine did not block the thalicarpine effect, although injected histamine was prevented from causing its usual depressor response in the dog (Fig. 3).

The precipitous and long lasting hypotensive response to 10 mg./Kg. of thalicarpine was not altered by cervical vagotomy or atropine pretreatment. DMPP-induced pressor responses, known to be mediated through ganglionic stimulation, were the same during the prolonged hypotension produced by thalicarpine as they were at preinjection times. Similarly injected epinephrine (2–4 mcg./Kg.) produced the same rise in blood pressure during the intense thalicarpine hypotension as it did during preinjection times.

When thalicarpine was added to a bath with isolated rabbit ileum a prompt relaxation occurred. Upon washing, this effect was abolished and normal activity resumed. When methacholine chloride was introduced the characteristic spasm was seen; however, pretreatment with thalicarpine greatly reduced the methacholine-induced spasm of the ileum. This effect became less with time (Fig. 5). Normal contractions of the rat uterus were inhibited both in frequency and amplitude upon the addition of thalicarpine into the bath. Vasopressin-induced spasms of the uterus were completely blocked after thalicarpine. With repeated washing of the tissue the vasopressin effect returned (Fig. 6). Similar results were obtained on epinephrine-induced contractions of the rabbit aortic strip. After pretreatment with thalicarpine, in concentrations having no effect of their own (10–40 mcg./ml.), contractions

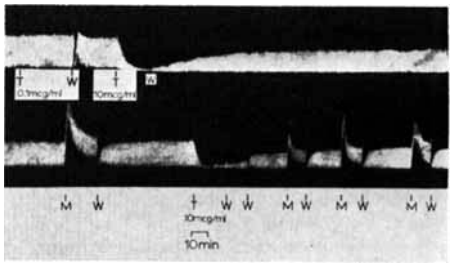

Fig. 5.—Continuous kymograph recording of the isolated rabbit ileum. The administration of thalicarpine caused a prompt relaxation of the ileum. Pretreatment with thalicarpine greatly reduced the methacholine-induced spasm of the ileum. Key: T, thalicarpine; M, methacholine 0.05 mcg./ml.; W, wash.

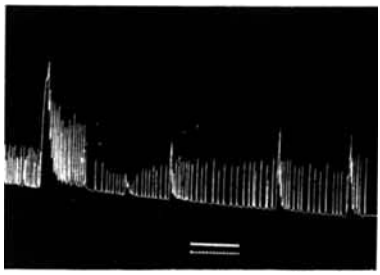

Fig. 6.—Kymograph recording of the isolated rat uterus. The administration of thalicarpine inhibited normal contractions of the uterus and greatly reduced the vasopressin-induced spasm of the uterus. Key: P, vasopressin 0.1 unit/ml.; T, thalicarpine 40 mcg./ml.; W, wash.

Fig. 4.—The response of the anesthetized, vagotomized dog to 2 mg./Kg. thalicarpine. Key: blood pressure in mm. of Hg; heart rate indicated by numbers above blood pressure tracing (beats/min.); Is, isoproterenol; DCI, dichloroisoproterenol; T, thalicarpine; Ach, acetylcholine; A, atropine.

due to epinephrine were greatly reduced, the effect being reversed with time and repeated washing of the tissue.

DISCUSSION

The administration of 2 mg./Kg. of thalicarpine to the anesthetized vagotomized dog produced a fairly long lasting pressor response with a gradual onset. If liberation of endogenous catecholamines was necessary for the response, then pretreatment with reserpine should block this action, since reserpine is known to deplete sympathetically innervated tissue of catecholamine stores and, thus, reduce the response of various organs to sympathetic nerve stimulation and to indirectly acting sympathomimetic amines (5–7). Thalicarpine-induced pressor activity was observed in the reserpinized dog, and thus, liberation of catecholamines does not appear necessary for the response. When the initial pressor activity to thalicarpine had been demonstrated and further administration of thalicarpine produced only depressor activity, norepinephrine infusion could not restore the pressor action. When the α-adrenergic receptor blocking agent, dibenamine, was given in a dose sufficient to block the pressor effect of injected epinephrine, the pressor activity of thalicarpine was not altered. Therefore, the increase in arterial blood pressure due to thalicarpine is not due to release of catecholamines or a direct α-receptor stimulant effect.

In dogs pretreated with DCI, thalicarpine pressor activity was observed, although positive chronotropic activity of control injections of isoproterenol was blocked (Fig. 4). DCI has been demonstrated to block the positive chronotropic and inotropic effects of adrenergic stimuli in dogs, while it is ineffective against similar responses produced by digoxin or theophylline (8).

Since the pressor activity of thalicarpine does not appear to be due to an epinephrine-like effect, the possibility exists that the effect may be similar to the cardiac action of digoxin which is not altered by DCI. Papaverine also has direct positive inotropic effects (9), and if one views the structure of thalicarpine as reported by Kupchan and Yokoyama (10), it is apparent that thalicarpine contains the basic moiety of papaverine. The possibility exists, therefore, that the pressor activity of thalicarpine may be due to a direct positive inotropic effect to increase cardiac output, especially since a positive chronotropic action was not always seen with thalicarpine.

Some transient direct stimulant effect on vascular smooth muscle might also be a possibility as the cause of the pressor action, although bath concentrations of thalicarpine up to 40 mcg./ml. produced no contraction of the isolated rabbit aortic strip. Vasopressin has been shown to be a potent vasoconstrictor in the intact animal but without effect on rabbit aortic strips (11); a similar situation may exist in the case of thalicarpine.

The rise in blood pressure produced by injections of DMPP during typical thalicarpine hypertension was of the same magnitude as that produced by control injections. DMPP has been shown to be a ganglionic stimulant (12), and if thalicarpine also possessed this activity, then an additive pressor action might be expected. Such was not the case and, therefore, ganglionic stimulation as a basis for the pressor effect of thalicarpine does not appear likely. Furthermore, if ganglionic stimulation is a factor a neural pathway would be involved and dibenamine, DCI, or reserpine pretreatment should have blocked the response.

The hypotension induced by repeated injections of 2 mg./Kg., or by a single injection of 10 mg./Kg. of thalicarpine, does not appear to be cholinergic in nature for atropine did not alter this effect, although it effectively blocked injected acetylcholine (Fig. 4). Cervical vagotomy also had no effect on this response to thalicarpine.

It has been reported that 10 mg./Kg. of thalicarpine caused death in the anesthetized cat (1). In our experiments the administration of this dose of thalicarpine to the dog, and doses of 5 mg./Kg. in the cat, never produced overt signs of toxicity, or death. Animals receiving this dose of thalicarpine appeared normal with respect to EKG and respiratory patterns, and in general appearance. Although the depressor response was intense and long lasting, the blood pressure did return to approximately preinjection levels (Fig. 2). We do not exclude the possibility, however, that this depressor effect may represent thalicarpine toxicity.

The observations that epinephrine and DMPP produced similar pressor effects during thalicarpine hypotension to those at preinjection times indicate that adrenergic blockade or ganglionic blockade are not prime causes of the potent hypotension.

Thalicarpine, in bath concentrations of 10–40 mcg./ml., produced a prompt relaxation of the isolated ileum and uterus. Spasms of the ileum produced by methacholine and those of the uterus produced by vasopressin, were greatly reduced by thalicarpine pretreatment (Figs. 5 and 6). Thalicarpine pretreatment of the isolated rabbit aortic strip also greatly inhibited epinephrine-induced contractions. This effect was reversed on all 3 preparations with time and repeated washings of the tissues. Thus our results indicate a nonspecific inhibition of smooth muscle activity and an ability to antagonize spasmogenic effects of drugs of diverse nature on various smooth muscle as an important pharmacological effect of thalicarpine. This peripheral inhibitory effect of smooth muscle plus bradycardia may well be the cause of the potent hypotension observed with higher doses of thalicarpine.

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Analogs of Tetrahydrofolic Acid XXXV

Hydrophobic Bonding to Dihydrofolic Reductase VII. Species Differences with Substituted 4,6-Diamino-1,2-dihydro-*s*-triazines

By B. R. BAKER* and BENG-THONG HO

Twenty-four 4,6-diamino-1,2-dihydro-*s*-triazines with varying substituents on the 1- and 2-positions have been compared as inhibitors of the dihydrofolic reductase from pigeon liver and from *E. coli* B. Since the 4,6-diamino-1,2-dihydro-*s*-triazine most probably binds to the enzyme in the same area as the pteridine ring of the substrate, dihydrofolate, little difference was seen in complexing of the 1, 2, 2-trimethyl derivative (VI); this result was anticipated since the active site of an enzyme cannot be varied appreciably and still have a functioning enzyme. In contrast, hydrophobic bonding to the enzymes by alkyl or aryl groups on the 1- and 2-positions of the inhibitors could be anticipated to show differences in binding between the two species, since it should be possible for the relatively nonfunctional hydrophobic region on the enzyme to be quite variable from species to species and still allow the enzyme to function. The biggest differences between the two species were observed when substituents were attached to the 1-phenyl group of 4,6-diamino-1-phenyl-2,2-dimethyl-1,2-dihydro-*s*-triazine (I) or larger groups than methyl were introduced on the 2-position. Differences in conformational requirements for hydrophobic bonding to the two enzymes are presented.

THAT ALKYL or aryl groups attached to the 5-position of pyrimidines or the 1-position of 1,2-dihydro-*s*-triazines can give strong hydrophobic bonding to dihydrofolic reductase has recently been discovered (1); further studies (2) substantiated the proposal that the aryl groups were also bonded hydrophobically rather than by a charge transfer complex with the enzyme. Some of the conformational aspects of hydrophobic bonding to the dihydrofolic reductase from pigeon liver have also been investigated (3). The hydrophobic bonding region is most probably not in the area where dihydrofolate is complexed (4, 5); if it is adjacent to the 4-position or 8-position of dihydrofolate when the latter is complexed with dihydrofolic reductase (6), this hydrophobic region would therefore be in a nonfunctional part of the enzyme just adjacent to the active site. Furthermore, it might be expected that outside of the active site, one hydrophobic amino acid could be replaced by another hydrophobic amino acid without affecting the function of the active site, whereas amino acid replacement within the active site could be expected to be much more sensitive with respect to enzyme activity (7). *Ergo*, it can be anticipated that there should be species differences in the nonfunctional hydrophobic area presumed to be adjacent to the active site of dihydrofolic reductase. Some aspects of hydrophobic bonding to the dihydrofolic reductase from *E. coli* B and their relationship to the dihydrofolic reductase from pigeon liver is the subject of this paper. Furthermore, 2 previous studies—one from this laboratory (8) and one by Burchall and Hitchings (9, 10)—on the effect that substitution on the 5- and 6-positions of pyrimidines has on the comparative inhibition of dihydrofolic reductases from different sources can now be better envisioned when considered in the light of differences in hydrophobic bonding.

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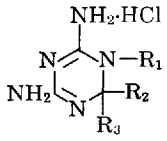
RESULTS AND DISCUSSION

Four discrete series of dihydro-*s*-triazines with variants at either the 1-position or 2-position were compared as inhibitors of the dihydrofolic reductase from pigeon liver and from *E. coli* B; the results are reported in Table I.

Series A.—*1-$C_6H_5(CH_2)_n$-Variants.*—The syntheses of the compounds in this series have been previously described (12). With both enzymes insertion of 1 methylene group (II) between the 1-aryl group and the *s*-triazine of I was detrimental to binding, being 10-fold with the *E. coli* B enzyme and thirtyfold with the pigeon liver enzyme; thus with both enzymes, when the aryl group is out of the plane of the triazine, a loss in binding occurs. By comparison of the 1-benzyl-*s*-triazine (II) with the 1-methyl-*s*-triazine (VI), it can be seen that the phenyl group of II gives little binding to the *E. coli* enzyme, but a 22-fold increment in binding to the pigeon liver enzyme, presumably due to the phenyl group, still remains. The next higher homolog, phenylethyl (III), can now have the phenyl ring nearly coplanar with the *s*-triazine ring; as a result, activity is better than benzyl (II) with both enzymes. On the *E. coli* B enzyme, the phenethyl (III) side-chain is just as effective as the phenyl side-chain (I); in contrast, phenethyl (III)

TABLE I.—INHIBITION OF DIHYDROFOLIC REDUCTASES BY



Compd.	R_1	R_2	R_3	μM Concn. for 50% Inhibition[a] Pigeon Liver[b]	*E. coli* B[c]	Pigeon Liver: *E. coli* B
I	$-C_6H_5$	CH_3	CH_3	0.11[d]	3.0	1/27
II	$-CH_2C_6H_5$	CH_3	CH_3	3.3[d]	34	1/10
III	$-(CH_2)_2C_6H_5$	CH_3	CH_3	0.71[d]	3.8	1/5.3
IV	$-(CH_2)_3C_6H_5$	CH_3	CH_3	0.028[d]	0.063	1/2.2
V	$-(CH_2)_4C_6H_5$	CH_3	CH_3	0.041[d]	0.21	1/5.1
VI	$-CH_3$	CH_3	CH_3	74[e]	48	1.5
VII	$-C_2H_5$	CH_3	CH_3	200[e]	62	3.5
VIII	$-C_3H_7$-*n*	CH_3	CH_3	11[e]	4.7	2.3
IX	$-C_4H_9$-*n*	CH_3	CH_3	0.36[e]	2.1	1/5.8
X	$-C_5H_{11}$-*i*	CH_3	CH_3	0.058[e]	0.69	1/12
XI	$-C_6H_{13}$-*n*	CH_3	CH_3	0.32[e]	0.92	1/2.9
XII	$-C_8H_{17}$-*n*	CH_3	CH_3	0.14[e]	0.27	1/1.9
XIII	$-C_6H_4-C_6H_5$-*p*	CH_3	CH_3	160[f]	5.8	28
XIV	$-C_6H_4-C_6H_5$-*m*	CH_3	CH_3	1.3[f]	1.0	1.3
XV	$-C_6H_4-(C_4H_9$-*n*$)$-*p*	CH_3	CH_3	0.064[f]	7.2	1/110
XVI	9-fluorenone-2-yl	CH_3	CH_3	85[d]	1.5	57
XVII	$-C_6H_4CC_6H_5$-*m* (O)	CH_3	CH_3	1.1[d]	0.61	1.8
XVIII	$-C_6H_4Cl$-*m*	CH_3	CH_3	0.0085[g]	0.60[g]	1/71
XIX	$-C_6H_4CH_2C_6H_5$-*p*	CH_3	CH_3	0.062[f]	4.8	1/77
XX	$-C_6H_4CH_2C_6H_5$-*m*	CH_3	CH_3	0.019[d]	0.28	1/15
XXI	$-C_6H_4CH_2CH_2C_6H_5$-*m*	CH_3	CH_3	0.024[d]	0.092	1/3.5
XXII	$-(CH_2)_4C_6H_5$[h]	*p*-$AcNHC_6H_4-$	H	0.62[d]	0.41	1.5
XXIII	$-C_6H_4Cl$-*m*	*p*-$AcNHC_6H_4-$	H	190[d]	180	1.0
XXIV	$-C_2H_5$	$-C_6H_5$	H	15,000[d]	310	50

[a] The technical assistance of Maureen Baker and Karen Smith with these assays is acknowledged. [b] A 45–90% saturated ammonium sulfate fraction prepared from pigeon liver acetone powder and assayed with 6 μM dihydrofolate and 12 μM TPNH as previously described (11) at pH 7.4 in Tris buffer. [c] *E. coli* B cell walls were broken with a French pressure cell at 20,000 p.s.i. A 45–90% saturated ammonium sulfate fraction was prepared as previously described (11), then assayed with 6 μM dihydrofolate and 30 μM TPNH. [d] Data from *Reference 12*. [e] Data from *Reference 1*. [f] Data from *Reference 2*. [g] Data from *Reference 8*. [h] Picrate salt.

is 6.5-fold less effective than phenyl (I) with the pigeon liver enzyme.

Maximum effectiveness against both enzymes is shown with the phenylpropyl side-chain (IV), with phenylbutyl (V) being 1.5–3 times less effective. However, the increment between phenyl (I) and phenylpropyl (III) is much larger (48-fold) with the *E. coli* enzyme than with the pigeon liver enzyme (fourfold); most of this difference is due to the fact that the 1-phenyl (I) binds to the pigeon liver enzyme 27-fold better than the *E. coli* B enzyme, possible reasons for which are discussed later. Furthermore, the greatest specificity toward the pigeon liver enzyme (27-fold) is shown by the 1-phenyl side-chain (I), but none of the compounds in this series are more effective on the *E. coli* B enzyme than the pigeon liver enzyme.

Series B.—*1-Alkyl Variants.*—The synthesis of these compounds has been previously described (1). With straight-chain alkyl substituents maximum hydrophobic bonding with the pigeon liver enzyme occurred in *n*-butyl (IX) with *n*-hexyl (XI) being equal and *n*-octyl (XII) perhaps twofold better; in contrast, with the *E. coli* B enzyme, *n*-hexyl (XI) was 2.3-fold better than *n*-butyl (IX) and *n*-octyl (XII) was 3.4-fold better than *n*-hexyl (XI). Extension of the series would be required to reveal whether maximum straight-chain hydrophobic bonding to the *E. coli* B enzyme had occurred at *n*-octyl.

Branching of the *n*-butyl group (IX) to isoamyl (X) gave a sixfold increment in binding on the pigeon liver enzyme, and X was the most effective alkyl group found for the enzyme from this source (1, 3); the isoamyl group (X) gave a threefold increment in binding over the *n*-butyl group (IX) with the *E. coli* enzyme, but—in contrast to the pigeon liver enzyme—the isoamyl group (X) was still less effective than the *n*-octyl group (XII). Whether chain-branching farther out the chain than the C-3 branch of isoamyl (X) would give a still better inhibitor of the *E. coli* B enzyme is as yet unknown.

It was previously noted (1) that the increment in binding between methyl (VI) and *n*-butyl (IX) to the pigeon liver enzyme was 200-fold; this is well within the 1000-fold (10-fold per methylene) that is theoretically possible. However, it was pointed out that there was a twentyfold increment in binding in proceeding from ethyl (VI) to *n*-propyl (VIII) and a thirtyfold increment in *n*-propyl (VIII) to *n*-butyl (IX), both of which are above the 10-fold increments theoretically possible. The fact that methyl to butyl gave only a 200-fold increment—which is within the theoretical 1000-fold

increment in binding for 3 methylene groups—was rationalized by assuming a conformational change in the enzyme; if this conformational change in the enzyme required no more than 900 cal./mole, then this 900 cal./mole could be supplied by the five-fold difference between the 200-fold increment observed in proceeding from methyl to butyl and the 1000-fold increment theoretically possible. Thus, the greater than 10-fold increment in binding between *n*-propyl and ethyl or *n*-butyl and *n*-propyl is still energetically possible including the energy required for a conformational change in the pigeon liver enzyme. With the *E. coli* B enzyme only the change between ethyl and *n*-propyl gave greater than a 10-fold increment (13-fold) which indicates that little or no conformational change in this enzyme need take place for maximum hydrophobic binding; it should be further noted that the maximum increment in hydrophobic bonding by straight-chain alkyl groups with the *E. coli* enzyme is only 180-fold (between methyl and *n*-octyl) whereas the pigeon liver enzyme has a maximum increment of 530-fold between methyl and *n*-octyl groups.

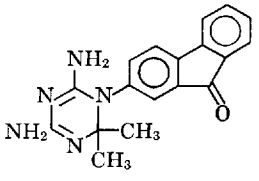
In this series, the isoamyl-*s*-triazine (X) shows the most specificity toward the pigeon liver enzyme, being 12-fold more effective than on the *E. coli* B enzyme; the most specific compound toward the *E. coli* B enzyme is the ethyl-*s*-triazine (VII) which is 3.5-fold more effective than on the pigeon liver enzyme.

Series C.—*1-Phenyl with m- and p-Substituents.*—The synthesis of these compounds and their inhibition on pigeon liver dihydrofolic reductase have been previously described (2, 12). In this series is seen the largest spread in specificity between the two enzymes in both directions. The *E. coli* B enzyme is relatively insensitive to *m*- or *p*-substituents, the maximum variation being between 7.2 μM for the *p*-(*n*-butyl)-substituent (XV) to 0.6 μM for the *m*-benzoyl (XVII) and *m*-chloro (XVIII) substituents, a 12-fold spread; in contrast, the pigeon liver enzyme is extremely sensitive to substitution, varying from 0.0085 μM for the *m*-chlorophenyl group (XVIII) to 160 μM for the *p*-biphenyl group (XIII) at the 1-position of the *s*-triazine, a 19,000-fold spread. As a result the *p*-(*n*-butyl)-phenyl-*s*-triazine (XV) is 110-fold more effective on the pigeon liver enzyme than the *E. coli* B enzyme; similarly, the *m*-chlorophenyl-*s*-triazine (XVIII) and the *p*-(benzyl)phenyl-*s*-triazine (XIX) are 71 and 77-fold, respectively, more effective on the pigeon liver enzyme. In contrast, the *p*-biphenyl-*s*-triazine (XIII) and the 9-fluorenon-2-yl-*s*-triazine (XVI) are 28 and 57-fold more effective, respectively, on the *E. coli* B enzyme. Note that the benzoyl group of XVII causes a 10-fold loss in binding to the pigeon liver enzyme, but a fivefold gain in binding to the *E. coli* B enzyme. When the *m*-benzoylphenyl group of XVII is cyclized to the 9-fluorenon-2-yl group as in XVI, only a 2.5-fold loss in binding occurs in the *E. coli* B enzyme, but a further 77-fold loss in binding to the pigeon liver enzyme occurs; thus, the pigeon liver enzyme cannot tolerate a large flat substituent at the 1-position, but the *E. coli* B enzyme can. Reduction of the ketone of XVII to give the *m*-benzyl derivative (XX) causes little change with the *E. coli* B enzyme, but 46-fold better inhibition is seen with the pigeon liver enzyme.

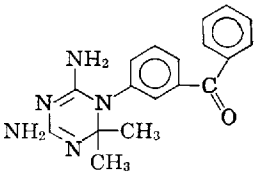
The most active and one of the most specific compounds for the pigeon liver enzyme in this series is *m*-chlorophenyl-*s*-triazine (XVIII) at 0.0085 μM. The most active compound in this series for the *E. coli* B enzyme is the *m*-phenethylphenyl-*s*-triazine (XXI) at 0.092 μM, but this compound has no specificity toward the *E. coli* B enzyme since XXI is even more effective on the pigeon liver enzyme.

Series D.—*Variants at the 2-Position.*—The synthesis of these compounds and their evaluation on pigeon liver dihydrofolic reductase have been previously described (12). Structural change at the 2-position by introduction of larger groups has a much more detrimental effect on binding to the pigeon liver enzyme than the *E. coli* B enzyme. Note that replacement of the 2,2-dimethyl group of the 1-phenylbutyl-*s*-triazine (V) by 2-(*p*-acetamidophenyl) (XXII) reduces binding to the pigeon liver enzyme by a factor of 15-fold, but reduces binding to the *E. coli* enzyme by only a factor of twofold. Similarly, the same 2-substituent exchanged on the 1-(*m*-chlorophenyl)-*s*-triazine (XVIII) to give XXIII causes a 19,000-fold loss in binding to the pigeon liver enzyme, but only an 850-fold loss to the *E. coli* B enzyme; it should be noted that this structural change giving a 1,2-diaryl-*s*-triazine reduces coplanarity of at least one phenyl and possibly both phenyls with the *s*-triazine ring (13). When the 2, 2-dimethyl group of the 1-ethyl-*s*-triazine (VII) is replaced by phenyl to give XXIV, it results in only a fivefold reduction in binding to the *E. coli* B enzyme, but a 68-fold reduction in binding to the pigeon liver enzyme occurs. A study of other 2-substituents with IV and V could well lead to higher affinity to the *E. coli* B enzyme than the pigeon liver enzyme since the latter is more sensitive to structural change at this 2-position; note that XXIV, although it is a poor inhibitor, is fiftyfold more effective on the *E. coli* B enzyme than the pigeon liver enzyme and that no compound in this series shows the converse order of affinity.

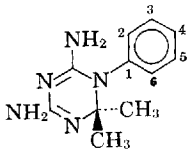
Relative Topography of Hydrophobic Bonding to the Two Enzymes.—The difference in binding to the two enzymes can give considerable insight into the differences in topography of the hydrophobic regions of the two enzymes. It is well established that the phenyl group of I*a* must be nearly coplanar with the triazine ring for maximum binding (13) to the enzyme since introduction of an *o*-chloro group causes a great reduction in the affinity of the inhibitor to both enzymes due to the restricted rotation of the 1-phenyl-*s*-triazine system imposed by the *o*-chloro group (8, 12). Since there is strong experimental support for the 1-phenyl group being hydrophobically bonded to the enzyme (2), it is a reasonable assumption that the 1-phenyl group of I*a* and a 1-alkyl group of VIII*a* are bonded in the same



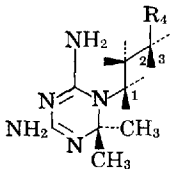
XVI



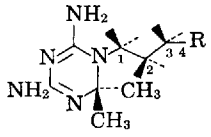
XVII



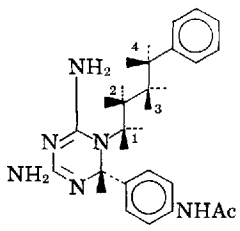
Ia



Va, R = $C_6H_5CH_2$
VIIIa, R = H
IXa, R = CH_3



IVb, R = $C_6H_5CH_2$
VIIIb, R = H
IXa, R = CH_3



XXIIa

region. The alkyl group can then be staggered in the plane of the triazine either away from the 2-position as in VIII*a* or toward the 2-position as in VIII*b*. Since XXII and XXIV can still bind reasonably well to both enzymes, a downward-stagger of the VIII*b* type is not possible due to the 2-phenyl group of XXII occupying the same space as the C_2-methylene group of the side chain. With the upward-staggered conformation, there is no steric interaction between the C_2-methylene group and the 2-phenyl, but there is now a possible steric interaction between the C_1-methylene group and the 2-phenyl group; this C_1-methylene steric interaction does not occur if the 2-phenyl group is in a plane perpendicular to the triazine, but is strong if the plane of the phenyl group is moved 90°. It follows that the *E. coli* B enzyme has more tolerance for the 2-phenyl group approaching a plane perpendicular to the triazine than the pigeon liver enzyme, since little activity is lost in the *E. coli* B enzyme when VII is converted to XXIV or V is converted to XXII; the large reduction in affinity to the pigeon liver enzyme indicates that this enzyme cannot tolerate the 2-phenyl group approaching a plane perpendicular to the *s*-triazine ring.

Note that the *n*-propyl-*s*-triazine (VIII) is nearly as effective as the 1-phenyl-*s*-triazine (I) on the *E. coli* B enzyme, but that I is 100-fold more effective than VIII on the pigeon liver enzyme. Models clearly indicate that C_1, C_2, and C_3 of the phenyl group exactly overlap the C_1, C_2, and C_3 groups of an alkyl side chain when they have conformations I*a* and VIII*a*, respectively. Since the 1-propyl-*s*-triazine (VIII*a*) binds almost as well as the 1-phenyl-*s*-triazine (I*a*) to the *E. coli* enzyme, it is reasonable to propose that most of the phenyl bonding of I*a* occurs through the C_1, C_2, and C_3 carbons; the remaining 1.6-fold difference between I*a* and VIII*a* might be due to slight additional bonding by the C_4 or C_6 carbons of the phenyl or the C_1, C_2, and C_3 carbons of the phenyl of I*a* may hydrophobically bond a little better than the corresponding carbons of VIII*a;* this 1.6-fold difference, however, is practically negligible.

The 100-fold better binding of the 1-phenyl of I*a* than the 1-propyl of VIII*a* to the pigeon liver enzyme suggests that at least 2 more carbons of the benzene ring—in addition to C_1, C_2, and C_3—are complexed hydrophobically to this enzyme; one of these carbons is most probably C_4 since the isoamyl group (X) with its skewed C_4 and C_4' carbons has been shown to have both of these carbons hydrophobically bonded (3).

Both the *n*-octyl (XII) and the phenylbutyl-*s*-triazine (IV*a*) are better—and about equally effective—inhibitors of the *E. coli* B enzyme than the 1-phenyl-*s*-triazine (I*a*) by a factor of 11-to 14-fold. Furthermore, XII and IV*a* are about 10-fold better inhibitors than the *n*-butyl-*s*-triazine (IX*a*); since the terminal four carbons of XII can only increase affinity to the enzyme by hydrophobic bonding of one or more of these terminal carbons, it follows that the phenyl group of IV*a* does likewise. In contrast, the pigeon liver enzyme has only a twofold greater affinity for the octyl group of XII than the butyl group of IX*a*, but the phenylbutyl group of IV*a* is complexed eightfold better than the butyl group of IX*a;* it follows that the additional hydrophobic bonding to the pigeon liver enzyme past the *n*-butyl group requires the relatively flat interaction that can occur with a benzene ring, but the *E. coli* B enzyme can bind either a flat or staggered group in this area. The greater tolerance to type of group attached to the 1-phenyl substituent by the *E. coli* B enzyme has also been pointed out under *Series C*.

Of further interest is that there is less than a twofold difference in binding to the two enzymes with the 1-methyl-*s*-triazine (VI), but there is a 12-fold difference with the *i*-amyl-*s*-triazine (X) and even larger differences in both directions with XV and XVI. (See *Series C*.) Since it is highly probable that the 4,6-diamino-*s*-triazine moiety binds within the normal complexing region for the pteridine ring of the substrate, dihydrofolate, and since the active site of an enzyme has a considerable limitation on structural change which will still allow the enzyme to be operable, little difference should be seen in binding of the 4,6-diamino-*s*-triazine moiety to the enzyme. In contrast, large differences in hydrophobic bonding were anticipated and found since this is a nonspecific part of the enzyme which could readily vary from species to species and still leave an operable enzyme.

Further examples of species differences in the ability of dihydrofolic reductase to bind 2,4-diamino-heterocycles that have varying hydrophobic groups have been collated by Burchall and Hitchings (9); their observations fit into the differences in hydrophobic bonding that are described here. They also noted that the biggest species differences existed when substituents on the 1-phenyl group or 2-position of the *s*-triazine were varied.

Although differences in hydrophobic bonding to the dihydrofolic reductase between invading organisms such as bacteria and protozoa on the one hand, and mammalian enzymes on the other hand, are sufficiently large to enjoy a chemotherapeutic advantage with reversible inhibitors *in vivo* (9), such differences between a cancer cell and the host are most probably too small to have any chemotherapeutic advantage with reversible inhibitors of the 2,4-diamino-heterocycle type. By utilizing both hydrophobic bonding to dihydrofolic reductase

and the bridge principle of specificity with active-site-directed irreversible inhibitors (14), it should be possible to magnify immensely any small difference between the hydrophobic areas of the tumor tissue and host dihydrofolic reductases; this small difference would be unusable or even undetectable with reversible inhibitors. Since active-site-directed irreversible inhibitors of the dihydrofolic reductase from pigeon liver have been found which utilize the hydrophobic bonding region (15, 16), only a small difference in the hydrophobic region of a tumor tissue dihydrofolic reductase would be sufficient for high specificity—such as a change of a valine for a leucine or a slightly different conformation in the hydrophobic region caused by a single amino acid exchange in another region. Such a small difference could be exploited by attaching a bridging moiety to the hydrophobic bonding moiety of an inhibitor so that the subsequent attack by the bridging group to form a covalent bond to a nucleophilic site on the enzyme is subject to proper juxtapositioning by the hydrophobic area on the enzyme. Such studies are currently being pursued.

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Effect of Certain Drugs on Perfused Human Placenta VI

Serotonin Antagonists

By CHARLES O. WARD and RONALD F. GAUTIERI

The antiserotonin action of several compounds was investigated in the vasculature of the isolated perfused human placenta. Average onset, duration of action, and per cent decrease at maximal antagonism were used to discern the antiserotonin capability of the smallest effective dose of each compound necessary to antagonize the pressor action of serotonin. Their ability to antagonize the vasoconstrictor effect of serotonin, in decreasing order of effectiveness, was: cyproheptadine, LSD, diphenhydramine, chlorpromazine, promethazine, promazine, and dibenamine. Cyproheptadine, which had a relatively short duration of action, caused the greatest decrease to the pressor action of serotonin, while chlorpromazine and diphenhydramine exhibited the longest duration of action. The mechanisms by which these agents antagonized the vasopressor effect of serotonin are attributed to a blockade of α-adrenergic receptors, competition for specific receptor sites, and/or direct negative musculotropic action. It is suggested that the human placenta, rather than tissues of other species, may serve as the organ of choice to evaluate the potential effectiveness of serotonin antagonists useful in therapeutics.

ALTHOUGH the vasoconstrictor property of serotonin has been known since 1869 (1), the interplay between its physiologic and pathologic functions, as well as the elucidation of its pharmacologic mechanisms, awaited the development not only of reliable assays for its estimation in biologic tissues, but also techniques for determining the potency of agents specifically antagonistic to it.

Many compounds have been shown to antagonize the stimulant effect of serotonin on smooth muscle, and among the several organs used to demonstrate this phenomenon are the gastric fundus of the rat (2), estrogen-primed rat uterus (3), isolated rabbit ear (4), sheep artery rings (5), and the guinea pig ileum (6). The effect of serotonin on diuresis (7) and blood pressure (8) in the intact animal also has been used to show the antiserotonin effect of these agents. While these techniques are generally sensitive to minute doses of serotonin, and though the results are usually reproducible in the particular procedure

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The authors express sincere thanks to the members of the Obstetrical Staff, Temple University Hospital, for the generous supply of placentas, to Drs. David E. Mann, Jr., and E. J. Larson for their valuable suggestions and discussions, and to Mr. J. C. Tatnall for his technical assistance.
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TABLE I.—COMPARISON OF SEROTONIN ANTAGONISM BY SEVERAL COMPOUNDS IN PERFUSED HUMAN PLACENTAL VESSELS

Drug	Dose	Expt., No.	Av. Max. Antagonism, min.	Range of Duration of Action, min.	Av. Decrease at Max. Antagonism, %
Chlorpromazine	0.5–1.0 mg.	3	20.0	21–79	67.8
Promazine	0.5–1.0 mg.	5	14.0	9–46	47.4
Promethazine	0.5–2.0 mg.	6	16.2	11–49	52.7
LSD	100.0–200.0 mcg.	6	10.8	11–30	75.6
Cyproheptadine	100.0 mcg.	3	11.0	17–28	86.2
Diphenhydramine	10.0 mg.	3	31.7	24–63	68.9
Dibenamine	2.0 mg.	4	26.3	17–52	46.4

employed, conflicting results occur often enough to make it difficult to predict the effect of these antagonists in humans.

Variation among the several species of animals used is considerable, both in response to serotonin and to its antagonists as well. For example, the intravenous injection of serotonin in dogs increases the heart rate and blood pressure, while the rabbit responds to similar doses with hypotension and bradycardia (8). The gramine derivatives, medmain and methylmedmain, are serotonin antagonists on the rat uterus but have no effect on antidiuresis (7). Morphine is ineffective as a serotonin antagonist on strips of the gastric fundus of the rat (2), the isolated rat uterus, and the isolated rabbit ear, but has potent antiserotonin actions on the guinea pig ileum (6). The ergot alkaloids, however, are poor serotonin antagonists on the guinea pig ileum (9), but give excellent results in other tissues, including sheep artery rings (5), the isolated rat uterus (3), and the blood vessels of the isolated human placenta (10). Hydralazine increases serotonin-induced contractions of the guinea pig gut, with only slight, if any, effect on the rat intestine (11). On the other hand, the vasoconstrictor action of serotonin on the blood vessels of the human placenta is occasionally antagonized by hydralazine (12). Another adrenergic blocking agent, dibenamine, has also been shown to exhibit serotonin-like and antiserotonin actions, in that it inhibits diuresis when given alone to the intact rat, then blocks the antidiuretic effect of subsequent doses of serotonin (13).

The obvious conclusion is that no one animal preparation can be used to assay for all serotonin antagonists, and even if one were available, this would not be an exact indication that the same effects would be noted when the drugs were given to man.

Serotonin has been implicated as a causative factor in the toxemias of pregnancy (14, 15), certain forms of mental disease (16), allergic disorders (17), and collagen diseases (18); in the toxemias of pregnancy the toxic effects of serotonin are thought to be the result of a constriction of the blood vessels of the placenta with resulting ischemia (15).

Thus, it is imperative that a method for evaluating the therapeutic effects of serotonin antagonists in man be available. Therefore, it was the purpose of this investigation to evaluate the antiserotonin activity of several known serotonin antagonists on the vasculature of the human placenta in order to predict more accurately the degree of antagonism of these agents in human tissue.

MATERIALS AND METHODS

Full-term human placentas, obtained from the hospital 15 to 20 min. after normal delivery, were used throughout this investigation. Each was transported to the laboratory in a glass container filled with approximately 1 L. of Tyrode's solution preheated to 38°.

The apparatus employed and the procedures involved in the preparation and perfusion of the placentas, in recording and maintaining the perfusion pressure, and in measuring the inflow and outflow volumes of the perfusate have been described in previous papers (12, 19). In 26 successful placental preparations, each lasting 1–4 hr., a total of 30 experiments were performed. The results obtained from 4 placentas were discarded because the response to a test dose of serotonin was erratic.

Throughout this investigation the agonist, serotonin, was administered before the various antagonists for the purpose of establishing a standard response to its vasoconstrictor effect; the antagonists were administered 5 min. after the pressor action of serotonin subsided. Responses to subsequent doses of serotonin, after the various antagonists, were then compared to the initial response to serotonin at the start of each experiment. The per cent decrease at maximal antagonism of the pressor effect of serotonin was calculated and used to compare the relative antagonistic capability of the compounds employed. The average onset of maximal antagonism, as well as the time interval (duration of action in Table I) before subsequent doses of serotonin would produce pressor responses that approximated half that of the initial standard response, were also used as bases of comparison. The doses of serotonin, administered after the antagonist, were injected at approximately 8–10-min. intervals and were based on those doses used in previous investigations in this laboratory (12,

19), either 25 or 50 mcg. In the later perfusions the dose of 50 mcg. was adhered to because this appeared to give more consistent responses.

One objective of the investigation was to observe the relative antagonism of the compounds employed utilizing the smallest dose which exhibited serotonin antagonism. This dose, for the antagonists, was not always the same in every placenta. Also, because the antagonists varied widely in their inherent ability to antagonize the pressor action of serotonin, they could not be compared on an equidosage basis and still show antiserotonin activity.

The following known serotonin antagonists were injected into the arterial side of the perfusion, in a volume of distilled water not exceeding 2.0 ml., except where noted: 5-hydroxytryptamine creatinine sulfate (serotonin), 0.01%; chlorpromazine hydrochloride,[1] 0.025%; promethazine hydrochloride,[2] 0.05%; promazine hydrochloride,[3] 0.05%; cyproheptadine hydrochloride,[4] 0.01%; D-lysergic acid diethylamide-25[5] (LSD-25), 0.01%; diphenhydramine hydrochloride,[6] 1.0%; dibenamine hydrochloride,[7] 0.4% in 95% U.S.P. alcohol. In addition, 95% U.S.P. alcohol was injected alone to observe the effect of this vehicle on the placental vessels.

RESULTS

The following results, which are summarized in Table I, were obtained on the vasculature of full-term human placentas perfused at pressures between 60 and 108 mm. Hg. This range of perfusion pressures corresponds to inflow rates of 58 to 72 ml. of perfusion fluid [Tyrode's solution modified by the addition of 0.525% polyvinylpyrrolidone (Plasdone C)] per min.

Chlorpromazine.—In doses of 0.5–1.0 mg., chlorpromazine caused a maximum decrease of 52.0 to 85.0% (average 67.8) compared to the standard pressor effect of an initial 25.0-mcg. dose of serotonin. In the 3 experiments performed, the maximum antagonism of serotonin occurred an average of 20 min. (range 10–28) after the administration of chlorpromazine. Approximately 21–79 min. elapsed before further doses of serotonin gave an increase in pressure approximately equal to half the pressure increase produced by the initial dose of serotonin (28.0–119.2 mm. Hg) (Fig. 1). In 2 additional preparations, subsequent doses of serotonin failed to produce pressure increases that even approximated half that caused by initial doses of serotonin.

Promazine.—In a total of 5 experiments, 0.5–1.0 mg. of promazine produced an average maximum antagonism of serotonin of 38.2–72.2% (average 47.4). The antagonistic effect of promazine became maximal at an average of 14 min. (range 9–19) after administration and lasted 9–46 min. before a standard dose of serotonin (25.0–50.0 mcg.) caused an increase in pressure in the placental vessels that was about half that caused by the same dose of serotonin initially (11.0–123.6 mm. Hg) (Fig. 2).

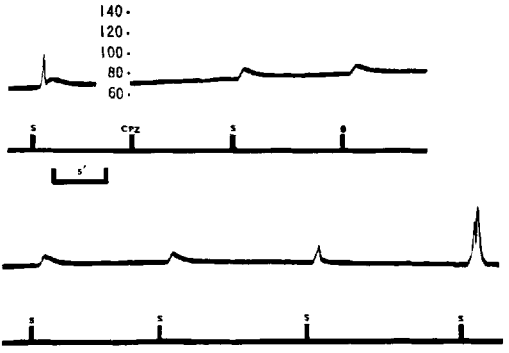

Fig. 1.—Antagonism of serotonin by chlorpromazine (top and bottom). Key: S, serotonin (25 mcg.); CPZ, chlorpromazine (1.0 mg.).

Promethazine.—Promethazine, in doses of 0.5–2.0 mg., produced an average maximum decrease to the pressor effect of 25.0–50.0 mcg. of serotonin of 52.7% (range 16.7–80.1). The maximum antagonism became evident at an average of 16.2 min. (range 11–30) after promethazine was given and lasted from 11–49 min. before the same dose of serotonin gave a response approximately half that of the initial dose (4.8–258.0 mm. Hg). The results presented above were obtained from 6 experiments on 5 separate placental preparations (Fig. 3).

LSD.—Doses of 100.0–200.0 mcg. of LSD reduced the pressor effect of 50.0 mcg. of serotonin by an average of 75.6% (range 28.3–100.0) at maximal antagonism. This effect of the antagonist was observed to reach a maximum at an average of 10.8 min. (range 6–16) after administration and lasted from 11–30 min. before subsequent doses of serotonin produced increases in pressure equal to about half those produced by initial doses (7.8–74.8 mm. Hg). In 4 of the 6 experiments performed, LSD produced an increase in pressure itself equivalent to 1.2–4.4 mm. Hg (Fig. 4).

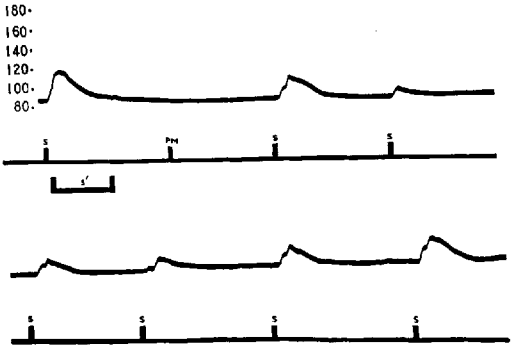

Fig. 2.—Antagonism of serotonin by promazine (top and bottom). Key: S, serotonin (25 mcg.); PM, promazine (0.5 mg.).

[1] Solution used prepared from commercially available ampul. Marketed as Thorazine by Smith Kline & French.
[2] Solution used prepared from commercially available ampul. Marketed as Phenergan by Wyeth.
[3] Solution used prepared from commercially available ampul. Marketed as Sparine by Wyeth.
[4] Supplied through the courtesy of Merck, Sharp and Dohme Laboratories, West Point, Pa. Marketed as Periactin.
[5] Supplied through the courtesy of Sandoz Pharmaceuticals, Hanover, N. J.
[6] Solution prepared from commercially available ampul. Marketed as Benadryl by Parke Davis.
[7] Supplied through the courtesy of Givaudan Corp., Delawanna, N. J.

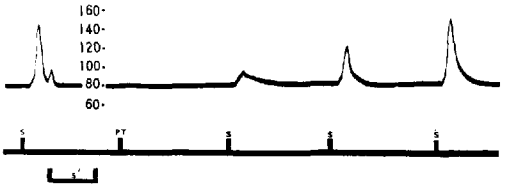

Fig. 3.—Antagonism of serotonin by promethazine. Key: S, serotonin (25 mcg.); PT, promethazine (1.0 mg.).

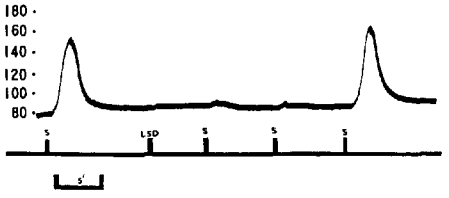

Fig. 4.—Antagonism of serotonin by LSD. Key: S, serotonin (50 mcg.); LSD, lysergic acid diethylamide (200 mcg.).

Fig. 5.—Antagonism of serotonin by cyproheptadine. Key: S, serotonin (50 mcg.); CP, cyproheptadine (100 mcg.).

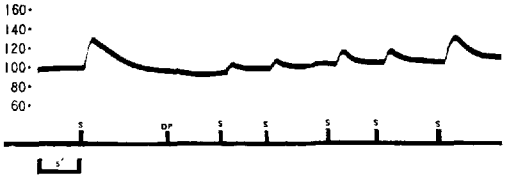

Fig. 6.—Antagonism of serotonin by diphenhydramine. Key: S, serotonin (50 mcg.); DP, diphenhydramine (10 mg.).

Cyproheptadine.—In 3 experiments 100.0 mcg. of cyproheptadine produced an average of 86.2% (range 82.7–92.4) maximum decrease in comparison to the pressor effects of 50 mcg. of serotonin. The maximum serotonin antagonism of cyproheptadine was noted at an average of 11 min. (range 10–12) after administration and lasted 17–28 min. before the administration of another dose of serotonin gave pressure increases equivalent to about half those produced by the control dose (13.6–53.8 mm. Hg) (Fig. 5). In one perfusion, 50.0-mcg. doses of serotonin subsequent to the administration of 100.0 mcg. of cyproheptadine failed to produce an increase in pressure about equal to half that produced by the initial dose of the agonist.

Diphenhydramine.—Diphenhydramine, in a dose of 10.0 mg., produced an average 68.9% decrease (range 63.6–71.4) to the pressor effects of serotonin at maximal antagonism. In the 3 experiments performed, the average maximal antagonism occurred an average 31.7 (range 14–49) min. after administration and lasted 24–63 min. before subsequent doses of serotonin gave responses equivalent to about half the original pressure increase (19.8–46.2 mm. Hg) caused by the initial 50-mcg. dose of serotonin. In 2 of the 3 preparations, diphenhydramine elicited an initial decrease in pressure ranging from 3.1–6.6 mm. Hg (Fig. 6).

Dibenamine.—In a total of 4 experiments, 2.0 mg. of dibenamine caused an average maximum decrease in the response to serotonin of 46.4% (range 14.2–92.8). The average maximum antagonism occurred 26.3 min. (range 7–52) after administration and lasted from 17–52 min. before subsequent doses of serotonin produced about half the pressure increase caused by the control dose of 50 mcg. (7.0–47.0 mm. Hg). In all of the experiments performed, the administration of dibenamine alone caused an increase in pressure ranging from 2.0–5.0 mm. Hg. The administration of a volume of 95% U.S.P. ethyl alcohol (0.5 ml.), equal to that used to dissolve a 2-mg. dose of dibenamine hydrochloride, caused a comparable increase in pressure in 4 separate experiments. In 2 of the 4 experiments utilizing dibenamine, it was noted that the first dose of serotonin subsequent to the antagonist elicited a pressure increase greater than the control response at the beginning of the perfusion. However, all other injections of serotonin resulted in diminished responses.

DISCUSSION

When the antagonists, utilizing their smallest effective dose, were compared on the basis of the per cent that they decreased the pressor response of the placental vessels to serotonin at maximal antagonism, their ability to antagonize the vasoconstrictor effect of serotonin, in decreasing order of effectiveness, was: cyproheptadine, LSD, diphenhydramine, chlorpromazine, promethazine, promazine, and dibenamine. As can be seen from Table I, the onset of maximal antagonism for most of the compounds tested was between 10 and 20 min. after administration, with only diphenhydramine and dibenamine having a more prolonged onset. The range of duration of action, based upon the previously stated criterion, varied greatly, with chlorpromazine being by far the longest acting, as would be expected from the known localization of this compound in body tissues (20). Cyproheptadine, while the most effective antiserotonin agent tested, had the shortest duration of action. Chlorpromazine, in 2 perfusions, and cyproheptadine, in 1, blocked serotonin's vasoconstriction so effectively that subsequent doses never elicited pressor responses that approximated half those produced by the initial standard doses of serotonin during the biological life of the preparation. In all of the perfusions performed the phenomenon of tachyphylaxis to the pressor action of serotonin was never observed. In most instances the effect of the antagonist reached a maximum after 10–31 min.; subsequently the pressor response

to successive doses of serotonin began to approximate half that of the control response.

In a preliminary investigation, leading to the present study, no dose-response relationship to serotonin could be demonstrated in the perfused placental preparation. Consequently, it was not possible to classify the compounds employed as specific or nonspecific antagonists.

Because previous investigations (12, 21) have alluded to the concept that placental vessels contain both α- and β-adrenergic receptors, according to Ahlquist's classification (22), and because it has been postulated that the α receptors are stimulated by serotonin (12), the vasoconstrictor effect of this agonist, as well as the actions of the various antagonists, can be interpreted in the light of these observations, as well as by the well-known stimulant effect of serotonin on most smooth muscle. The phenothiazines used in this investigation, chlorpromazine, promazine, and promethazine, possibly owe their antiserotonin action to a blockade of the α-adrenergic receptors in the placental vessels, since they have been shown to possess adrenergic blocking properties (23). Dibenamine and possibly LSD share this mechanism with the phenothiazines, and a similar interpretation can be applied to the understanding of their antiserotonin action in this preparation. LSD, however, has been shown to have a structural similarity to serotonin, notably the indole configuration, and thus it may compete with serotonin for receptor sites in the smooth muscle (24). In addition, LSD has a stimulant effect on smooth muscle similar to that of serotonin (24) which again may be attributed to its structural similarity to serotonin. This was probably the case in this investigation, as LSD produced pressure increases equivalent to 1.2–4.4 mm. Hg in most of the experiments performed. Cyproheptadine (25) and diphenhydramine (26) both have a negative musculotropic action in other systems, and it is plausible to credit their antiserotonin action in the placental vessels to a similar mechanism. Diphenhydramine and cyproheptadine were both shown to be strong serotonin antagonists in this investigation, being more potent than dibenamine and the phenothiazines which acted predominantly on receptor sites. In fact, diphenhydramine, in 2 of the 3 perfusions, caused a decrease in pressure immediately after it was injected which varied between 3.1 and 6.6 mm. Hg.

In 2 of the 4 perfusions performed using dibenamine, doses of serotonin, subsequent to this antagonist, gave pressor responses greater than the original control response, indicating that the dibenamine was potentiating the vasoconstrictor action of serotonin. However, dibenamine has been shown to possess serotonin-like actions in other preparations, such as its antidiuretic effect in the rat (13); therefore, a similar effect in the placental vessels cannot be disregarded at this time. The administration of dibenamine in a vehicle of 95% ethyl alcohol always caused an initial increase in pressure, ranging from 2.0 to 5.0 mm. Hg; this is due to the vasoconstrictor effect of the alcoholic vehicle since in this, and previous investigations (27), alcohol has been shown to cause vasoconstriction of the placental vessels.

By following the procedures outlined in this investigation, we have evaluated the relative effectiveness of several agents that are antagonistic to serotonin. Consequently, we can hypothesize that those agents which were found to be the most effective serotonin antagonists in this organ may have some therapeutic efficacy in the diseases in which abnormal amounts of serotonin play a significant role in their pathologic manifestations as well as their etiology. The primary example of such a disease is toxemia of pregnancy since the clinical manifestations of this malady are thought to be the result of the vasoconstrictor effect of serotonin on the blood vessels of the placenta (14, 15).

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Azulene Analogs of Pharmacologic Agents

By TULLY J. SPEAKER* and GLENDEN D. REDMAN

The synthesis of a series of azulene-1-carboxylic acid esters is described. These esters are to be screened for pharmacologic activity.

AZULENE, with a resonance energy of about 46 Kcal./mole can be classified as aromatic with respect to resonance stability (1, 2) and is the only known bicyclic nonbenzenoid hydrocarbon of this type. Azulene has been unequivocally shown to undergo electrophilic substitution at the 1-position (3).

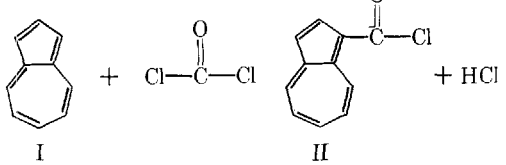
Until the development of an efficient method for the preparation of azulene in 1956 by Ziegler (4), utilization of this nonbenzenoid nucleus was severely restricted, at least in part by economic factors.

Investigation of the pharmacodynamic activity of azulene and azulene derivatives, principally hydrocarbons, has to date been restricted in the main to examination of the antiphlogistic (5, 6), antiallergic (7), and bacteriostatic (8) properties of these compounds or mixtures of them obtained from chamomile and similar oils.

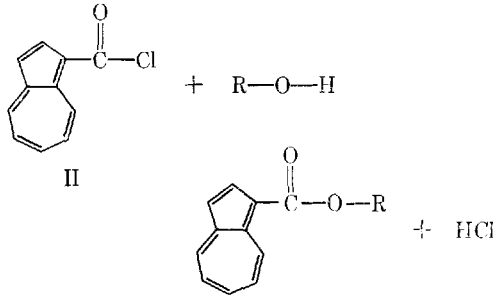
It seemed of importance, therefore, to undertake a systematic study of the effects of replacing benzenoid nuclei in structures known to exhibit pharmacologic activity with the nonbenzenoid aromatic nucleus of azulene.

Because the aromatic portions of local anesthetics are essential to a high level of local anesthetic activity, it seemed reasonable to initiate this study by preparing several azulene compounds which might be expected to be local anesthetics were their aromatic portions benzenoid rather than nonbenzenoid.

The room temperature reaction of azulene (I) with phosgene in the absence of Friedel-Crafts catalysts described by Triebs *et al.* conveniently provided the acid chloride of azulene-1-carboxylic acid (II), and this acid chloride on reaction with appropiate alcohols afforded the corresponding esters (III) in yields averaging about 90% of theoretical, based on azulene.



I II

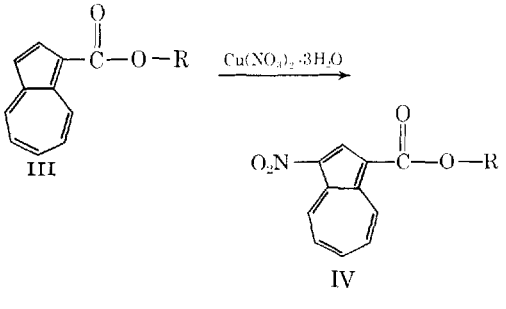


II III

The high π-electron density of the 1- and 3-positions of the azulene nucleus calculated by the linear combination of atomic orbitals-molecular orbital method of Hückel (10) suggests that a 3-substituent corresponds closely in electronic behavior to a *para* substituent in a phenyl nucleus.

Accordingly, it was of interest to prepare several 3-substituted esters for this study.

Treating an azulene-1-carboxylic acid ester with cupric nitrate in a mixture of acetic acid and acetic anhydride after the method of Anderson (11) provided a route to the corresponding 3-nitro ester (IV) and reduction of the 3-nitro



III IV

esters yielded the relatively unstable 3-amino esters (V).

Attempts to chlorocarboxylate 1-nitroazulene were unfruitful.

Some esters substituted in the alcohol-derived portion are poorly stable under even these mild nitration conditions, but the relative stability of the Ω-haloalkanol esters to these nitration conditions offered a method for introduction of more labile groups into the alcohol-derived portion of the 3-substituted esters.

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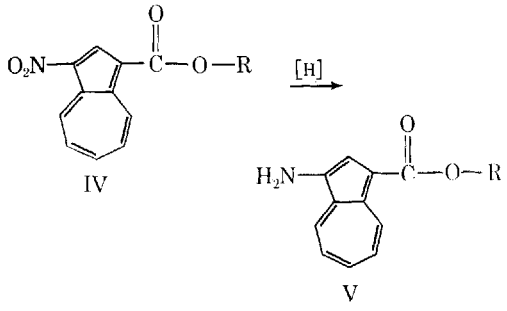


EXPERIMENTAL

Azulene for this study was at first prepared in this laboratory after the method of Ziegler (4) and was later obtained from Terra Chemicals, Inc.

Alcohols available from Distillation Products Industries, Division of Eastman Kodak Co., were obtained from that source. The others were prepared in this laboratory.

All melting points were determined using a Fisher-Johns melting point apparatus and are corrected.

All spectral data were obtained using a Beckman DB spectrophotometer and Distillation Products Industries spectro grade methanol as solvent.

Gases were obtained from the Matheson Co., Inc.

Chromatographic alumina was Fisher Scientific Co. No. A540.

Esters of Azulene-1-carboxylic Acid.—Each of the esters of unsubstituted azulene-1-carboxylic acid was prepared by the following procedure adapted from Triebs *et al.* (9). A solution of 1.282 Gm. (0.01 mole) of azulene in 50 ml. of dry toluene was saturated with phosgene while cooled in a bath of running tap water and allowed to stand 1 hr. (Failure to cool the solution increased the amount of tarry material formed and significantly lowered yields of ester.) During this time the solution changed from a dark blue color to a dark red-violet color. The excess phosgene was removed by gentle refluxing under a slight vacuum for about 30 min.

A solution of 0.010 mole of the appropriate alcohol in dry toluene was added dropwise to this solution of azulene-1-carboxylic acid chloride. The reaction mixture was allowed to stand 30 min., treated with 20 ml. of dilute ammonium hydroxide, and transferred to a separator. The reaction vessel was rinsed with an additional 10 ml. of toluene and the rinsings were added to the funnel. The reaction mixture was successively extracted with 10-ml. portions of ammonium hydroxide until the aqueous portion showed no blue color. The wet toluene was allowed to evaporate spontaneously in a stream of cool air. (Early experiments suggested that yields fall drastically if the esters are heated at steam bath temperatures.)

The residue was triturated with approximately 5 Gm. of alumina and packed above an alumina chromatography column (1.8 × 15 cm.), and the chromatogram was developed first with ligroin to remove any unreacted azulene and then with toluene. The red-violet eluates were collected, and solvent was allowed to evaporate spontaneously as before. Most of these esters were oils.

Alkanolamine esters were converted to their corresponding hydrochlorides in the usual manner. The amine hydrochlorides were hygroscopic.

3-Nitroazulene-1-carboxylic Acid Esters.—Each of the 3-nitroazulene-1-carboxylic acid esters was prepared by the following procedure adapted from the work of Anderson *et al.* (11). Increasing the quantity of reagents by a factor of 10 introduced no new difficulties.

A chilled slurry of 0.241 Gm. (0.001 mole) of finely powdered cupric nitrate, $Cu(NO_3)_2 \cdot 3H_2O$, in 3 ml. of acetic anhydride was added to a mechanically stirred solution of 0.001 mole of an azulene-1-carboxylic acid ester in a mixture of 5 ml. of acetic acid and 1 ml. of acetic anhydride maintained at temperatures below 5°. The transfer of the slurry was facilitated by the use of an additional 2 ml. of chilled acetic anhydride.

The mixture was stirred for 10 min. and poured into 50 ml. of ice water and extracted with three 10-ml. portions of chloroform. The combined chloroform extracts were washed 3 times with 10-ml. portions of water, evaporated to dryness in a stream of cool air, and the residue was triturated with about 5 Gm. of alumina.

This triturate was packed above an alumina chromatography column (1.8 × 15 cm.). Elution with toluene removed the unreacted ester as a red-violet band and slowly displaced an orange band from a closely following dark brown region. The column was extruded and the orange region packed above a fresh alumina column. The new column was eluted with 20% ether in toluene and evaporation of the orange eluate in a stream of cool air gave orange to red needles of the 3-nitro esters.

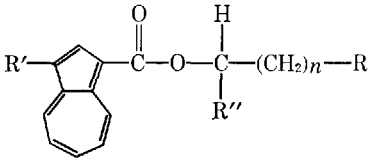
3-Aminoazulene-1-carboxylic Acid Esters.—Each of the 3-aminoazulene-1-carboxylic acid esters was prepared by the following procedure adapted from that of Anderson *et al.* (3). Over a 5-min. period 0.5 Gm. of zinc dust was added to a stirred solution of 0.001 mole of the appropriate 3-nitroazulene-1-carboxylic acid ester and 0.2 Gm. of sodium acetate in 10 ml. of acetic acid. The mixture was stirred 1 hr. and diluted with 30 ml. of distilled water. The resulting blue mixtures were decanted from any unreacted zinc and extracted with three 20-ml. portions of ether. The emerald-green ether extracts were combined and washed with aqueous sodium hydroxide (5% w/v) until free of acid. The ether solution was extracted with dilute hydrochloric acid until the aqueous extracts were colorless.

The combined acid extracts were neutralized with ammonium hydroxide and extracted with two 20-ml. portions of ether. The ether was allowed to evaporate spontaneously, and the residue was triturated with about 5 Gm. of alumina. The triturate was packed above an alumina chromatography column and eluted with 20% ether in toluene. It gave only a single red band. Spontaneous evaporation of the eluate in a stream of cool air gave the free 3-aminoazulene-1-carboxylic acid esters. The amines were converted to their hydrochlorides in the usual manner.

Alkanolamine Ester of 3-Nitroazulene-1-carboxylic Acid.—The Ω-halo ester of 3-nitroazulene-1-carboxylic acid was converted to the corresponding *N,N*-dialkylaminoalkanol ester of 3-nitroazulene-1-carboxylic acid by the following procedure.

A solution of 0.073 Gm. (0.001 mole) of diethylamine in 10 ml. of ether was added dropwise to a

TABLE I.—ESTERS OF AZULENE-1-CARBOXYLIC ACIDS



R	R′	R″	*n*	Yield, %	M. p., °C.	Formula	Anal. Calcd.	Anal. Found	λ_{max}	Log ε
H	H	H	1	93	Oil	$C_{13}H_{12}O_2$	C, 77.97 H, 6.04	77.82 6.40	232	4.70
Cl	H	H	1	86	60–61	$C_{13}H_{11}ClO_2$	C, 64.07 H, 4.55	64.21 4.37	310	4.63
Dimethylamino	H	H	1	92	105 dec.	$C_{15}H_{17}NO_2$. HCl	N, 5.00	4.89	304	4.74
Diethylamino	H	H	1	93	127	$C_{17}H_{21}NO_2$. HCl	N, 4.55	4.61	228	3.33
2-Methylpiperidino	H	H	2	87	122	$C_{20}H_{25}NO_2$. HCl	N, 4.02	3.98	240	3.11
Cyclohexylamino	H	CH_3	1	88	108	$C_{20}H_{25}NO_2$. HCl	N, 4.02	4.09	315	4.50
H	NO_2	H	1	64	150–152	$C_{13}H_{11}NO_4$	N, 5.71	5.66	292	4.61
Cl	NO_2	H	1	52	108–110	$C_{13}H_{10}ClNO_4$	N, 5.00	4.94	292	3.24
Diethylamino	NO_2	H	1	50	183–184	$C_{17}H_{20}N_2O_4$. 2HCl	N, 7.19	7.10	243	4.51
H	NH_2	H	1	83	190–191	$C_{13}H_{13}NO_2$	N, 5.56	5.55	219	4.60
Diethylamino	NH_2	H	1	78	163–165	$C_{17}H_{22}N_2O_2$. 2HCl	N, 7.79	7.74	240	4.62

mechanically stirred solution of 0.279 Gm. (0.001 mole) of the 2-chloroethanol ester of 3-nitroazulene-1-carboxylic acid in 20 ml. of ether, and the mixture was heated at reflux temperature for 1 hr. The ether was decanted from the resulting gummy blue solid, and the residue was dissolved in 20 ml. of distilled water. The solution was transferred to a separator, and the solution was made alkaline with ammonium hydroxide. The solution was extracted with 20-ml. portions of ether, and the combined ether extracts were washed with a little water. The ether extract was allowed to evaporate spontaneously, and the residue was triturated with about 3 Gm. of alumina. The triturate was packed above an alumina chromatography column and eluted with 20% ether in toluene. Only a single red-orange band appeared. It was eluted and spontaneous evaporation of the solvent in a stream of cool air gave the *N,N*-diethylamino derivative, which was converted to the corresponding hydrochloride in the usual manner.

The results are reported in Table I together with some of the physical properties of these new compounds.

Upon preliminary pharmacologic testing some of these compounds were found to exhibit local anesthetic activity.

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Increasing Dissolution Rates and Gastrointestinal Absorption of Drugs *Via* Solid Solutions and Eutectic Mixtures II

Experimental Evaluation of a Eutectic Mixture: Urea–Acetaminophen System

By ARTHUR H. GOLDBERG*, MILO GIBALDI, and JOSEPH L. KANIG

The binary system of APAP and urea was found to be a simple eutectic mixture with negligible formation of solid solutions. Solubility studies indicated that urea increased significantly the solubility of APAP. Dissolution rate studies were conducted with APAP alone, and in fused and physical mixtures with urea. An unusual dissolution phenomenon was found to exist in the system and is considered. In all cases the presence of urea enhanced the dissolution rate of APAP by virtue of a microenvironmental solubilization. The fused mixture at the eutectic composition was found to give similar, but prolonged enhancement of dissolution rate as compared to the physically mixed sample.

It is well established that dissolution is frequently the rate-limiting step in the gastrointestinal absorption of a drug from a solid dosage form. The relationship between solution rate and absorption is particularly distinct when considering drugs of low solubility. Consequently, numerous attempts have been made to modify the dissolution characteristics of certain drugs in an effort to attain more rapid and more complete absorption.

Bruner and Tolloczko (1) were the earliest workers to demonstrate that dissolution rate was a function of the surface area exposed to the dissolution medium. Accordingly, a drug will dissolve more rapidly when its specific surface area is increased, *i.e.*, when its particle size is decreased.

Levy (2) has considered a number of methods by which a drug may be presented to the gastrointestinal fluids in finely divided form. The most direct method is the utilization of microcrystalline or micronized particles which may be administered in any one of a number of dosage forms. A second method involves the administration of solutions from which, upon dilution with gastric fluids, the dissolved drug will precipitate in the form of very fine particles. A more unique way of obtaining microcrystalline dispersions of a drug in gastrointestinal fluids has been recently suggested by Sekiguchi *et al.* (3, 4). This approach involves the administration of a eutectic mixture composed of the drug and a substance which dissolves readily in water.

Sekiguchi and co-workers (3, 4) have proposed that when the eutectic mixture is exposed to the gastrointestinal fluids, the soluble carrier dissolves rapidly, leaving the insoluble drug in an extremely fine state of subdivision. More recently Goldberg *et al.* (5) presented theoretical arguments which attempted to demonstrate that the results obtained by Sekiguchi (3, 4) were due to the formation of solid solutions rather than simple eutectic mixtures. The existence of solid solutions in the eutectic mixture precluded a direct evaluation of the role of particle size reduction in the enhancement of dissolution. Owing to the physical-chemical characteristics of a solid solution, a very rapid rate of dissolution is to be expected when an insoluble drug is dissolved in a soluble carrier (5).

The purpose of this investigation was to compare the dissolution rate of a *simple eutectic mixture*, composed of the drug and a readily dissolving substance, with the dissolution rate of the drug alone. A study of a eutectic mixture, uncomplicated by the presence of solid solutions, permits a more realistic evaluation of the utility of the eutectic system in enhancing dissolution.

EXPERIMENTAL

A study was initiated to find a poorly soluble drug that would form a simple eutectic mixture with a water soluble carrier. The carriers investigated met the following criteria: (*a*) soluble in water; (*b*) physiologically inert; (*c*) a melting point of not more than 200°; (*d*) thermal stability up to its melting point; (*e*) a relatively low vapor pressure.

A number of drugs were employed that met the following criteria: (*a*) poor water solubility; (*b*) therapeutically significant; (*c*) thermal stability up to its melting point; (*d*) relatively low vapor pressure; (*e*) melting point of not more than 250°.

Screening Procedure.—In order to select combinations which would lend themselves to more detailed investigation, an initial screening procedure

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* Research fellow under a grant from the Bristol-Myers Co., New York, N. Y. Present address: University of Michigan, Ann Arbor.

was developed to permit rapid evaluation. Equal quantities (3 Gm.) of carrier and drug were physically mixed and placed, along with a thermometer, into a test tube. The tube was immersed into a temperature controlled silicone[1] bath, preset to the temperature of the melting point of the lower melting component. The mixture was constantly stirred to facilitate distribution of heat and the temperature of the silicone bath was slowly raised, if necessary, until complete melting occurred. The following observations were made during each such screening procedure: (*a*) temperature at which mixture started to melt; (*b*) temperature at which discoloration, gas evolution, or fumes occurred (if such phenomena did occur); (*c*) temperature at which complete melting was effected; and (*d*) the number of distinct phases present in the melted mixture.

The fused liquid was then immediately poured onto Ferrite plates[2] and the following evaluations made: (*a*) length of time for crystallization to occur; (*b*) physical state of mass (crystalline or amorphous); and (*c*) length of time required for the amorphous mixtures to change to a crystalline form.

Solid-Solid Interaction.—On the basis of results obtained in the screening procedure, the combination of APAP[3] and urea was selected for further study. This mixture formed a homogeneous liquid when fused, exhibited a melting point lower than that of the lower melting component, and recrystallized to a crystalline solid immediately upon quenching in air under ambient conditions.

A phase diagram of APAP–urea was made using the cooling curve method, employing blends of APAP and urea in the following mole fractions: 0.9–0.1; 0.8–0.2; 0.7–0.3; 0.6–0.4; 0.5–0.5; 0.4–0.6, and 0.2–0.8. The blended mixtures were placed into the inner tube of a Beckman molecular weight apparatus with the stirring loop, thermometer, and stopper in place. The tube was then immersed into a temperature controlled silicone bath. The temperature was slowly raised to effect fusion, and to allow the temperature of the melt to rise to about 10° above its melting point. The entire inner tube was then placed into the outer jacket of the Beckman apparatus which was immersed in a water bath maintained at 60°. The top of the stirring loop was attached to the arm of a U.S.P. tablet disintegration apparatus, which raised and lowered the stirring loop at the rate of 30 times per minute within the fused drug-carrier combination.

During the cooling process, temperature was plotted *versus* time, and two discontinuities in the curve were noted. The first change of slope (at the higher temperature) is comparable to the melting point of that component in the mixture which is in excess of the eutectic composition, and the second change in slope (at the lower temperature) corresponds to the eutectic temperature. The critical temperatures thus obtained were plotted *versus* composition on a second graph.

A second phase diagram of urea–APAP was prepared using a microthermal technique.[4] Various ratios of the materials were weighed, intimately mixed, and placed on a microscope slide. The slide was covered with a cover slip, and sealed with silicone grease,[5] to minimize any possible loss due to sublimation. This preparation was heated until fusion occurred, and then allowed to cool. The cooled crystalline mass was then reheated at the rate of 4°/min. by means of a heating stage attached to a microscope fitted with polarizing lenses. Using 100 magnification power, observations were made of the melt.

The first change noted occurs at the eutectic temperature. At this point the solid appears to disappear partially, and small liquid areas become apparent. These areas of liquid continue to increase in size until all of one component dissolves in the eutectic melt. The crystals remaining constitute that component which is present in excess of the eutectic composition. As the temperature increases, the component in excess slowly disappears from view. The temperature at which the last bit of crystal disappears is taken as the melting point of the component in excess. A phase diagram was constructed by plotting both temperatures (where melting first appears, and the final melting point) *versus* ratio of components (per cent w/w).

Interaction in Aqueous Solution.—To determine any possible interaction between drug and carrier in aqueous solution, solubility studies were performed. An excess amount of drug was placed in 60-ml. test tubes equipped with screw caps and containing 30 ml. of an aqueous solution of the carrier in varying concentrations. The content of each tube was equilibrated at 37° in a Gyrotory incubator shaker.[6]

At the end of this period, 1-ml. samples were withdrawn by means of a filter pipet. Each milliliter was diluted to 100 ml. with water, and 0.5 ml. of this dilution was added to 0.5 ml. of distilled water, and diluted to 10 ml. with acidified methanol (1% v/v solution of 0.1 *N* HCl in methanol). The absorbance of each diluted sample was determined at 242 mμ using a recording spectrophotometer.[7] A blank of 10% water in acidified methanol was employed. The absorbance was read and the amount of APAP present was determined by dividing the absorbance by the slope (obtained by the method of least squares) of a previously constructed Beer's law curve. Preliminary investigation had indicated that urea in no way interfered with the assay procedure.

Dissolution Studies.—The dissolution studies were conducted using the tape method (6). This method employs a strip of plastic adhesive,[8] tautly affixed to a metal frame. The powdered sample to be evaluated is dusted on the adhesive surface in a monoparticulate layer. The entire frame is then immersed below the surface of the dissolution medium which is stirred by a paddle attached to an overhead, constant speed motor rotating at 53.5 r.p.m.

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[1] 550 Fluid, Dow Corning Corp., Midland, Mich.

[2] 10 × 14 in., chrome plated, Apollo Co., Inc., New York, N. Y.

[3] A common synonym for acetaminophen or *N*-acetyl-*p*-aminophenol.

[4] The authors gratefully acknowledge the expert assistance of Miss Marie Jones, microscopist, Bristol-Myers Division, Hillside, N. J., in this phase of the work.

[5] Hi-vacuum grease, Dow Corning Corp., Midland, Mich.

[6] Model G-25, New Brunswick Scientific Co., New Brunswick, N. J.

[7] Model DB, Beckman Instrument Co., Mountainside, N. J.

[8] Scotch Brand Magic Mending Tape, 3/4 in. wide, Minneapolis Minnesota Mining Co., Minneapolis, Minn.

TABLE I.—APAP SAMPLES EMPLOYED IN DISSOLUTION STUDIES

Sample	Compn., mg. APAP	Urea	Mesh Size	Method of Prepn.
1	30	...	50–60	Fusion
2	30	...	100–120	Fusion
3	30	23.8	50–60	Fusion
4	30	23.8	50–60	Physical mixture
5	30	35.7	50–60	Physical mixture

Table I lists the various APAP samples used in the dissolution studies. Samples 1–3 were prepared by fusing APAP or a blend of APAP and urea. When complete liquefaction occurred, the homogeneous melt was immediately cast on Ferrite plates under ambient conditions. The congealed solid was then crushed using a mortar and pestle. The resulting powder was subsequently classified by means of a Syntron shaker operated at 90 v. for 2 min. The desired fraction was then collected. Samples 4 and 5 were prepared by first fusing, congealing, and sizing the individual components and then blending the two materials in the indicated proportions.

The dissolution fluid consisted of 400 ml. of distilled water maintained at 37° in a 600-ml. beaker which was immersed in a constant-temperature water bath. The particles to be tested were weighed and dusted onto the tape. The frame was then positioned in the dissolution apparatus and, at time zero, dropped below the surface of the dissolution fluid. One-milliliter samples were withdrawn at 1-min. intervals for 5 min. Each sample was diluted to 10 ml. with acidified methanol and assayed spectrophotometrically as previously outlined.

RESULTS AND DISCUSSION

Solid-Solid Interactions.—The phase diagrams obtained by the cooling curve method and the microthermal technique closely coincided. An analysis of the phase diagram presented in Fig. 1 (which was prepared by the microthermal technique) reveals the existence of a binary system which closely approximates a theoretical eutectic mixture. No solid solubility was detected. Figure 1 shows the eutectic composition to be 52% APAP and 48% urea, and the eutectic temperature to be 115°.

The phase diagram prepared by the cooling curve method indicated a eutectic composition of 55.7% APAP and 44.3% urea. The eutectic temperature was found to be somewhat lower (110°) than the temperature observed when employing the microthermal technique. This finding is probably the result of supercooling. The small discrepancy in eutectic composition between each method of phase diagram preparation is attributable to the sublimation of urea from the open system employed in the cooling curve technique.

Interaction in Aqueous Solution.—Equilibrium solubility experiments were conducted to determine the extent of interaction between drug and carrier in solution. As may be noted in Fig. 2, urea was found to interact strongly with APAP in aqueous solution. This interaction was manifested by a linear increase in the solubility of APAP to the

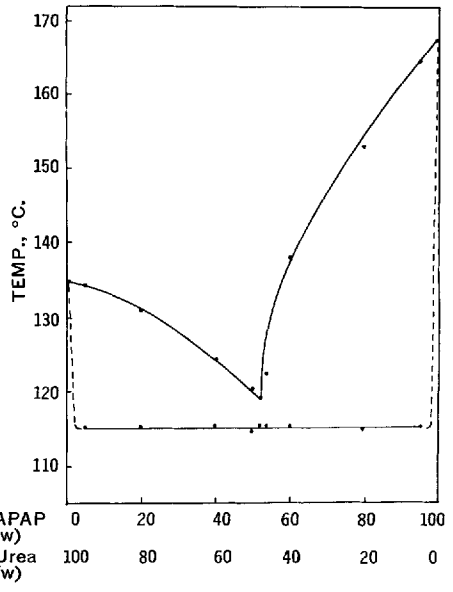

Fig. 1.—Phase diagram for urea–APAP system prepared by a microthermal technique.

extent of 0.23 Gm./Gm. of urea. The significance of the solubilization effect will be considered in the discussion of dissolution rates which follows.

Dissolution Studies.—According to the Noyes-Whitney equation, the dissolution rate of a solid is a function of the surface area presented to the dissolution medium as well as the concentration gradient existing between the solid–liquid interface and the bulk of the dissolution medium (7). Under conditions of constant surface area, as is observed in the dissolution of a material from a planar surface, the dissolution rate should follow zero-order kinetics

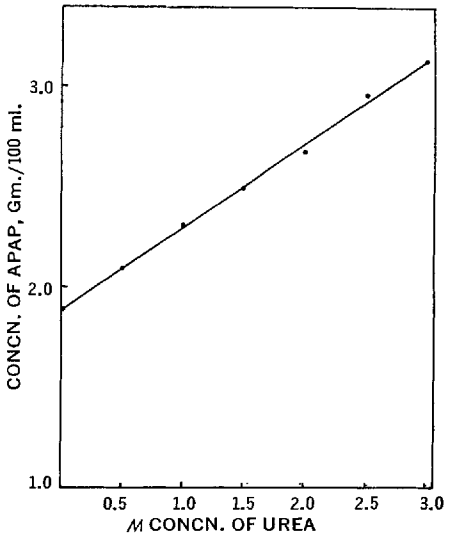

Fig. 2.—Aqueous solubility of APAP as a function of urea concentration.

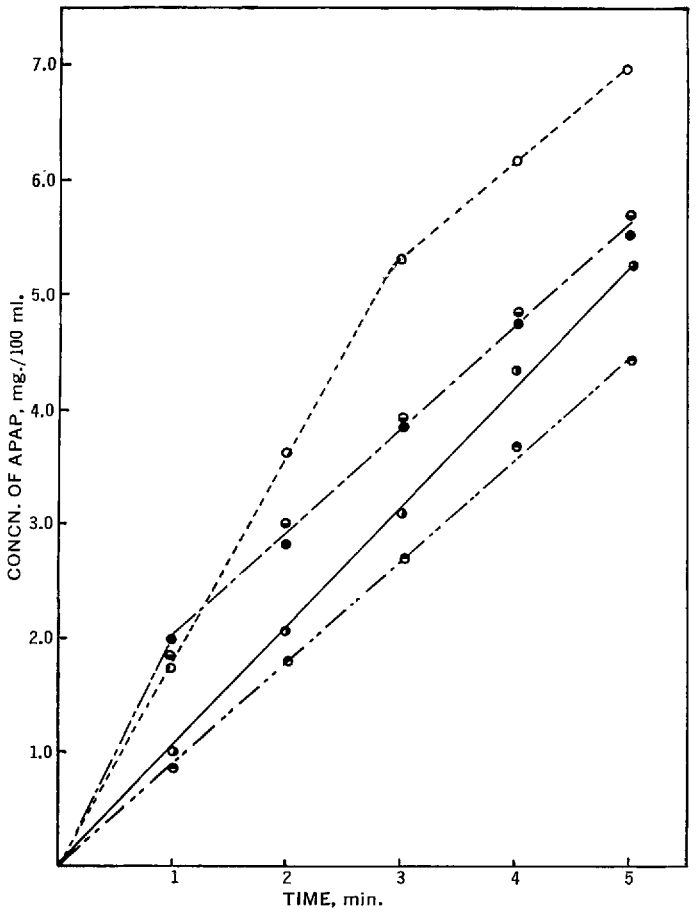

Fig. 3.—Dissolution rates of APAP and APAP–urea as determined by the tape method. Key: ◓, APAP, 50 60 mesh; ◑, APAP, 100–120 mesh; ●, APAP–urea, physical mixture at eutectic composition; ◒, APAP–urea, physical mixture, urea present in excess of eutectic composition; O, APAP–urea, fused eutectic.

provided the concentration gradient is maintained essentially constant by keeping the dissolution medium sufficiently dilute. On the other hand, in those situations where only the concentration gradient may be maintained constant, *e.g.*, in particulate dissolution, the dissolution rate is proportional to the surface area. Accordingly, the dissolution of particulate solids has been found to adhere to the "cube root law" which represents a mathematical model to account for the decrease in surface area during the dissolution process (8).

The results of the dissolution studies in this investigation appeared to differ considerably from theoretical expectations. Previous studies (6), utilizing the tape method, showed reasonable agreement with the "cube root law" indicative of particulate dissolution. Dissolution of the APAP samples, however, seemed to agree with a planar surface dissolution model. As may be noted in Fig. 3, dissolution curves were of two distinct types: either linear for the entire experimental period or biphasic, consisting of two linear segments. Both samples of pure APAP followed pseudo-zero kinetics over the 5-min. period. When the data obtained with the pure APAP were tested for fit to the "cube root law," it was found that the coarser sample (50–60 mesh) showed reasonably good agreement while the 100–120 mesh sample demonstrated significant curvature and deviation. Based on these observations, it was concluded that particulate density, *i.e.*, the number of particles per unit area of tape, exerted an influence on the dissolution process.

It has been theorized that the dissolution process involves the formation of a thin layer or film of saturated solution at the solid-liquid interface and the diffusion of molecules from this layer to the bulk solution (1). Applying this concept to the dissolution of particulates from the tape, it is apparent that as long as the particles are farther apart than the thickness of the diffusion layer, the "cube root law" would be obeyed. However, if the particulate density is increased so that the diffusion layer of each particle "overlaps" with that of its neighboring particle, it is reasonable to expect dissolution to approach a planar surface model. The latter situation was observed with the 100–120 mesh APAP.

When particles of pure APAP were physically mixed with particles of urea, a biphasic dissolution curve was obtained (Fig. 3). Theoretically, in a system measuring only particulate dissolution the urea would not influence the dissolution rate of the APAP since each particle would dissolve independently. However, the initial dissolution rate of APAP (from time zero to 1 min.) from both physically mixed samples was significantly greater than the dissolution rate of APAP alone. These findings further support the possibility that the diffusion layers of the urea and APAP particles

"overlap." When the sample is first introduced into the dissolution medium, the urea rapidly goes into solution and quickly attains a very high microenvironmental concentration in the "mixed" diffusion layer of both particles. The presence of urea increases the solubility of APAP in the diffusion layer and thereby increases the dissolution rate of the drug. When the eutectic mixture was studied, a biphasic dissolution curve again resulted, demonstrating an increased initial dissolution rate of APAP.

These findings coupled with the results obtained with the pure APAP samples indicate that the dissolution process, under these experimental conditions, adheres to a mixed mathematical model, *i.e.*, intermediate to a planar surface dissolution model and a particulate dissolution model. This rather unusual situation does not, however, negate the value of the system for comparing the relative dissolution rate of APAP from the various samples. In each case, with the exception of the pure APAP (100–120 mesh), the quantity of the drug, the particle size of the sample, and the approximate monolayer density were maintained constant. Moreover, the mechanism of dissolution remained the same regardless of the sample tested, as manifested by the pseudo zero-order kinetics observed in part or *in toto* in each dissolution run. It should nevertheless be noted that the use of the tape method for determining true particulate dissolution is dependent on maintaining a small monolayer area in relation to the area of tape on which the particles are placed.

The results of the dissolution studies permitted the calculation of pseudo zero-order rate constants. (Table II.) The pure APAP sample (50–60 mesh) yielded a rate constant of 0.89 mg./min. while the 100–120 mesh sample of pure drug showed a rate constant of 1.07 mg./min., showing an increase of approximately 20%. The physically mixed sample of APAP and urea, corresponding to the eutectic composition, demonstrated an initial rate constant (0–1 min.) of 1.99 mg./min., about twice that of pure APAP of comparable particle size. The second phase of this dissolution curve showed a rate constant of 0.90 mg./min. This second rate constant is the same as that of pure APAP of equal particle size (0.89 mg./min.). This suggests that urea has left the microenvironment at about 1 min. Verification of this conclusion was provided by microscopic examination of the sample; after 1 min., the tape is depleted of urea and only APAP particles remain.

When the amount of urea in the physically mixed sample is increased beyond the eutectic composition an initial rate constant (0–1 min.) of 1.85 mg./min. is found, comparing favorably to the initial rate constant found with the other physically mixed sample. The second phase of the dissolution (1–5 min.) had a rate constant of 0.83 mg./min., again quite close to that of pure APAP of equal mesh size. The urea concentration in the mixed diffusion layer is apparently close to the saturation level since initial dissolution rates were essentially independent of the quantity of urea in the sample. In addition, once the urea is completely depleted from the diffusion layer, APAP particles of equal size remain on the tape and dissolution is identical to the dissolution of pure APAP.

The fused eutectic mixture shows an initial rate constant (0–3 min.) of 1.72 mg./min., reasonably close to the initial rate constants found with the physically mixed samples. The second phase of the dissolution curve yielded a rate constant of 0.81 mg./min., again in close agreement with the rate constant of pure APAP particles of 50–60 mesh.

These results indicate that particle size reduction of APAP by eutectic formation with urea does not increase the dissolution rate of the drug. It has been observed in the present dissolution system that decreasing the particle size 50% yields a 20% increase in the dissolution rate of APAP. According to Findlay (9), the APAP in the eutectic should appear as fine-grained crystals. However, no evidence of any significant particle size reduction was observed. The initial rate of dissolution of APAP from the eutectic was found to be the same or slightly less than that of the physical mixture. The major difference between the physical mixture and the eutectic was one of duration of effect. It apparently required a longer period for the urea to diffuse completely from the eutectic than from the physical mixture, thereby increasing the duration of the solubilizing effect of the urea on the APAP.

Table II.—Pseudo Zero-Order Dissolution Rate Constants for APAP Alone and in Fused or Physical Mixtures with Urea

Sample	Description	Initial Rate Constant	Time Interval, min.	Second Rate Constant	Time Interval, min.
1 APAP	Fused, 50–60 mesh	0.89	0–5	...	...
2 APAP	Fused, 100–200 mesh	1.05	0–5	...	...
3 APAP–urea	Fused mixture, eutectic composition	1.72	0–3	0.81	3–5
4 APAP–urea	Physical mixture, eutectic composition	1.99	0–1	0.90	1–5
5 APAP–urea	Physical mixture, urea present in excess of the eutectic composition	1.85	0–1	0.83	1–5

A second major reason for believing that particle size reduction was not achieved in the eutectic is provided by the second rate constant, which approximated the rate constant for the 50–60 mesh APAP. This result suggests that the urea is leached from the mixed eutectic particle leaving a matrix of APAP with an effective surface area comparable to that of a 50–60 mesh particle of APAP. Therefore, the value of eutectic formation as a means of enhancing dissolution rate still remains somewhat dubious. An enhancement of dissolution rate by virtue of simple eutectic formation alone is yet to be demonstrated.

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Increasing Dissolution Rates and Gastrointestinal Absorption of Drugs *Via* Solid Solutions and Eutectic Mixtures III

Experimental Evaluation of Griseofulvin–Succinic Acid Solid Solution

By ARTHUR H. GOLDBERG*, MILO GIBALDI, and JOSEPH L. KANIG

The phase diagram for the griseofulvin and succinic acid system showed a eutectic mixture with considerable solid solubility of griseofulvin in succinic acid. Evaluation of the dissolution rates of critical samples indicated that the solid solution dissolved 6.5–7 times faster than the pure material.

THE UTILIZATION of finely subdivided or micronized particles as a means of increasing the rate of dissolution of a drug has been considered frequently (1–4). Various methods of achieving particle size reduction have been reviewed by Levy (5). Sekiguchi *et al.* (6, 7) have suggested that particle size reduction, as a means of increasing gastrointestinal absorption of a drug, may be achieved through eutectic formation between a poorly soluble drug and a rapidly soluble carrier. Goldberg *et al.* (3, 4) have proposed that the increased absorption and dissolution rates found by Sekiguchi *et al.* were a function of the solid solutions present in the samples tested, rather than eutectic formation *per se*. Goldberg *et al.* (3) have also noted that the dissolution rate of a poorly soluble drug from a solid solution with a soluble carrier should be considerably faster than any other physical form of the drug, including the soluble eutectic and micronized forms. The purpose of this study was to evaluate experimentally the role of solid solutions in increasing dissolution rates.

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* Research Fellow, under a research grant from the Bristol-Myers Co., New York, N. Y. Present address: College of Pharmacy, University of Michigan, Ann Arbor.

EXPERIMENTAL

The experimental procedures for selection of drugs and carriers, including the initial screening techniques, were reported previously (4). The system selected for this investigation consisted of the highly insoluble antifungal agent, griseofulvin,[1] with succinic acid as the carrier. The phase diagram for the binary system was prepared by the microthermal technique (4).

To determine any possible interaction between drug and carrier in aqueous solution, solubility studies were undertaken. An excess amount of drug was placed in 60-ml. test tubes equipped with screw caps and containing 30 ml. of an aqueous solution of the carrier in varying concentrations. The contents of each tube were equilibrated at 37° in a Gyrotory incubator shaker.[2]

At the end of this period, 1-ml. samples were withdrawn by means of a filter pipet. The griseo-

[1] Generously supplied by the Schering Corp., Bloomfield, N. J.
[2] Model G-25, New Brunswick Scientific Co., New Brunswick, N. J.

A second major reason for believing that particle size reduction was not achieved in the eutectic is provided by the second rate constant, which approximated the rate constant for the 50–60 mesh APAP. This result suggests that the urea is leached from the mixed eutectic particle leaving a matrix of APAP with an effective surface area comparable to that of a 50–60 mesh particle of APAP. Therefore, the value of eutectic formation as a means of enhancing dissolution rate still remains somewhat dubious. An enhancement of dissolution rate by virtue of simple eutectic formation alone is yet to be demonstrated.

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Experimental Evaluation of Griseofulvin–Succinic Acid Solid Solution

By ARTHUR H. GOLDBERG*, MILO GIBALDI, and JOSEPH L. KANIG

The phase diagram for the griseofulvin and succinic acid system showed a eutectic mixture with considerable solid solubility of griseofulvin in succinic acid. Evaluation of the dissolution rates of critical samples indicated that the solid solution dissolved 6.5–7 times faster than the pure material.

THE UTILIZATION of finely subdivided or micronized particles as a means of increasing the rate of dissolution of a drug has been considered frequently (1–4). Various methods of achieving particle size reduction have been reviewed by Levy (5). Sekiguchi *et al.* (6, 7) have suggested that particle size reduction, as a means of increasing gastrointestinal absorption of a drug, may be achieved through eutectic formation between a poorly soluble drug and a rapidly soluble carrier. Goldberg *et al.* (3, 4) have proposed that the increased absorption and dissolution rates found by Sekiguchi *et al.* were a function of the solid solutions present in the samples tested, rather than eutectic formation *per se*. Goldberg *et al.* (3) have also noted that the dissolution rate of a poorly soluble drug from a solid solution with a soluble carrier should be considerably faster than any other physical form of the drug, including the soluble eutectic and micronized forms. The purpose of this study was to evaluate experimentally the role of solid solutions in increasing dissolution rates.

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EXPERIMENTAL

The experimental procedures for selection of drugs and carriers, including the initial screening techniques, were reported previously (4). The system selected for this investigation consisted of the highly insoluble antifungal agent, griseofulvin,[1] with succinic acid as the carrier. The phase diagram for the binary system was prepared by the microthermal technique (4).

To determine any possible interaction between drug and carrier in aqueous solution, solubility studies were undertaken. An excess amount of drug was placed in 60-ml. test tubes equipped with screw caps and containing 30 ml. of an aqueous solution of the carrier in varying concentrations. The contents of each tube were equilibrated at 37° in a Gyrotory incubator shaker.[2]

At the end of this period, 1-ml. samples were withdrawn by means of a filter pipet. The griseo-

[1] Generously supplied by the Schering Corp., Bloomfield, N. J.
[2] Model G-25, New Brunswick Scientific Co., New Brunswick, N. J.

TABLE I.—COMPOSITION OF GRISEOFULVIN SAMPLES USED IN DISSOLUTION STUDIES

Sample	Compn.	Amt., mg.	Form	Particle Size, μ
1	Griseofulvin[a]	10.0	Crystalline	250–300
2	Griseofulvin	10.0	Eutectic	250–300
	Succinic acid	5.2		
3	Griseofulvin	10.0	Physically mixed	250–300
	Succinic acid	5.2		250–300
4	Griseofulvin	10.0	Solid soln.	250–300
	Succinic acid	40.0		
5	Griseofulvin	10.0	Physically mixed	250–300
	Succinic acid	40.0		250–300
6	Griseofulvin	10.0	Micronized	1–10

[a] Particle size enlargement of pure griseofulvin was obtained by crystallization from methanol. All other samples were fused. Griseofulvin, however, showed signs of decomposition upon heating to its melting point.

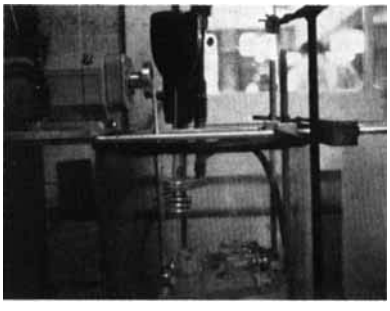


Fig. 1.—Oscillating bottle apparatus used for determining dissolution rates.

fulvin–succinic acid sample was diluted to 10 ml. with methanol and assayed spectrophotometrically at 292 mμ using a recording spectrophotometer.[3] A blank of 10% water in methanol was employed. The absorbance was read and the amount of griseofulvin present was determined by dividing the absorbance by the slope (obtained by the method of least squares) of a previously constructed Beer's law curve. Preliminary investigation had indicated that succinic acid did not interfere with the assay procedure. No shift in the absorbance peak was observed and solutions containing succinic acid gave the same absorbance as solutions containing an equal quantity of griseofulvin alone in the solvent.

The dissolution rate studies were conducted by 2 different methods. The apparatus used for one study is shown in Fig. 1. The various samples listed in Table I were screened, weighed, and placed into 10-ml. multiple-dose vials, sealed with an elastomer stopper, and capped with a metal ferrule having a hole in the center, exposing a portion of the top of the elastomer. Ten milliliters of water at 37° was injected by syringe through the stopper at time zero. The vial was immediately placed on the oscillating platform assembly in the water bath. The platform, in motion, described a 15° arc, 14 times per minute. Destructive sampling was used for the assays at 3, 5, 10, 15, and 20 min. after introduction. A sample was withdrawn by means of a syringe and rapidly filtered under vacuum using a Millipore[4] filter assembly. A 2-ml. sample of the filtrate was diluted to 10 ml. with methanol and assayed spectrophotometrically as previously described. A minimum of 2 experiments were conducted with each sample at each time interval.

The second method employed for evaluating the comparative dissolution rates was the tape method (8). This procedure involves accurately weighing and quantitatively transferring the screened material to be tested to a taut, adhesive surface and, in turn, introducing the resulting monoparticulate layer to the dissolution medium. Four hundred milliliters of distilled water, maintained at 37°, served as the dissolution fluid. Stirring rate was maintained at 130 r.p.m. by means of a constant speed motor. The stir paddle employed was 2 times the width of the stir paddle previously reported (8). The increased speed and wider stir paddle was necessitated by the extremely low solubility of griseofulvin.

Samples 1, 2, 4, and 5 of Table I were evaluated by the tape method. Sampling times were 0.5, 1.5, 3.0, 5.0, and 10 min. At each time interval 10-ml. samples were withdrawn by means of a filter pipet and immediately replaced with 10 ml. of dis-

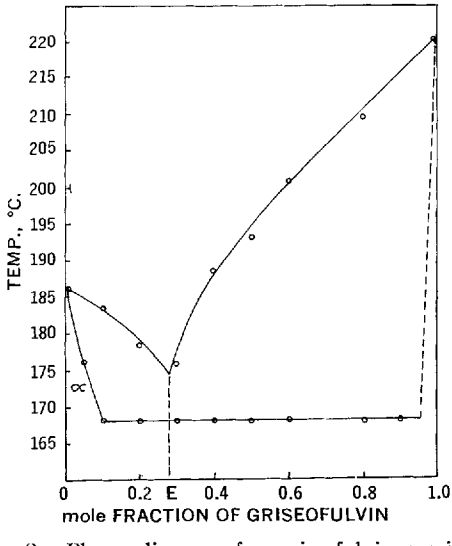


Fig. 2.—Phase diagram for griseofulvin–succinic acid mixtures obtained by microthermal techniques.

[3] Model DB, Beckman Instrument Co., Mountainside, N. J.

[4] Filter No. HA, 0.45 μ, white, plain, 25 mm. Millipore, Inc., Bedford, Mass.

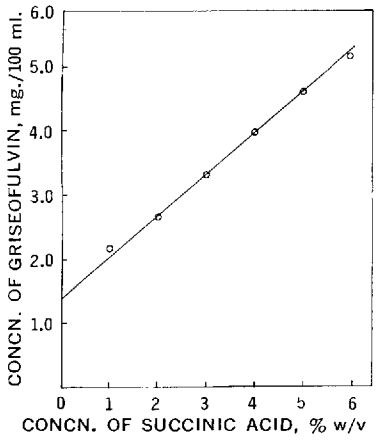


Fig. 3.—Effect of succinic acid on the aqueous solubility of griseofulvin.

tilled water preheated to 37°. Griseofulvin concentration was determined spectrophotometrically. All values were corrected to account for drug removed by prior sampling.

RESULTS AND DISCUSSION

The phase diagram obtained with the griseofulvin–succinic acid mixtures is shown in Fig. 2. The diagram clearly indicates the existence of a significant solid solution region consisting of griseofulvin and succinic acid. Little solubility of succinic acid in griseofulvin in the solid state is evidenced. Further inspection of the phase diagram reveals that the mixture exhibits a well-defined eutectic point. The eutectic mixture has a composition of 0.71 mole of succinic acid and 0.29 mole of griseofulvin, corresponding to about 55% griseofulvin and 45% succinic acid.

The eutectic mixture consists of 2 physically separable phases. One phase is almost pure griseofulvin, while the other phase is a saturated solid solution of griseofulvin in succinic acid. The composition of the saturated solid solution is 10 moles per cent griseofulvin and 90 moles per cent succinic acid. This corresponds to about 25% griseofulvin dissolved in succinic acid. Because the ratio of griseofulvin to succinic acid at the eutectic point is thermodynamically fixed, it can be shown by simultaneous equations that the eutectic mixture is composed of 60% solid solution and 40% of almost pure griseofulvin. Since the solid solution is 25% griseofulvin, 15 parts of the 55 parts of griseofulvin found in 100 parts of eutectic mixture is in the form of a solid solution. This corresponds to 27.3% of the griseofulvin.

It has been our experience that when 2 compounds exhibit solid state interactions they frequently demonstrate interaction in aqueous solution. This phenomenon has been observed with *N*-acetyl-*p*-aminophenol and urea (4), niacinamide and ascorbic acid (9), and in sulfathiazole–urea and chloramphenicol–urea systems (10). Similarly, griseofulvin and succinic acid were found to display

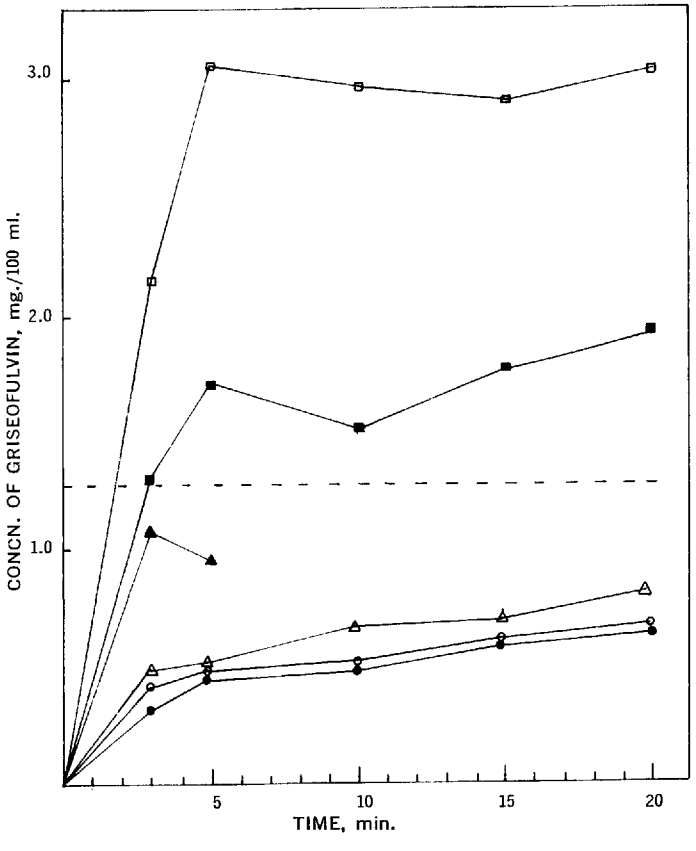


Fig. 4.—Dissolution rates of various griseofulvin and griseofulvin–succinic acid samples as determined by the oscillating bottle method. Key: ●, griseofulvin, crystalline; ▲, griseofulvin, micronized; ■, eutectic mixture; ○, physical mixture at eutectic composition; □, solid solution; △, physical mixture at solid solution composition. The dotted line indicates the equilibrium solubility of griseofulvin in water.

TABLE II.—DISSOLUTION STUDIES OF GRISEOFULVIN IN FUSED AND PHYSICAL MIXTURES WITH SUCCINIC ACID EMPLOYING THE VIAL METHOD

Sample	Form	Relative Dissolution Rate 3 min.	5 min.
Griseofulvin	Crystalline	1.0	1.0
Griseofulvin, Succinic acid	Physical mix, eutectic compn.	1.4	1.0
Griseofulvin, Succinic acid	Physical mix, solid soln. compn.	1.5	1.1
Griseofulvin	Micronized	3.5	2.1
Griseofulvin, Succinic acid	Fused, eutectic mixture	4.2	3.7
Griseofulvin, Succinic acid	Fused, solid soln.	6.9	6.7

a solution phase interaction manifested by a linear increase in the solubility of griseofulvin with increasing concentration of succinic acid. The solubility data are presented in Fig. 3.

As noted in the experimental section of this report, 2 methods were employed to evaluate the relative dissolution rates of the various samples listed in Table I. The results obtained using the oscillating vial method are shown in Fig. 4 and Table II. Table II contains relative dissolution rate data which were calculated by determining the amount of griseofulvin dissolved from a particular sample and dividing this figure by the amount of griseofulvin dissolved from the pure crystalline sample at the same time interval. At 3 min., the ratio of griseofulvin solid solution to that of the control is almost 7, indicating that the initial rate of dissolution of griseofulvin from a solid solution containing 20% griseofulvin is in the order of 7 times greater than griseofulvin alone.

The amount of dissolved griseofulvin from the solid solution at 5 min. was 2.4 times the equilibrium solubility. It is well known that reducing the particle size of a material to a very fine state of subdivision often results in supersaturation (11). Theoretically, griseofulvin is released from the solid solution in a molecular state and it is reasonable to expect such supersaturation.

It is not possible to attribute the observed increased dissolution rate to a local effect since the dissolution rate of all physical mixtures of succinic acid and griseofulvin was about the same as that of pure griseofulvin. In addition, no sample contained a sufficient amount of succinic acid to produce a bulk solubilizing effect. The increased dissolution rate of the micronized form as compared to the crystalline form of griseofulvin can be related to only one factor: decreased particle size and increased surface area. It may be concluded that since the solid solution dissolves twice as fast as the micronized form, the griseofulvin must be released in a particle size that is much smaller than the

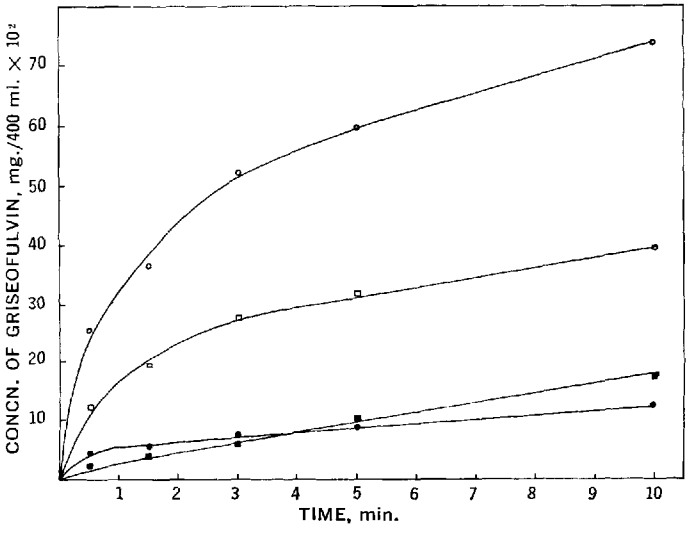
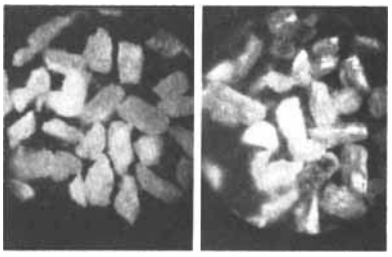


Fig. 5.—Dissolution rates of griseofulvin and griseofulvin–succinic acid samples as determined by the tape method. Key: ■, physical mixture at solid solution composition; ●, griseofulvin, crystalline; □, eutectic mixture; ○, solid solution.

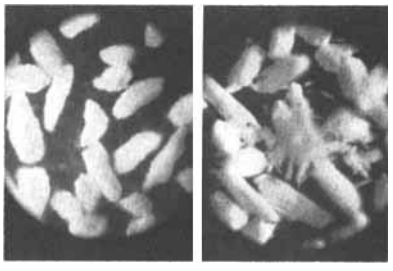
TABLE III.—DISSOLUTION STUDIES OF GRISEOFULVIN IN FUSED AND PHYSICAL MIXTURES WITH SUCCINIC ACID EMPLOYING THE TAPE METHOD

Sample	Form	Relative Dissolution Rate 0.5 min.	1.5 min.	3.0 min.	5.0 min.	10.0 min.
Griseofulvin	Crystalline	1.0	1.0	1.0	1.0	1.0
Griseofulvin, Succinic acid	Physical mix, solid soln. compn.	0.6	0.8	0.9	1.2	1.5
Griseofulvin, Succinic acid	Fused eutectic mixture	2.6	3.7	3.6	3.8	3.3
Griseofulvin, Succinic acid	Fused solid soln.	5.6	6.9	6.8	7.0	6.2



A B

Fig. 6.—Photomicrographs of crystalline griseofulvin before (*A*) and after (*B*) exposure to the dissolution medium.



A B

Fig. 7.—Photomicrographs of the griseofulvin–succinic acid eutectic mixture initially (*A*) and after 26 min. in the dissolution medium (*B*).

micronized particles theoretically approaching molecular size.

The eutectic mixture, at 3 min., dissolved 4.2 times faster than the crystalline griseofulvin, and 1.2 times faster than the micronized powder. This result is expected from the predictions of Sekiguchi *et al.* (6, 7). However, since the eutectic mixture is composed of 60% of the solid solution, it is reasonable to assume that the increased dissolution rate is attributable primarily to the presence of this solid solution in the eutectic sample. If decreased particle size does exert an effect, this effect appears inconsequential as compared to the effect of solid solution formation.

Although it may be fortuitous, the eutectic mixture which consists of 60% solid solution shows a rate at 3 min. which is just 60% that of the solid solution. Since it has been shown (4) that formation of a eutectic mixture *per se* may not increase the rate of dissolution, it is interesting to speculate that this is not just coincidental, but a physically meaningful result.

The results of the dissolution rate studies conducted with the tape method are shown in Fig. 5 and Table III. Inspection of the data indicates excellent correlation between the 2 dissolution methods. The griseofulvin from the solid solution was again found to dissolve 6–7 times faster than the pure material.

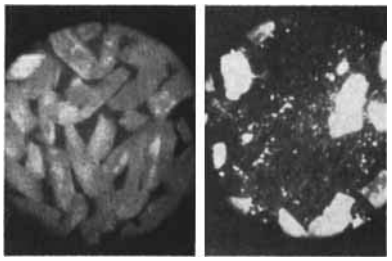
When employing the tape method in an earlier study (4), with a physical mixture of APAP and urea, a distinct local effect of the urea on the APAP was noted. In the present investigation, despite the solubilizing effect of the succinic acid on griseofulvin, no local effect was noted. The supersaturation that was found when using the vial method was absent from the data obtained with the tape method. This may be the result of differences between the 2 methods in the amount of drug excess and in the type of agitation employed.

The use of the tape method permitted the preparation of photomicrographs of the particles before and after the dissolution study. A light microscope, equipped with polarizing lenses, was employed at a magnification of 150×, in conjunction with a Polaroid Land camera.

Figure 6, *A*, shows the particles of pure griseofulvin before dissolution. Figure 6, *B*, shows the same particles after a 26-min. exposure to the dissolution medium. The only difference seems to be the disappearance of the microcrystals that appear to be associated with the griseofulvin crystals in Fig. 6, *A*.

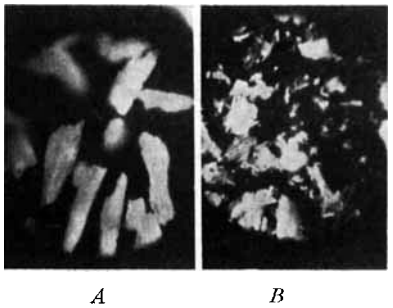
Figure 7, *A* and *B*, are photomicrographs of the griseofulvin–succinic acid eutectic mixture before and after a 26-min. dissolution study. Fragmentation of the particles is evident in Fig. 7, *B*. This is probably the result of preferential loss of the more soluble components of the individual particulates.

Figure 8, *A*, shows the particles in a physical mixture of griseofulvin and succinic acid. The striking similarity between the two crystals may be observed. After a 26-min. period in the dissolution medium (Fig. 8, *B*) only the griseofulvin crystals appeared to remain on the tape with little or no



A B

Fig. 8.—Photomicrographs of a physical mixture of griseofulvin and succinic acid present in a ratio corresponding to the solid solution composition. *A* shows both crystals; *B* shows the same area of the tape after a 26-min. exposure to the dissolution fluids; only the griseofulvin crystals remain on the tape.



A B

Fig. 9.—Photomicrographs of the griseofulvin–succinic acid solid solution before (*A*) and after (*B*) exposure to the dissolution medium.

change in size or shape. Figures 9, *A* and *B*, are photomicrographs of the solid solution containing 20% griseofulvin. Before dissolution the particles appeared somewhat more elongated and irregular than the other samples considered. After exposure to the dissolution fluids (Fig. 9, *B*) the particles seem to be sintered and greatly reduced in size.

The photomicrographs serve to support graphically the quantitative findings of the dissolution rate studies. The most rapidly soluble forms of griseofulvin, *i.e.*, the solid solution and the eutectic mixture, manifest the most dramatic alterations in their crystalline nature after exposure to the dissolution medium.

The therapeutic advantages of the griseofulvin–succinic acid solid solution are presently under investigation in our laboratories. Extrapolation of the *in vitro* findings suggests the possibility that this form will provide more rapid and complete absorption of the drug, permit a reduction in dosage, and conceivably provide a more uniform therapeutic response. Of greater significance is the fact that this investigation has demonstrated a method of physical modification which could prove more important than micronization in enhancing the absorption and therapeutic effect of many water-insoluble drugs.

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The Antitumor Agent, 1,3-Bis(2-chloroethyl)-1-nitrosourea

By TI LI LOO*, ROBERT L. DION, ROBERT L. DIXON†, and DAVID P. RALL

The new potent antitumor agent, 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), is most stable at pH 4. In acid and in solutions above pH 7, it decomposes rapidly. In plasma, BCNU has a half-life of 20 min. *in vitro*, and less than 15 min. *in vivo*. Its alkylating action is not caused by the slow hydrolysis of the chlorine. BCNU is 80 per cent bound to human plasma protein at 0°. When administered intravenously to the dog, it enters the cerebrospinal fluid (CSF) readily and disappears speedily from the plasma and the CSF. The total amount of unchanged drug excreted in the urine in 4 hr. is less than 0.1 per cent of the dose. Heating at 43° for 5 hr. converts BCNU partly into 1,3-bis(2-chloroethyl)urea.

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Chemically, although considered to be an alkylating agent, BCNU differs from typical derivatives of 2-chloroethylamine in having several reaction sites in addition to the carbon–chlorine bond which are potentially liable to attack by a variety of reagents under normal physiological conditions. Besides, the resultant transient chemical species may undergo further extensive biotransformations. These interesting considerations have prompted the authors to undertake a study on some of the chemical and pharmacological properties of BCNU. The present paper summarizes the results of these studies.

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This article is dedicated to the late Dr. E. K. Marshall, Jr., Professor Emeritus of Pharmacology, Johns Hopkins University School of Medicine, Baltimore, Md. The senior author gratefully acknowledges the counsel of Dr. Marshall on numerous occasions. The present work would have been impossible without the colorimetric method for sulfanilamide of Bratton and Marshall (1).

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EXPERIMENTAL

BCNU.[1]—A sensitive and reproducible colorimetric method for the determination of BCNU has been described (8). The rate of decomposition was calculated by measuring the amount of unchanged BCNU after incubation with buffers or biological fluids. For the recovery of BCNU, buffers were treated the same way as biological fluids. Also, at pH above 8, the very fast rate of decomposition of BCNU necessitates the continuous direct measurement of absorbance at 230 mμ, the $\lambda_{max.}$ of BCNU. However, because of low molecular extinction (about 6,000), the direct measurement of absorbance is far less sensitive than the colorimetric method. In the range of 10–50 mcg./ml., a satisfactory linear relationship was found to exist between the concentration of BCNU and its absorbance at 230 mμ. However, the absorbance at 230 mμ assigned to $\pi \rightarrow \pi^*$ transition (9) could be attributed to the excitation of —N=N— as well as to that of —N=O. The decrease in absorbance at 230 mμ is a measurement of not only BCNU, but also its primary rearranged product (namely, the diazoester, see below). The colorimetric method is devoid of such ambiguities.

Determination of Chloride.—Microtitration of chloride was performed with an automatic Cotlove titrator (10). Nitrite anion derived from the nitroso group of BCNU was found not to interfere with the titration.

Protein Binding.—Protein binding was determined by ultrafiltration using the Toribara apparatus (11). The experiments were carried out at 0° because of the extreme instability of BCNU in plasma. A solution of BCNU in human plasma was prepared. An aliquot was kept at 0° throughout the duration of the experiment as a control. A measured volume of the remaining solution was placed in a cellulose sac (Visking Corp.) in the Toribara apparatus and spun at 2,200 r.p.m. for 2.5 hr. Further prolongation of centrifugation did not affect the results. After centrifugation, the BCNU in the control plasma, the plasma in the cellulose sac, and the ultrafiltrate were determined.

Plasma Level and Urinary Excretion in the Dog.—While under anesthesia with sodium pentobarbital,[2] female mongrel dogs weighing 10–16 Kg. received BCNU *via* the femoral vein, 10 mg./Kg., in a minimal volume of 50% ethanol. Samples of blood, 5 ml. each, were drawn from the opposite femoral artery with syringes wetted with heparin solution. Plasma was separated by centrifugation at 0°. Urine samples were collected by catheterization. Cerebrospinal fluid (CSF) (1.5 ml.) was sampled *via* the cisterna magna. All biological fluids were immediately frozen in a dry ice bath until analyzed. It is often desirable to prepare plasma and urine standards of the same animal as previously explained (8). Triplicate determinations were run whenever possible.

To achieve a constant plasma level, constant drug infusion was performed after an intravenous priming dose of 10 mg./Kg. The infusion solution was prepared by dissolving an appropriate amount of BCNU in a minimal volume of 95% ethanol and diluting with 0.2 *M* acetate buffer of pH 4.1. The latter was chosen because it is nearly isotonic and because BCNU is most stable (see below) at pH 4. The concentration of the infusion solution was such that the animal received 20 mg. of BCNU/Kg./hr. at a constant infusion rate of 0.5–1 ml./min.

Paper Chromatography.—Solutions of BCNU were applied to strips of Whatman No. 3 mm. paper and developed, ascending flow, by either 0.1 *M* sodium acetate buffer of pH 4.4 or petroleum ether of b.p. 100–115°. The instability of BCNU limits the solvent system to the above. Depending on BCNU concentration, the spots could be made visible on the dried paper by any of the following procedures. (*a*) Direct illumination with ultraviolet light of 254 mμ. (*b*) Spraying first with a solution of 50 mg. of sulfanilamide in 0.2 *N* hydrochloric acid, and 5 min. later with a solution of 0.3 Gm. of *N*-(1-naphthyl)ethylenediamine dihydrochloride in 100 ml. of 95% *n*-butanol. (*c*) Spraying with a diphenylamine–palladium chloride reagent (12) consisting of 5 parts of 1.5% diphenylamine in ethanol and 1 part of 0.1% palladium chloride and 0.2% sodium chloride in water, followed immediately by irradiation with ultraviolet light of 254 mμ.

In acetate buffer system, BCNU gave an R_f value of 0.73 and in petroleum ether, 0.78.

Attempted Isolation of BCNU Metabolite from Dog Urine.—Following an intravenous injection of BCNU at 10 mg./Kg., the urine of a dog was collected for 3 hr. It was extracted 6 times with ether, using one-third its volume of ether each time. The combined ether extracts were dried over anhydrous magnesium sulfate and concentrated in an atmosphere of nitrogen at 0°. The presence of BCNU in the residual oil was confirmed by paper chromatography. However, other spots on the chromatogram were not identified. The oil resisted attempts at crystallization.

Thermal Decomposition of BCNU in Petroleum Ether.—BCNU, 500 mg., was dissolved in 50 ml. of petroleum ether (b.p. 30–60°) and refluxed at 43° (internal temperature) for 5 hr. The solvent was removed by vacuum distillation in a water bath at room temperature. After refrigeration overnight, the yellowish semisolid was triturated with ether and centrifuged. The supernatant was discarded and the residual solid was recrystallized twice from 30 ml. of ether containing 3 ml. of methanol. The fine colorless leaflets had an m.p. of 127–1:8°, undepressed by authentic 1,3-bis(2-chloroethyl)urea (13). The yield was 50 mg., 38%.

Anal.—Calcd. for $C_5H_{10}Cl_2N_2O$: C, 32.45; H, 5.45; Cl, 38.32; N, 15.14. Found: C, 32.52; H, 5.39; Cl, 37.97; N, 14.86.

RESULTS AND DISCUSSION

Stability of BCNU.—Table I summarizes the authors' results. The stability of BCNU is profoundly influenced by the pH of the solution. It is evident that BCNU is most stable in petroleum ether or in aqueous solution at pH 4. In acetate buffer of pH 4 at room temperature, its half-life exceeds 8 hr.

In strong acid (pH below 1), the rate of decomposition is too fast to measure. In fact, the ready

[1] Supplied by Merck, Sharp and Dohme Research Laboratories, Rahway, N. J., through the courtesy of the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda, Md.

[2] Marketed as Nembutal by Abbott Laboratories, North Chicago, Ill.

TABLE I.—STABILITY OF BCNU: FIRST-ORDER RATE CONSTANTS AND HALF-LIFE PERIODS[a]

pH	Medium	Concn. of BCNU,[b] $mM \times 10^2$	Temp., °C.	$k \times 10^3$ min.$^{-1}$	$t_{1/2}$, min.
1	0.1 *N* HCl[c]	2.57	37	3.41	202
2.2	0.01 *M* K_2SO_4	2.34	37	1.66	416
2.2	0.01 *M* K_2SO_4	18.72	37	1.47	470
4.4	0.1 *M* NaOAc	2.57	37	1.35	511
5.4	Dist. water[c]	2.34	37	2.69	257
5.7	Urine[c]	2.34	37	2.48	278
6.0	0.1 *M* phosphate	2.34	37	2.20	314
6.8	0.1 *M* Tris	2.34	37	6.14	112
7.1	Plasma[c]	2.34	37	40.0	17
7.4	0.1 *M* barbiturate	2.34	37	13.3	52
7.4	0.1 *M* phosphate	2.34	37	13.3	52
7.5	0.005 *M* phosphate	18.72	37	20.4	34
7.8	Ringer's solution	2.34	37	26.3	26
8.0	0.1 *M* phosphate	18.72	25	4.51	152
8.0	0.01 *M* phosphate	18.72	25	4.89	141
8.2	0.1 *M* Tris	18.72	25	4.00	170
8.2	0.01 *M* Tris	18.72	25	4.70	147
8.2	0.001 *M* Tris	18.72	25	3.82	181
8.3	0.1 *M* NH_4HCO_3	18.72	25	6.29	110
8.3	0.01 *M* NH_4HCO_3	18.72	25	7.73	89
8.8	0.1 *M* borate	9.36	25	12.0	57
8.8	0.1 *M* borate	18.72	25	12.0	57
9.3	0.1 *M* Tris	14.04	25	29.5	23
	Petroleum ether[c]	14.04	50	0.94	730

[a] Average of three experiments. [b] Solutions more concentrated than 2.57×10^{-2} m*M* were studied by direct spectrophotometric measurement of absorbance at 230 mμ. [c] Unbuffered solutions.

liberation of nitrous acid by BCNU in strong acidic solution constitutes the basis for its colorimetric determination. Between pH 1 and pH 4, the decomposition is first order with respect to BCNU. Its relative stability at pH 1 (half-life longer than 3 hr.) agrees with the observation that BCNU is effective by mouth.

From pH 4.4 to pH 7.8, the decomposition still follows first-order kinetics; however, as explained below, the mechanism is probably not the same as in the more acidic media. The results of some typical experiments are graphically represented in Fig. 1. In the pH range of 4 to 9 the rate varies directly with pH,[3] but it is independent of the nature and concentration of the buffer. It is naturally affected by temperature: at room temperature (25°) the rate of decomposition is about 35% slower than at body temperature (37°). Also, at pH above 9.3, or even at pH 8, but at 37° instead of 25°, the decomposition of BCNU becomes complex kinetically, and the rate is not only very fast, but also no longer first order. Its half-life at pH 10 is not much more than 5 min.

In urine, BCNU is about as stable as in water of comparable pH. But with plasma, the case is entirely different. It decomposes at a rate 3 times faster than in phosphate buffer of pH 7.4, and its *in vitro* half-life in plasma is about 20 min. Although fast, the decay still obeys first-order kinetics, in distinct contrast with the *in vivo* situation. In the latter case, the decay of BCNU is not only somewhat faster, but follows complex kinetics because of biotransformation and excretion. In Ringer's solution, however, the decomposition is unremarkable. Clearly, plasma accelerates the destruction of BCNU *in vitro*.

[3] A rate expression could be empirically derived,

$$-\frac{d(\text{BCNU})}{dt} = k(\text{BCNU})/(\text{H}^+)^{0.3}$$

at 25° for the range pH 4 to pH 9.

The stability of BCNU was also studied in two organic solvents, namely, methanol and petroleum ether (b.p. 100–115°) by direct spectrophotometry. In the former at 50°, the decomposition of BCNU is rapid and the kinetics is complicated. On the

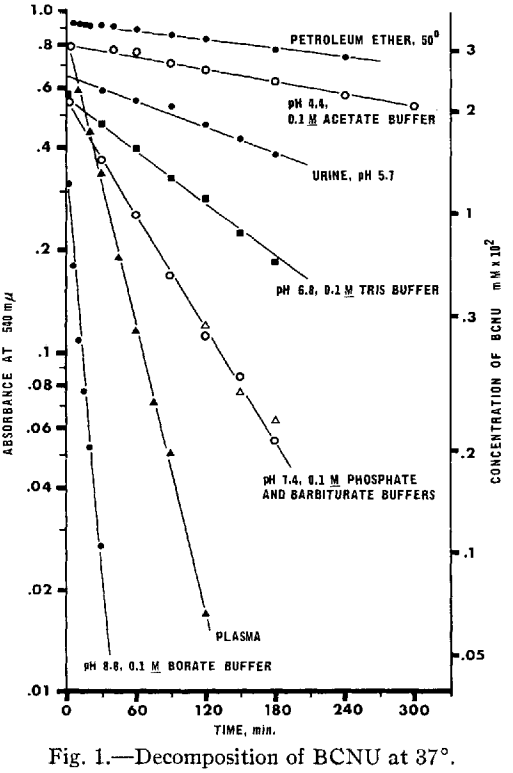


Fig. 1.—Decomposition of BCNU at 37°.

other hand, BCNU appears to be relatively stable in the latter even at 50°, and the decay obeys first-order law.

Alkaline Hydrolysis of BCNU.—It is difficult, perhaps impossible, to ascertain meaningfully the true rate of hydrolysis of the chloro- group in BCNU because of the extreme instability of BCNU in alkaline solution. However, since BCNU is generally considered an alkylating agent, we undertook a study of the rate of release of chloride ion by BCNU in order to compare it with other well-known alkylating agents. As expected, a semilog plot of either chloride liberated or fractional BCNU unhydrolyzed *versus* time fails to reveal any kinetic trend. The rate of hydrolysis is relatively slow; no more than 22% of the theoretical amount of chlorine was set free by incubation of BCNU at 25° for 90 min. in 0.1 *M* borate buffer of pH 9.8 at concentrations ranging from 0.187 m*M* (40 mcg./ml.) to 2.34 m*M* (500 mcg./ml.). At 37° in 0.1 *M* phosphate buffer of pH 7.4, a 2.34 m*M* solution of BCNU showed 31.6% of hydrolysis after more than 5 hr. The same solution exhibited 87% hydrolysis only after heating at 50° for 24 hr. As stated above, these rates could not really pertain to BCNU because under the experimental conditions BCNU has a half-life of about 5 min. to less than 1 hr. Obviously, in spite of the common biochemical effects (14) and cross resistance shared by BCNU and other alkylating agents (4, 15, 16), BCNU does not belong to the group of alkylating agents such as bis(2-chloroethyl)methylamine. If it is an alkylating agent at all, it must owe the alkylating action to one of its degradation products.

Protein Binding.—With a solution of 9.36×10^{-5} *M* of BCNU in human plasma, the results of a typical experiment are shown in Table II.

The percentage of BCNU bound to plasma protein is: (0.760–0.170)/0.760 × 100 or 78%, and the percentage recovery is: (0.760 × 4.5) + (0.170 × 0.5) × 100/(0.784 × 5.0) or 109%. On the basis of three experiments, the average extent of binding of BCNU with human plasma protein is about 80% at 0°.

The fraction of plasma protein involved in drug binding usually is albumin. Assuming an albumin concentration of 5×10^{-4} *M* in human plasma (17, 18), and also there is only one single binding site for BCNU per molecule of human plasma protein, the association constant, *K*, of the plasma protein–BCNU complex is easily estimated:

$$K = \frac{(P \cdot \text{BCNU})}{(P_f)(\text{BCNU})} = \frac{0.80 \times 9.36 \times 10^{-5}}{(5 \times 10^{-4} - 0.80 \times 9.36 \times 10^{-5})(0.20 \times 9.36 \times 10^{-5})}$$

$$= 9.4 \times 10^{3}\ M^{-1} \text{ at } 0°$$

which is comparatively small.

TABLE II.—RESULTS OF TYPICAL EXPERIMENT

	Absorbance at 540 mμ	Vol., ml.
Control	0.784	5.0
Inside	0.760	4.5
Ultrafiltrate	0.170	0.5

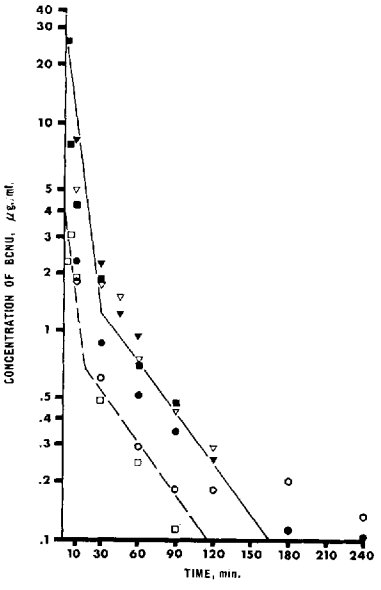


Fig. 2.—Plasma and CSF levels of BCNU in the dog. Key: ○, dog 1; □, dog 2; ▽, dog 3. (Solid symbols indicate plasma; open symbols indicate CSF.)

Plasma Level and Urinary Excretion in the Dog.—The rapid decline of BCNU in the plasma of the dog after a single intravenous injection is shown in Fig. 2. For the first 30 min. the fall of plasma levels in the three animals does not deviate too much from an exponential pattern and the half-life in plasma seems to be less than 15 min. Although protein binding by BCNU appears extensive, the association constant is relatively small. Consequently, even

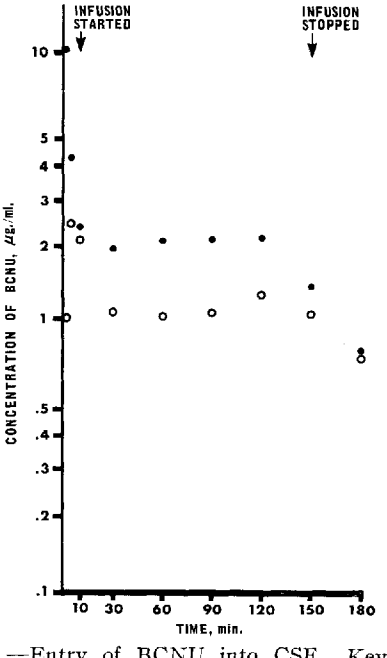
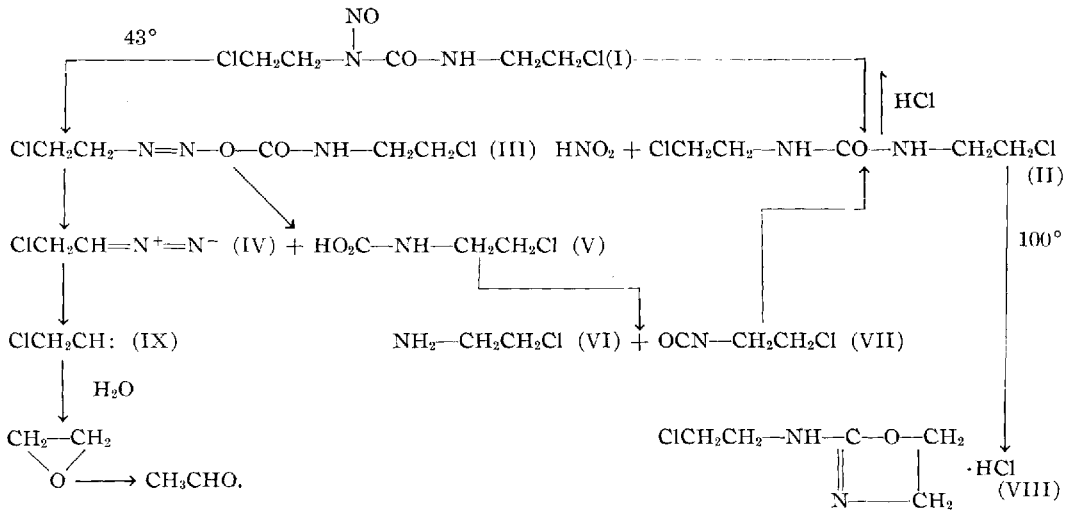


Fig. 3.—Entry of BCNU into CSF. Key: ●, plasma; ○, CSF.



Scheme I

though an increased proportion of drug would be bound as the total amount of BCNU decreases, this estimation of half-life in plasma is essentially correct (17). Extrapolation to zero time gives a zero time plasma level of about 30 mcg./ml. Since the dose of BCNU in these experiments was 10 mg./Kg., the volume of distribution is therefore 33% of body weight, in other words, somewhat larger than the volume of extracellular water. This is unexpected because BCNU, being highly soluble in lipids, appears to penetrate cellular membranes freely. In fact, BCNU makes its appearance in the CSF and the blood stream simultaneously. Furthermore, its concentration in the former falls at about the same rate as in the latter.

In view of the exceptional chemical reactivity of BCNU, it is necessary to maintain a steady plasma level by infusion before a meaningful estimate of the extent of entry into the CSF could be made. Figure 3 illustrates the results of such an experiment. After a constant plasma level of the drug has been achieved, the CSF level of BCNU reaches about 48% that of plasma (average of three experiments), confirming the screening data and clinical experience that BCNU readily penetrates the blood-brain barrier. At first sight this ratio appears to be higher than the percentage of free BCNU in plasma (20%). However, it should be recalled that protein binding was determined with human plasma at 0° and besides, the cellulose sac used is only a crude facsimile of cell membrane.

Ten minutes after a single intravenous injection, BCNU begins to appear in the urine. The excretion reaches a peak at about 2 hr. and then gradually tapers off until 3 hr. later when a detectable amount of the drug was present. Nevertheless, in no case is the total amount of unchanged BCNU excreted in 4 hr. in excess of 0.1% of the dose.

That the colorimetric method actually measures unchanged BCNU in plasma, CSF, and urine is supported by paper chromatography. When applied on Whatman No. 3 mm. paper and developed by the systems described above, the ether extracts of these biological fluids exhibit spots with R_f values identical to those obtained from ether extracts of the same fluid after the addition of authentic BCNU.

Doubtlessly BCNU undergoes extensive catabolism in the body since the percentage of unchanged BCNU recoverable from urine is so low. Although its biotransformation must await further studies, some indications could be gleaned from its thermal decomposition.

Thermal Decomposition of BCNU in Petroleum Ether.—The authors chose to undertake the study in a nonpolar solvent, namely, petroleum ether, so as to minimize secondary reactions. The isolation of 1,3-bis(2-chloroethyl)urea (II), after heating BCNU at 43° for 5 hr., suggests the postulated sequence of events in Scheme I.

The proposed 4-centered thermal rearrangement of BCNU (I), a nitrosamide, to the diazoester (III), is well known (19, 20). This is perhaps also the first step in the decay of BCNU in aqueous solutions above pH 4. The diazoester (III), incapable of but a fleeting existence, at once decomposes by an α-elimination analogous to the case of *N*-(*n*-butyl)-*N*-nitrosotrimethylacetamide (21) to give the diazoethane (IV) and the carbamic acid (V). The fate of the diazoethane (IV) cannot be defined by this experiment. However, the isolation of acetaldehyde from an aqueous solution of BCNU after refluxing (22) implies that the acetaldehyde originates from an analogous ephemeral intermediate, 2-chloroethyldiazoic acid, $ClCH_2CH_2N{=}NOH$, *via* ethylene oxide. Possibly, therefore, in this work the diazoethane (IV), on losing nitrogen, becomes transformed into the reactive carbene (IX) which also hydrolyzes to acetaldehyde. In aqueous solution, the intermediate steps would be similar.

It is not clear exactly how 1,3-bis(2-chloroethyl)-urea (II) is derived from the carbamic acid (V); the over-all reaction, nevertheless, must result from 2 moles of V through the loss of 1 mole each of water and carbon dioxide. The authors suggest 2-chloroethyl isocyanate (VII)[4] as a possible inter-

[4] Dr. R. E. Larson independently made the same suggestion that 2-chloroethyl isocyanate might be one of the intermediate biotransformation products of BCNU.

mediate not only because the addition of 2-chloroethylamine (VI) to the postulated 2-chloroethyl isocyanate (VII) explains very well the formation of 1,3-bis(2-chloroethyl)urea (II), but also because the delayed hepatoxicity of BCNU strongly resembles that of α-naphthyl isocyanate. In any event, the thermal decomposition of BCNU (I) to afford the original unnitrosated 1,3-bis(2-chloroethyl)urea (II) cannot be explained by the simple loss of a nitroso group directly from BCNU for the following reasons: first of all, such a homolysis is unprecedented; second, and most important, when 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea undergoes a like reaction in petroleum ether (b.p. 66°–75°) at 67° overnight, the product is 1,3-dicyclohexylurea [m.p. 224°, identical to an authentic sample (23) in every respect] and not 1-(2-chloroethyl)-3-cyclohexylurea, m.p. 130°–132° (24).

It has also been shown that 1,3-bis(2-chloroethyl)-urea (II) readily cyclizes in boiling water to 2-(2-chloroethylamino)-2-oxazoline (hydrochloride) (VIII) (25). However, we have no evidence that this has occurred at 43° in petroleum ether. Whether this is true *in vivo* remains to be seen.

SUMMARY

1. BCNU is most stable at pH 4. In acid as well as in aqueous solutions of pH greater than 7 it is shortlived. Its half-life in plasma is about 20 min. *in vitro* and less than 15 min. *in vivo*.

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3. BCNU is 80% bound to human plasma protein at 0°.

4. In the dog, after a single intravenous injection, BCNU enters the CSF immediately and then disappears rapidly from both the blood stream and the CSF. No more than 0.1% of unchanged drug is excreted in 4 hr. Nothing is known concerning its biotransformation *in vivo*.

5. Refluxing of BCNU at 43° in petroleum ether for 5 hr. converts it partly into 1,3-bis(2-chloroethyl)urea. The mechanism of this reaction is discussed.

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Retention, Excretion, and Distribution of 2,3,5-Triiodobenzoic Acid and Its Metabolites in the Rat

By R. D. ICE, C. E. BRECKINRIDGE, JR., and J. E. CHRISTIAN

The synthesis of 2(^{131}I),3,5-triiodobenzoic acid (TI*BA), starting with 2-amino-3,5-diiodobenzoic acid and sodium iodide (^{131}I) is described. The rate of excretion, biological half-life, and metabolites of this compound in rats were studied. Four metabolites and TIBA were found in the urine.

In soybeans, TIBA affects plant morphology and flowering response (1, 2). When properly used it increases bean production through a better utilization of photosynthate (3). Since soybeans are used for animal and human consumption, the question arises as to the environmental health hazards of TIBA and its metabolites.

TIBA was originally synthesized by Wheeler and Johns (4). Anthranilic acid was reacted with iodine monochloride and the resultant product diazotized and reacted with potassium iodide. The synthesis of TI*BA has been de-

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Retention, Excretion, and Distribution of 2,3,5-Triiodobenzoic Acid and Its Metabolites in the Rat

By R. D. ICE, C. E. BRECKINRIDGE, JR., and J. E. CHRISTIAN

The synthesis of 2(^{131}I),3,5-triiodobenzoic acid (TI*BA), starting with 2-amino-3,5-diiodobenzoic acid and sodium iodide (^{131}I) is described. The rate of excretion, biological half-life, and metabolites of this compound in rats were studied. Four metabolites and TIBA were found in the urine.

In soybeans, TIBA affects plant morphology and flowering response (1, 2). When properly used it increases bean production through a better utilization of photosynthate (3). Since soybeans are used for animal and human consumption, the question arises as to the environmental health hazards of TIBA and its metabolites.

TIBA was originally synthesized by Wheeler and Johns (4). Anthranilic acid was reacted with iodine monochloride and the resultant product diazotized and reacted with potassium iodide. The synthesis of TI*BA has been de-

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scribed by Munakata and Nakai (5). They followed the procedure of Wheeler and Johns substituting sodium iodide (^{131}I) for potassium iodide.

Saz *et al.* (6–8) have reported that oral doses have been given to man without apparent toxic effects and were still being excreted 10 days after administration. Goldberg *et al.* (9) have reported the LD_{100} for white mice to be 1.25 Gm./Kg. The LD_{50} was 0.70 Gm./Kg. Crismer (10) reported a recovery of 40% in the bile and 12% in the urine of TIBA given i.v. to dogs. Knoefel and Huang (11) have reported that TIBA exerts its toxic action through its protein binding capacity and that TIBA was reabsorbed from the glomerular filtrate.

EXPERIMENTAL

Apparatus and Materials.—A large volume liquid scintillation detector, the Purdue University Small Animal Counter (PUSAC), described by Dunavant and Christian (12), was employed for whole body counting. Crystal scintillation counting, using a 2-in. thallium activated, sodium iodide, well crystal, was used for feces, urine, and tissue counting. All activity determinations were corrected for background, counter efficiency, and decay as required.

Thin-layer chromatograms were made 250 μ thick with a model 200 (L-2) spreader.[1] Five plates, 20 × 20 cm., were prepared from 15-Gm. Silica Gel G[2] mixed with 37 ml. of water. The chromatograms were heated 0.5 hr. at 110° before use. Solvent chambers lined with Whatman No. 1 filter paper were used. Each chamber was saturated with solvent before use.

Thick-layer chromatograms, 1.0-mm. thick, were prepared using a model 200-11[1] variable thickness spreader. Five chromatograms were prepared from 85 Gm. of Silica Gel G and 150 ml. of water. The plates were activated at 110° for 1 hr. prior to use. Three solvents systems were used. They were as follows: (*a*) normal propanol–ammonium hydroxide (28%)–water (10:1:1), (*b*) normal butanol–acetic acid–water (4:1:1), (*c*) methanol–water (75:25).

All solvent systems were prepared fresh, except for solvent system (*b*) in which metabolite analyses were done with the solvent system 30 or more days old. Each solvent was allowed to proceed 12–15 cm. on the chromatogram.

Unlabeled TIBA was located with bromocresol green spray[3] (0.1% in *n*-butanol). All labeled compounds were identified from autoradiograms using No Screen medical X-ray film. After exposure, the film was developed using Kodak liquid X-ray developer and fixed with Kodak liquid X-ray fixer[4] following the manufacturer's recommendations. Reagent grade chemicals were used. Sodium radioiodide was purchased from Iso/Serve, Inc., Cambridge, Mass.

Synthesis of TI*BA.—Following a procedure described by Breckinridge *et al.* (13), which is a modification of the method used by Olivier and Combe (14), 2.5 Gm. of 2-amino-3,5-diiodobenzoic acid[4] was dissolved in 10 ml. of sulfuric acid with continual cooling to 0°. One gram of sodium nitrite was added slowly. The reaction was stirred for 2 hr. This mixture was poured over 33 Gm. of ice and air was bubbled through the solution for 1 hr. While cold, it was filtered through Whatman No. 1 filter paper by gravity. Sodium iodide (^{131}I), 54.5 mc., in 1 ml. of NaOH was added. A carrier solution of 1 Gm. NaI in 1 ml. of water was used to rinse the isotope container and this was added to the mixture. The solution was heated for 30 min. Free iodine was neutralized with sodium hydrogen sulfite. The resultant precipitate was separated by filtering by suction with a Büchner funnel and a double layer of Whatman No. 1 filter paper. The precipitate was dissolved with a minimum amount of hot ethanol. Crystallization was accomplished by adding distilled water to the warm alcoholic solution with stirring until a saturation point was obtained. The solution was reheated to effect complete solution, covered, and set aside for crystallization. Needle shaped, light orange crystals with a melting point of 223.6–225.5° were obtained. The specific activity was 9.80 mc./mmole. Yields of 80–86% of theoretical were obtained.

Radio-Compound Purity.—Dilutions of synthesized TI*BA in 10 μl. of ethanol, prepared to give a total of 10^8 disintegrating atoms during a 15-hr. period, were chromatographed using thin-layer chromatography and solvent system (*b*), made fresh. Only one labeled compound, R_f 0.69, appeared on the autoradiograph and this gave an R_r value of 1.0 when compared with TIBA. Thin-layer chromatography with solvent system (*a*) gave three separate labeled compounds. The R_f of TIBA was 0.60 and the two impurities were located at R_f 0.39 and R_f 0.73. Successive recrystallization decreased the amounts of the impurities significantly, but the impurities could not be completely removed, probably because of continued decomposition resulting from the recrystallization procedure. Repeated thin-layer chromatograms of the synthesized TI*BA compared with a radioiodide ion standard indicated an R_r of 1.0 for the impurity at R_f 0.73. The impurity at R_f 0.39 was not identified.

Purification was accomplished using a thick-layer chromatographic technique. Thirty-six spots of the synthesized TI*BA and unlabeled TIBA control were placed across a thick-layered chromatogram 1.5 cm. from the base of the plate. Each spot contained 3 mg. of TI*BA in 100 μl. of ethanol. After developing, the control TIBA was located with bromocresol green spray. The Silica Gel G in the corresponding area to the control presumed to contain pure TI*BA was scraped off and extracted with four 10-ml. portions of hot ethanol, and filtered through a sintered-glass funnel. The ethanolic solution was chromatographed using the thin-layer technique and solvent system (*a*). Only one labeled compound (R_f 0.60) appeared on the autoradiogram of the chromatogram. To further prove the identity of the labeled compound, 250 mg. of unlabeled TIBA was added and the mixture, dissolved in hot ethanol, filtered through a sintered-glass filter and recrystallized. The crystals melted at 223–225° (the known melting point of TIBA) and contained the radioactivity. Accordingly, the

[1] Research Specialties Co., Richmond, Calif.
[2] Brinkmann Instrument, Inc., Westbury, N. Y.
[3] Research Specialties Co., Richmond, Calif.
[4] Eastman Kodak Co., Rochester, N. Y.

TABLE I.—WHOLE BODY RETENTION OF ORALLY ADMINISTERED 2,3,5-TRIIODOBENZOIC ACID

Rat Group 1[a]		Rat Group 2[b]		Rat Group 3[c]	
Time, hr.	% Retained[d]	Time, hr.	% Retained[d]	Time, hr.	% Retained[d]
0	100.0	0	100.0	0	100.0
12	78.0	4	83.3	6	85.9
24	56.8	8	62.8	12	59.0
36	37.7	12	46.8	18	41.9
48	30.1	16	33.5	24	34.1
60	24.3	20	26.8	36	16.8
72	22.1	24	23.1	48	14.9
84	19.1	36	13.5	60	11.4
96	17.2	42	12.1	84	9.1
120	16.0	48	11.2	96	8.9
144	15.0	60	10.2	...	...
168	14.4	...	...	...	...

[a] Seven, female, albino, Holtzman Rats; weight, 266–288 Gm.; temp., 21°; food and water *ad libitum*. [b] Six female, albino, Badger rats; weight, 165–175 Gm.; temp., 28°; food and water *ad libitum*. [c] Six, male, albino Holtzman rats; weight, 182–238 Gm.; temp., 28°; food *ad libitum*; 20 ml. water limit daily. [d] Per cent of administered dose.

TABLE II.—ACCUMULATED PER CENT[a] EXCRETION OF ORALLY ADMINISTERED 2,3,5-TRIIODOBENZOIC ACID

Rat Group 1[b]		Rat Group 2[b]			Rat Group 3[b]		
Time, hr.	Urine, %	Time, hr.	Urine, %	Fecal, %	Time, hr.	Urine, %	Fecal, %
12	21.4	4	12.3	...	6	14.2	...
24	40.2	8	28.6	...	12	37.2	0.8
36	53.8	12	38.3	0.5	18	51.2	...
48	63.0	16	48.4	...	24	57.1	2.2
60	68.4	20	54.5	...	36	69.8	3.2
72	70.4	24	58.2	1.6	48	71.2	3.5
84	73.4	36	66.9	2.6	60	73.7	3.7
96	74.0	42	67.9	...	84	75.0	4.0
120	76.7	48	68.5	2.7	96	75.3	4.1
144	77.6	60	70.2	2.9	...	...	...
168	78.8	...	...	...	...	...	...

[a] Per cent of administered dose. [b] Same rat group conditions as listed in Table I.

purified TI*BA was considered to be free of labeled compound impurities.

Whole Body Retention and Excretion.—Three groups of rats were studied. All doses were oral using an oral administration needle. Each rat was given a dose of 0.2 μc. representing 0.10 mg. of TI*BA per 0.5 ml. All doses were in a 50% ethanol and distilled water solution. The results were tabulated in per cent retained and appear in Table I. The data suggest two exponential components. A short biological half-life component is indicated from 11.8–17.9 hr. This may represent blood concentration of TI*BA. A long biological half-life component, 395–403 hr., was also indicated. This component may indicate thyroid involvement. Excretion studies were run concomitantly with whole body retention studies using the same rats and groupings. The feces was collected at 12-hr. intervals and the urine was collected at 4–12-hr. intervals. Results are shown in Table II. Urinary excretion was the primary mode of excretion, accounting for 70–78% of the administered dose. Fecal excretion accounted for 3–4% of the administered dose.

Distribution Study.—Two groups of six rats each were starved for 24 hr. prior to and during the study. An oral dose of TI*BA, as before, was administered. Immediately after administration a whole body count was made. Four hours later the rats were anesthetized with ether and a 1-ml. blood sample was obtained from the tail. The animals were sacrificed by over-anesthetizing with ether and the thyroid and kidneys completely excised. An aliquot of muscle tissue (from the right rear leg) and liver was also obtained. Each sample was weighed immediately after removal and a radioactivity determination made in the crystal scintillation counter. Table III presents the data on the distribution of TIBA in the blood, liver, kidney, thyroid, and muscle tissue.

The distribution study clearly indicates thyroid concentration of the labeled iodine atoms. Table III indicates the thyroid concentration was 12–18 times the concentration found in the blood. This probably is due to a partial breakdown of the TIBA to iodide ion which in turn is taken up by the thyroid. However, TIBA itself or other metabolites may be concentrated in this particular tissue.

Metabolite Studies.—A total of 12 Holtzman rats were given orally 6.75 μc. representing 2.9 mg. of TI*BA in 50% ethanol and distilled water solution. The rats were not permitted food or water

TABLE III. DISTRIBUTION OF 2,3,5-TRIIODOBENZOIC ACID 4 hr. AFTER ORAL ADMINISTRATION

Tissue	% of Dose/Gm. of Tissue	
	Rat Group 1[a]	Rat Group 2[b]
Blood	0.9	0.7
Thyroid	12.5	18.8
Kidney	1.2	0.5
Muscle	0.1	0.1
Liver	0.3	0.3

[a] Mean of six, female, albino Badger rats, 170–190 Gm. [b] Mean of six, male, albino Holtzman rats, 192–217 Gm.

TABLE IV.—RELATIVE PERCENTAGES OF METABOLITES FOUND IN THE URINE FOLLOWING THE ADMINISTRATION OF 2,3,5-TRIIODOBENZOIC ACID TO RATS[a]

Metabolites, R_f Values	Relative Abundance, %	S.E.$\bar{x}$[b]
0.81	26.3	±3.2
0.77	31.3	±3.0
0.52	10.4	±3.6
0.48	20.4	±1.6
0.39	11.6	±1.8

[a] Thick-layer chromatographic separation with *n*-butanol–acetic acid–water (4:1:1). [b] Standard error of the mean as determined from 10 separate determinations, five from each of the two separate urine samples collected from two separate groups of five animals each.

24 hr. prior to or during the experiment. A 60-μl. aliquot of whole urine collected over a period of 18 hr. was spotted on each of three thick-layer chromatographic plates along with standards of TI*BA and radioiodide ion. The remaining urine was acidified with 1 *N* HCl using litmus paper as the indicator, allowed to stand for 30 min., and then a 60-μl. aliquot was spotted on chromatography plates.

A chromatogram was developed in each of the three solvent systems. In solvent system (*b*) (30 days old) four metabolites plus TIBA were found. Radioiodide ion standard gave an R_r of 1.0 with a spot located at R_f 0.52. TI*BA standard gave an R_r of 1.0 for the spot at R_f 0.77. The spot at R_f 0.48 disappears in the hydrolyzed urine indicating a possible conjugate of TIBA. The spots at R_f 0.39 and R_f 0.81 were not identified.

A separation occurred in solvent system (*a*) indicating three metabolites plus TIBA. An R_r of 1.0 for TIBA at R_f 0.60 was obtained. An R_r of 1.0 for iodide ion at R_f 0.73 was obtained. A metabolite at R_f 0.45 disappeared upon acid hydrolysis indicating a possible conjugate of TIBA. The third metabolite was at R_f 0.52 and was not identified.

In solvent system (*c*) three metabolites were indicated plus a heavy concentration at R_f 0.83. Iodide ion and TI*BA standards had R_r values of 1.0 at R_f 0.83. The other three metabolites occurred at R_f's of 0.81, 0.77, and 0.73.

The metabolite experiment described above was repeated using 10 animals to obtain a quantitative evaluation of the metabolites present in the urine. The urine collected over a period of 18 hr. was pooled into two separate groups representing five animals in each group and chromatographed as previously described using solvent system (*b*). The separated labeled compounds were located on the chromatogram by autoradiography and the Silica Gel G containing each metabolite was quantitatively transferred to separate vials for counting in the well crystal scintillation counter. The relative percentage of each metabolite, based on the total activity found on the chromatogram, was calculated. The data are presented in Table IV.

SUMMARY AND CONCLUSIONS

1. Radioactive labeled 2(^{131}I),3,5-triiodobenzoic acid was synthesized and purified free of other radio-labeled compounds by thick-layer chromatographic separation followed by hot ethanolic extraction.

2. Whole body retention studies of TIBA showed a two component system—one with a biological half-life of 11.8–17.9 hr. and the second 395–403 hr.

3. Excretion studies showed that the primary route of excretion was urinary. Of the administered dose, 70–78% was excreted through the urine while only 3–4% was excreted through the feces.

4. Distribution studies in the blood, liver, kidney, muscle tissue, and thyroid after 4 hr. indicated an average thyroid concentration of 15 times the average blood concentration.

5. Metabolite studies using thick-layer chromatography indicated 4 metabolites plus TIBA in the urine. One of the metabolites was identified as iodide ion. Quantitative determination showed 31.3% TIBA and 10.4% iodide ion. Three unidentified metabolites were found to be present in the quantity of 26.3, 20.4, and 11.6%.

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Rapid, Sensitive Colorimetric Method for the Determination of Estrogens

By TIBOR URBANYI and C. R. REHM

A colorimetric method has been developed for the routine determination of estrogens in pharmaceutical preparations. The method is based on the formation of an azo dye from the condensation of diazotized 4-amino-6-chloro-*m*-benzene disulfonamide with estrogens which contain a phenolic hydroxyl group. The condensation product exhibits a red color with an absorption maximum at 500 mμ in alkaline solution. The most reproducible results are obtained when coupling is carried out at pH 5 buffered solution. The color formation obeys Beer's law over a range of 0.05–0.25 mg. of estrogens per ml. of sample solution. Optimum conditions for the color formation have been determined, and the application of this procedure to pharmaceutical products is given.

THE OFFICIAL U.S.P. procedure for the determination of ethinyl estradiol, estradiol benzoate, estradiol dipropionate, and estrone are based on the Kober reaction in which a violet color is developed on reaction of the estrogens in a sulfuric acid–phenol–iron reagent (1). The Kober reaction, in spite of many modifications over the years, still leaves much to be desired in an analytical procedure. The method is time-consuming, and the color development is critically dependent upon reagent composition, reaction time, and temperature. Furthermore, interference by other nonphenolic steroids can occur (2).

Colorimetric methods, based on the coupling of the phenolic hydroxyl group with diazotized amines, have been reported for a number of the estrogens. These include coupling with diazotized *p*-nitroaniline (3), with diazotized sulfanilic acid (4, 5) and its derivatives (6), and with tetrazotized dianisidine (7). These methods have been used primarily in the determination of estrogens in biological samples.

It has been observed in these laboratories that diazotized 4-amino-6-chloro-*m*-benzene disulfonamide couples with phenolic compounds to form products with extremely stable colors (8). The suitability of this reagent for the determination of a number of estrogens has been investigated. As a result of these studies, a rapid and sensitive colorimetric method for the determination of ethinyl estradiol, estradiol, estradiol esters, and estrone has been developed.

EXPERIMENTAL

A Beckman model DU spectrophotometer was used to determine the absorbance values reported. A Cary model 11 recording spectrophotometer was used to obtain the spectra presented.

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Determination of Ethinyl Estradiol and Other Phenolic Estrogens

Reagents and Solutions.—4-Amino-6-chloro-*m*-benzene disulfonamide, 0.2% methanolic solution; sodium nitrite, 1% aqueous solution; hydrochloric acid, 1 *N;* sulfuric acid, 18 *N;* sodium acetate, 2 *N* solution; sodium hydroxide, 0.1 and 1 *N* aqueous solutions; sodium hydroxide, 10% aqueous solution; sodium hydroxide, 10% in 80% methanol–water.

Standard Estrogen Solution.—Weigh accurately about 50 mg. of the estrogen into a 50-ml. volumetric flask and dissolve it in methanol. Make up to volume with methanol, then dilute 10.0 to 100 ml. with 0.1 *N* sodium hydroxide. Concentration: approximately 0.1 mg./ml.

Preparation of Assay Solutions.—*Solid Dosage Forms.*—Extract an accurately weighed sample of the powder or powdered tablets equivalent to about 5 mg. of the estrogen with three 10-ml. portions of methanol. Centrifuge each portion and filter the methanol extracts into a beaker and evaporate the methanol to dryness. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 50-ml. volumetric flask and dilute to volume with additional 0.1 *N* sodium hydroxide. This is the assay solution

Oil Solutions.—Pipet 5.0 ml. of 10% sodium hydroxide solution in 80% methanol–water into a 40-ml. centrifuge tube. Accurately pipet a volume of the oily sample solution containing approximately 5 mg. of estradiol or estradiol esters and shake mechanically for a minimum of 15 min., add 20 ml. of petroleum ether and 5 ml. of 10% aqueous sodium hydroxide, shake for 2 min., and centrifuge. Remove the lower layer with a syringe equipped with a blunt, 14-gauge needle and filter the solution through a paper filter into a 50-ml. volumetric flask. Add 10 ml. of 10% sodium hydroxide to the centrifuge tube, shake 2 min., centrifuge, and filter the lower layer to the flask as before. Add sulfuric acid dropwise until the solution is approximately neutral (test with indicator paper, pH between 6 and 8). Dilute the solution to volume with 0.1 *N* sodium hydroxide. This is the assay solution.

Color Development.—Into separate 10-ml. volumetric flasks pipet successively 1.0 ml. each of 4-amino-6-chloro-*m*-benzene disulfonamide, sodium nitrite, and hydrochloric acid solutions, and mix well. Allow to stand 1–2 min. and add 2.0 ml. of the stand-

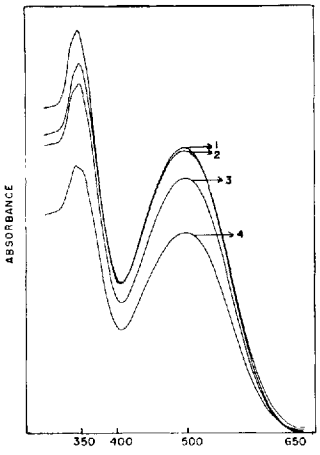

Fig. 1.—Absorption spectra of coupled products of: 1, estradiol; 2, estrone; 3, ethinyl estradiol; 4, estradiol dipropionate.

TABLE I.—MOLECULAR ABSORPTIVITIES

Compd.	E (500 mμ)
Estradiol	8.17×10^3
Ethinyl estradiol	7.93×10^3
Estradiol dipropionate	8.07×10^3
Estrone	8.04×10^3

TABLE II.—STABILITY OF COLOR OF COUPLING PRODUCT OF ESTRADIOL

Time, hr.	Absorbance at 500 mμ: Dark	Laboratory Lighting	Sunlight
0	.525	.525	.525
0.5	.525	.525	.525
1.0	.525	.525	.525
1.5	.525	.520	.525
2.0	.535	.535	.540
3.0	.530	.530	.540
4.0	.530	.530	.540

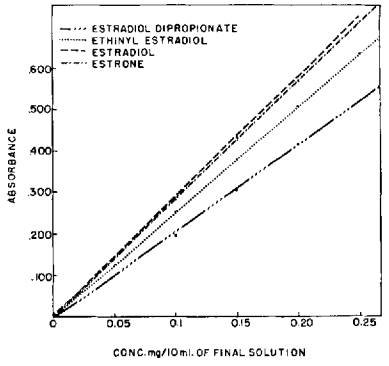

Fig. 4.—Plots of absorbance *vs.* concentration for diazo products of various estrogens.

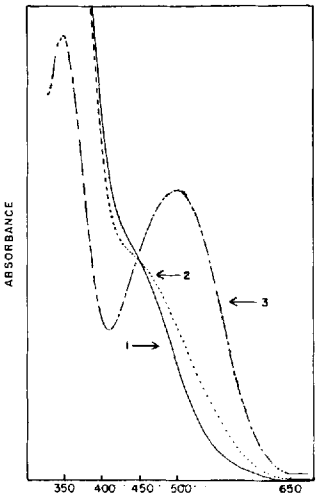

Fig. 2.—Effect of pH on the absorption spectrum of the coupled product of estradiol. Key: 1, pH 5.3; 2, pH 10.1; 3, pH 13.0.

ard solution, 2.0 ml. of the assay solution, and 2.0 ml. of 0.1 *N* sodium hydroxide solution to separate flasks. Add 2.0 ml. of sodium acetate solution to each flask and allow to stand exactly 6 min. Dilute each flask to volume with 1 *N* sodium hydroxide and determine the absorbance of the standard and sample solutions at 500 mμ in 1-cm. cells against the blank, using a suitable spectrophotometer.

DISCUSSION

The absorption spectra of alkaline solutions of the coupled products, resulting from the reaction of ethinyl estradiol, estradiol, estradiol dipropionate after saponification, and estrone with diazotized 4-amino-6-chloro-*m*-benzene disulfonamide are shown in Fig. 1. It is seen that similar spectra are obtained

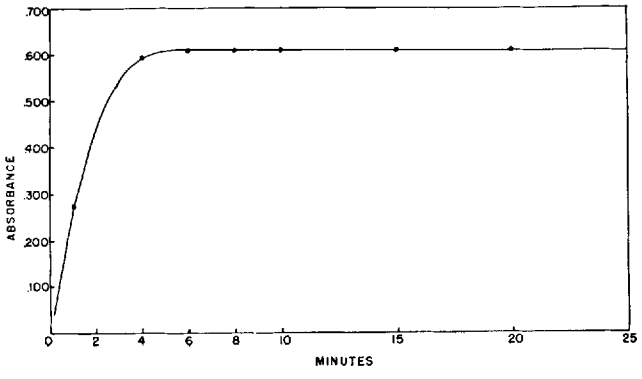

Fig. 3.—Absorbance of final solution *vs.* coupling reaction time for estradiol; coupling reaction carried out at pH 5.0.

TABLE III.—COLORIMETRIC DETERMINATION OF ESTROGENS IN THE PRESENCE OF COMMON EXCIPIENTS

Excipient	Estradiol	Ethinyl Estradiol	Estrone	Estradiol Dipropionate
Lactose				
Added, mg./Gm.	1.00	1.00	1.00	...
Found, mg./Gm.[a]	1.02	1.00	1.00	...
% Recovery	100	100	100	...
Starch				
Added, mg./Gm.	3.30	3.30	3.30	...
Found, mg./Gm.[a]	3.23	3.30	3.26	...
% Recovery	98	100	99	...
Sesame Oil				
Added, mg./Gm.	...	...	...	1.00
Found, mg./Gm.[a]	...	...	...	0.99
% Recovery	...	...	...	99

[a] Average of duplicate determinations.

for the four compounds, each exhibiting a broad absorption maximum at 500 mμ. Esters of estradiol exhibit spectra identical to that of estradiol when coupled with the reagent after saponification, as shown in Fig. 1. Saponification of mono- and diesters of estradiol proceeds rapidly in alkaline solution at room temperature and is complete in 10 min. or less in aqueous media which is 0.1 *M* or stronger in hydroxyl ion. The structure of the coupled products has not been determined; however, it is logical to assume that coupling occurs in one of the positions *ortho* to the phenolic hydroxyl group.

The molecular absorptivities of these four estrogens as calculated from the curves in Fig. 1 are listed in Table I.

It can be seen from these data that the molar absorptivities of the four compounds are nearly identical within experimental error.

The pH dependency of the absorption spectrum of the reaction product of estradiol with diazotized 4-amino-6-chloro-*m*-benzene disulfonamide is shown in Fig. 2.

It can be seen from these spectra that the red color of the coupled product is produced only in a highly alkaline medium (pH > 12). At pH 5 or below, the coupled product in solution exhibits a yellow color, the absorption spectrum exhibiting a shoulder at 450 mμ. At pH values between pH 5 and 12, varying shades of orange are produced. The absorption band at 500 mμ increases in intensity up to a pH of about 13; at pH values above this no further increase in intensity occurs. The spectra exhibit an isosbestic point at about 450 mμ as shown in Fig. 2. The absorption spectra of the coupled products of ethinyl estradiol and estrone with the diazotized disulfonamide exhibit similar pH dependencies.

The initial reaction between nitrous acid and 4-amino-6-chloro-*m*-benzene disulfonamide appears to be somewhat more complex than a simple diazotization reaction since it has been observed that approximately 2 moles of nitrous acid are consumed by the compound (8). Conditions for diazotization of the disulfonamide do not appear to be critical. Diazotization of the disulfonamide in 1 *N* hydrochloric acid is complete in 2 min. in the presence of excess nitrous acid. Destruction of the excess nitrous acid was not found to be necessary since no interference in the subsequent color development and measurement was observed. Coupling of the estrogens studied with diazotized 4-amino-6-chloro-*m*-benzene disulfonamide does not proceed readily in acid solution but was found to proceed rapidly when carried out at pH 5 or above. The most reproducible absorbance values were obtained when coupling was carried out at about pH 5. When coupling was carried out in alkaline solution, it was found that the resulting absorbance (color intensity) was critically dependent upon the rate of addition of alkali. This indicated a rapid destruction of the diazotized disulfonamide in highly alkaline solution. By coupling at pH 5, however, this problem is avoided and day to day variation in color intensity never exceeded 2%. This is consistent with the observation of Rehm and Smith, who found that coupling of the reagent with chromotropic acid under similar conditions of pH also gave highly consistent absorbance values (8). The effect of coupling time at pH 5 before the addition of alkali upon the absorbance of the final solution at 500 mμ is shown in Fig. 3. It is seen that color intensity increases during the first 6 min. and then remains constant over a period of at least 25 min. This indicates that the resulting diazo compound is quite stable at this pH.

The extreme stability of the red color as measured at 500 mμ is shown by the data in Table II. These data indicate no significant changes in absorbance of the final solution over a 4-hr. period in the dark and under normal laboratory illumination. An increase of about 3% in absorbance is noted after 2 hr. for solutions placed in direct sunlight.

The relationship between absorbance at 500 mμ and concentration was found to be quite linear for ethinyl estradiol, estradiol, estradiol dipropionate, and estrone as shown in Fig. 4.

The specificity of the method for these compounds in the presence of several frequently encountered excipients is demonstrated by the data shown in Table III. Recoveries of 98–100% were obtained for the various estrogens in the presence of lactose, starch, and sesame oil. Other phenols will, of course, interfere in the determination of these estrogens by this method as they would in the case of the Kober reaction; however, such interferences would not normally be expected.

The reproducibility, sensitivity, and rapidity of this procedure represent a considerable improvement over the presently used Kober reaction for the determination of these estrogens.

The application of this reaction to the determination of other compounds of pharmaceutical interest

containing phenolic hydroxyl groups such as pyridoxine will be presented in a future report.

SUMMARY

Diazotized 4-amino-6-chloro-*m*-benzene disulfonamide has been found to couple with various estrogens containing a phenolic hydroxyl group to yield a stable red color in alkaline solution.

This reaction is the basis of a rapid, sensitive, and reproducible method for the determination of estrogens such as estradiol, ethinyl estradiol, estrone, and estradiol dipropionate.

Commonly encountered excipients such as lactose, starch, and sesame oil were found not to interfere in the determination of these estrogens.

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Effects of Adsorbents on Drug Absorption II

Effect of an Antidiarrhea Mixture on Promazine Absorption

By DONALD L. SORBY and GRACE LIU

An antidiarrhea mixture containing attapulgite and citrus pectin was studied for its potential effect on the absorption of promazine from the human gastrointestinal tract. Test conditions were established so that results would have maximum applicability to the clinical use situation. Drug and adsorbent were not equilibrated prior to administration. Under these conditions, the antidiarrhea mixture decreased the rate and extent of absorption of the test drug. An *in vitro* adsorption study established that the antidiarrhea mixture had a strong affinity for the test drug. Results are in general agreement with the previous report in this series.

A PREVIOUS report (1) showed that activated charcoal and activated attapulgite both altered the absorption of promazine from the gastrointestinal tract. Prior to administration to human subjects, a 50-mg. quantity of promazine was equilibrated with the particular adsorbent material. The resulting test doses contained 38.5 mg. of the total 50-mg. quantity of promazine adsorbed to activated attapulgite or 24.7 mg. bound to activated charcoal. Under these conditions, activated attapulgite slowed the rate of absorption; however, it had no significant effect on the total availability of the drug. Activated charcoal, on the other hand, appeared not to release any of the adsorbed drug while within the gastrointestinal tract and only promazine, which was free in solution in the test dose at the time of administration, was absorbed. *In vitro* studies of desorption rates from adsorbates demonstrated that promazine release was rapid from activated attapulgite and very slow from activated charcoal.

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Several pharmaceutical products containing adsorbent materials are intended to control diarrhea by exerting their action within the gastrointestinal tract. In this respect they are thought to adsorb certain toxic amines produced by putrefaction or as by-products of bacterial metabolism, and thus prevent their undesirable actions on the human body (2). The question arises concerning whether the presence of such adsorbent materials within the gastrointestinal tract might also interfere with absorption of amine-type drugs, many of which are known to be strongly adsorbed *in vitro* by the materials included in antidiarrhea preparations (2–6).

The observed effects of adsorbents on drug absorption (1) are applicable only to the situation where drug and adsorbent are equilibrated before administration to test subjects. The question concerning whether the same effects will be obtained if drug and adsorbent do not meet until both are within the confines of the gastrointestinal tract was left unanswered.

The purpose of this research was to determine whether the presence of an adsorbent antidiarrhea mixture within the gastrointestinal tract would interfere with the absorption of an amine-type drug. Promazine hydrochloride (50

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The purpose of this research was to determine whether the presence of an adsorbent antidiarrhea mixture within the gastrointestinal tract would interfere with the absorption of an amine-type drug. Promazine hydrochloride (50

mg.) was used as the test drug. The antidiarrhea mixture contained in each 30-ml. vol. activated attapulgite (3 Gm.), activated attapulgite, colloidal (0.9 Gm.), and citrus pectin, 0.3 Gm.[1]

EXPERIMENTAL

Determination of Adsorption Isotherm.—Adsorption of promazine by activated attapulgite was reported previously (1). The antidiarrhea mixture contained additional ingredients, however, and it was thus necessary to study the interaction between promazine and the specific product.

The antidiarrhea mixture was diluted with a quantity of distilled water equal to twice its own weight. While the resulting suspension was vigorously stirred, 20-ml. aliquots were pipeted into 50-ml. glass-stoppered test tubes.

Visking cellulose dialysis casings ($^{18}/_{32} \times 8$ in.) were pretreated by soaking in distilled water at 90° for 10 hr. The water was changed several times during this period. The inside of each casing was then flushed with distilled water and the casings were returned to a warm water soak for an additional half hour. Just prior to use, excess water was expressed, and a knot was tied in one end of each casing to form a small bag.

A 10-ml. quantity of a solution containing either 10, 20, 30, 40, or 50 mg. of promazine hydrochloride in distilled water was pipeted into each dialysis bag, and a knot was tied to close the open end. Four bags were prepared for each concentration of promazine hydrochloride. Two of the four bags were placed into tubes containing the diluted antidiarrhea mixture. The remaining two bags were placed into tubes containing 20 ml. of distilled water. The tubes were stoppered and sealed with paraffin wax to prevent passage of water along the stoppers by capillary action. All tubes were placed in a rocker-shaker device totally immersed in a constant-temperature bath at 25.0°. Shaking proceeded for 5 days. Equilibrium was established by the end of this time period.

The dialysis bags were removed from the tubes and dried lightly with tissue. An appropriate aliquot of the solution inside the bag was removed, and the amount of promazine remaining in solution was determined by the method described previously (3). Control experiments showed that the antidiarrhea mixture contained no interfering materials at the levels of dilution used to assay for promazine. For systems containing only distilled water as the phase outside the dialysis bag, both external and internal solutions were assayed for promazine content.

The amount of material disappearing from solution in the systems containing no adsorbent was plotted *versus* the concentration of material remaining. This served as a calibration curve to provide a "dialysis cell adsorption value" for the samples containing adsorbent. The apparent amount of promazine adsorbed in systems containing the antidiarrhea mixture was calculated from the difference between the amount of promazine added initially and the amount present in solution at the end of the experiment minus the amount adsorbed by the dialysis cell.

Twenty-milliliter aliquots of the diluted suspension were pipeted into tared dishes, weighed, evaporated to dryness, and reweighed. From these data, it was possible to calculate the amount of water contributed to the dialysis system by the antidiarrhea mixture and also the weight of solids which it contained. This experiment also showed that there was a high degree of reproducibility in pipeting the diluted antidiarrhea mixture, even though there was some hold-back of material within the pipet.

The data obtained from the adsorption experiments were treated according to the Langmuir equation. Figure 1 shows the experimental values and the regression line calculated by the method of least squares. The constants for the Langmuir equation were calculated from the reciprocals of the slope and intercept values of the regression equation.

In Vivo Tests.—*In vivo* testing was conducted using an adult, male human, 31 years of age, weighing 80 Kg., and in apparent good

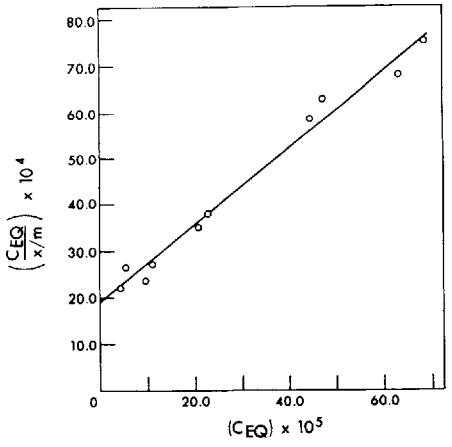

Fig. 1.—Langmuir isotherm for adsorption of promazine at 25.0° by the antidiarrhea mixture. C_{EQ} denotes the molar concentration of promazine in solution at equilibrium; x/m, the millimoles of promazine adsorbed per gram of solids in the antidiarrhea mixture.

[1] The antidiarrhea mixture was procured from a local source and was representative of the product as it appears in commerce.

TABLE I.—CUMULATIVE PROMAZINE EQUIVALENTS PRESENT IN TOTAL URINE SAMPLES AT THE END OF VARIOUS TIME INTERVALS FOLLOWING ADMINISTRATION OF TEST DOSAGE FORMS[a]

Time After Administration of Test Dose, hr.	Mean Promazine Equivalents Excreted — Test Dosage Form			
	Solution,[b] 25 mg.	Solution,[c] 37.5 mg.	Solution,[c] 50 mg.	Antidiarrhea[c] Mixture
1	83 ± 92[d]	96 ± 105[d]	121 ±78[d]	36 ± 61[d]
2	311 ± 167	478 ● 141	739 ± 265	159 ± 138
3	634 ± 311	1004 ± 180	1393 ± 303	333 ± 216
4	910 ± 443	1463 ± 191	1929 ± 411	561 ± 266
5	1114 ± 455	1764 ± 242	2233 ± 428	804 ± 324
6	1252 ± 518	1962 ± 311	2495 ± 480	1074 ± 389
8	1451 ± 590	2301 ± 392	2920 ± 496	1580 ± 355
10	1623 ± 678	2585 ± 492	3238 ± 452	2042 ± 453
12	1751 ± 792	2791 ± 567	3495 ± 434	2395 ± 596
15	1948 ± 882	3024 ± 640	3798 ± 437	2732 ± 754
18	2091 ± 931	3204 ± 700	4068 ± 448	2992 ± 884
24	2303 ± 955	3491 ± 793	4429 ± 589	3368 ± 1030
30	2411 ± 1032	3491 ± 793	4720 ± 747	3619 ± 1084
36	2445 ± 1137	3549 ± 774	4906 ± 721	3730 ± 1098

[a] For a definition of the promazine equivalent, see under *Analysis of Urine Samples*. [b] Mean of four experiments. [c] Mean of five experiments. [d] Plus-minus values represent the 95% confidence intervals about means.

health. The subject had participated in previous experiments (1) where he was designated as subject *A*.

The subject was assigned the various test doses in a random order determined through use of a table of random numbers. At least 1 week elapsed between administration of consecutive test doses. During the course of the study, there was no appreciable change in the amount of background material in the urine which was sensitive to the assay procedure. Thus, the 1-week delay between tests appeared to be adequate to avoid carryover effects between consecutive doses.

On the day prior to each experiment, the subject collected three separate urine samples to serve as blanks in the assay. Upon arising the morning of the test day, the subject voided his bladder, consumed 4 fl. oz. of water and, after waiting 0.5 hr., collected a urine sample also to be used as a blank in the assay. The subject immediately consumed the test preparation, rinsing the bottle with a small portion of water to insure complete transfer of the test dose. Urine samples were collected over the time intervals shown in Table I. No further food or drink was consumed for at least 1 hr. Consumption of food and water was *ad libitum* for the remainder of the test period. Immediately after collection, the volume of each urine sample was measured and recorded. An aliquot of the sample was frozen for assay at a later time. At the conclusion of each experiment, the frozen samples were returned to the laboratory, and after reaching room temperature they were assayed for their content of promazine excretion products.

When the experiment involved measurement of the effects of the antidiarrhea mixture, the subject consumed 1 fl. oz. of the undiluted preparation at bedtime the night before the test. Upon arising the next morning, the subject consumed an additional 1 fl. oz. of the antidiarrhea mixture. Finally, 0.5 hr. later, he took another 0.5 fl. oz. A solution containing 50 mg. of promazine hydrochloride in 45 ml. of distilled water was immediately consumed. Experiments were also carried out to measure excretion characteristics following doses of 25 mg., 37.5 mg., and 50 mg. of promazine hydrochloride in distilled water and with no antidiarrhea mixture present in the gastrointestinal tract. Data obtained from the various experiments are shown in Table I.

Analysis of Urine Samples.—Urine samples were analyzed by a procedure similar to the method used before (1). It was found previously that the yellow-colored urine pigments cause some interference with the assay procedure. This interference introduces a systematic but variable error into each assay since the intensity of the yellow color varies from sample to sample. The assay procedure was further modified in this experiment to better correct for this interference in order to obtain higher precision.

A relationship exists between the transmittance at 420 mμ of urine samples containing no promazine and the intensity of color developed at 515 mμ in these same samples when assayed by the method for determining promazine content of the urine. The assay procedure was thus modified in the following fashion. Four urine blanks were collected prior to each experiment. The transmittance of all samples, as well as urine blanks, was determined at 420 mμ before assay for promazine content.[2] The samples and blanks

[2] Per cent transmittance at 420 mμ was measured on the diluted urine sample immediately prior to passing it through the ion-exchange column.

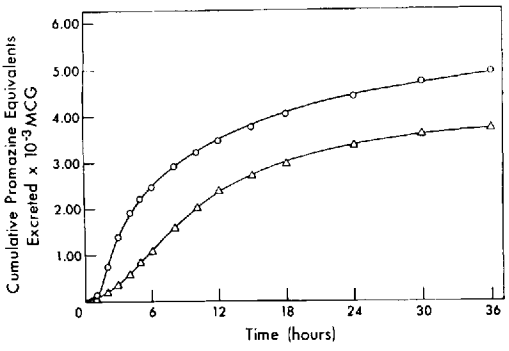

Fig. 2.—Cumulative amounts of promazine equivalents excreted in the urine following administration of promazine hydrochloride (50 mg.) in simple aqueous solution. Key: O, promazine administered alone; △, promazine administered immediately following antidiarrhea mixture. Points on each curve represent means from five separate experiments.

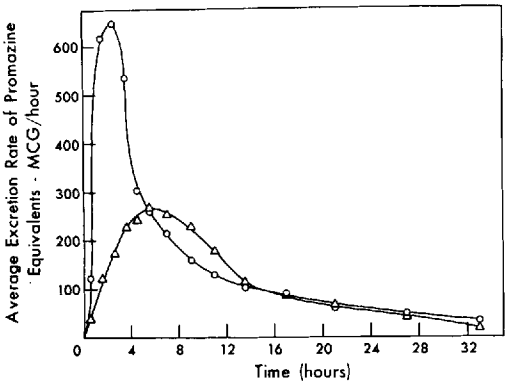

Fig. 3.—Average urinary excretion rate of promazine equivalents following administration of promazine hydrochloride (50 mg.) in simple aqueous solution. Key: O, promazine administered alone; △, promazine administered immediately following antidiarrhea mixture. Points on each curve represent means from five separate experiments.

were then assayed as described previously (1) for their apparent content of promazine excretion products. A blank correction curve was prepared by plotting the per cent transmittance at 420 mμ against per cent transmittance at 515 mμ after assay for each of the urine blanks. A blank correction was made for each test sample by comparing its per cent transmittance at 420 mμ with the correction curve. The probable color intensity at 515 mμ contributed by blank was therefore obtained for each test sample.

As discussed previously (1), due to the nonspecific nature of the assay procedure, it is necessary to express the results of assays on the basis of promazine equivalents. A promazine equivalent is defined as representing the amount of promazine hydrochloride which, if carried through the assay procedure, would give the same color intensity at 515 mμ as the urine sample in question. The excretion products in urine samples were quantitated by comparing their per cent transmittance at 515 mμ to a standard curve prepared by assaying known amounts of promazine hydrochloride dissolved in distilled water. The promazine equivalent representing the blank for a particular sample was subtracted from the assay value to obtain the actual amount of excretion product.

Treatment of Excretion Data.—Methods for treating urinary excretion data were described previously (1). Cumulative amounts of promazine equivalents excreted up to the end of each time interval and the average excretion rates during each time period were calculated. Statistical comparisons of data were made by a t test for independent sample means. Two-tail t values were used in assessing the level of significance of all data. The results of the various calculations are presented in Figs. 2, 3, and 4 and in Tables I and II.

DISCUSSION

Adsorption of promazine by the contents of the antidiarrhea mixture appears to conform to the Langmuir equation (Fig. 1). The equation for the regression line shown in Fig. 1 is

$$\frac{C_{EQ}}{x/m} = 8.31\ C_{EQ} + 1.90 \times 10^{-3}$$

where C_{EQ} is the concentration of promazine

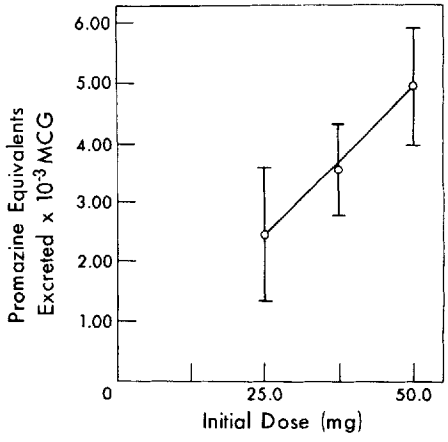

Fig. 4.—Relationship between initial dose of promazine hydrochloride and cumulative promazine equivalents excreted in the urine after 36 hr. Points represent means of five separate experiments for the 50.0 and 37.5 mg. doses and four separate experiments for the 25.0-mg. dose. Bars denote 95% confidence intervals about means. Differences between means are significant at the $P = 0.05$ level by the t test.

TABLE II.—RESULTS OF STATISTICAL COMPARISONS OF EXCRETION DATA FOR PROMAZINE, 50 mg., IN SOLUTION ADMINISTERED WITH AND WITHOUT ANTIDIARRHEA MIXTURE

Time After Administration of Test Dose, hr.	Comparison of Cumulative Amount Excreted[a]	Midpoint Time of Collection Period, hr.	Comparison of Average Excretion Rate[b]
1	$0.05/P/.025$	$^1/_2$	$0.05/P/0.025$
2	$0.001/P$	$1^1/_2$	$0.001/P$
3	$0.001/P$	$2^1/_2$	$0.001/P$
4	$0.001/P$	$3^1/_2$	$0.005/P/0.001$
5	$0.001/P$	$4^1/_2$	$0.30/P/0.25$
6	$0.001/P$	$5^1/_2$	$0.90/P/0.80$
8	$0.001/P$	7	$0.50/P/0.40$
10	$0.001/P$	9	$0.20/P/0.10$[c]
12	$0.005/P/0.001$	11	$0.25/P/0.20$[c]
15	$0.005/P/0.001$	$13^1/_2$	$0.70/P/0.60$
18	$0.02/P/0.01$	$16^1/_2$	$P/0.90$
24	$0.05/P/0.025$	21	$0.90/P/0.80$
30	$0.05/P/0.025$	27	$0.70/P/0.60$
36	$0.05/P/0.025$	33	$0.50/P/0.40$

[a] These comparisons pertain to plots shown in Fig. 2. [b] These comparisons pertain to plots shown in Fig. 3. [c] The variance ratio of these comparisons exceeded the critical value at the $P = 0.05$ level.

remaining in solution at equilibrium, and x/m is the amount of promazine adsorbed per unit weight of adsorbent, *i.e.*, the specific adsorption. The reciprocal of the slope yields a Langmuir adsorption constant which predicts the maximum amount of promazine which can be adsorbed. At that point, the surfaces of all adsorbing materials in the preparation supposedly are saturated with the adsorbed substance. The value of this constant is 0.120 mmole/Gm. of solids in the antidiarrhea mixture.[3] This amount corresponds to 38.5 mg. of promazine adsorbed per gram of solids. While the extent of adsorption is not always directly proportional to the total adsorbent in a system (7), it appears that the usual 1-fl. oz. dose of the antidiarrhea mixture would be capable of adsorbing nearly all of a 50-mg. dose of promazine hydrochloride. It should be pointed out that the degree of dilution by gastric fluids and by water in the test dose, as well as the effects of the various gastrointestinal contents, could make the adsorption characteristics quite different in the *in vivo* situation. These *in vitro* data do show, however, a high potential for adsorption of promazine by the antidiarrhea mixture. The quantity of antidiarrhea mixture consumed by the test subject would be more than adequate to adsorb all of the test dose under conditions similar to the *in vitro* experiment.

Data presented in Tables I and II and in Figs. 2 and 3 amply demonstrate the fact that the antidiarrhea mixture interferes with absorption of the test drug from the human gastrointestinal tract. Both the absorption rate and the over-all extent of absorption, as judged by urinary excretion data, are modified when the antidiarrhea mixture is present. The delay in the time of peak urinary excretion rate (Fig. 3) suggests that, for a period of time, the apparent absorption rate constant for promazine is altered. Such an effect was also seen previously (1) when promazine pre-equilibrated with activated attapulgite was administered to test subjects. These results differ from those obtained previously in that the total amount of drug absorbed is also significantly decreased. This may be seen in both Figs. 2 and 3. Judged by the 36-hr. cumulative excretion, the relative availability of promazine was 76% after administration with the antidiarrhea mixture. The same value can also be arrived at independently by comparing the 36-hr. cumulative excretion after the antidiarrhea mixture to Fig. 4. This amount of excretion product would be obtained from an initial dose of 38 mg. of promazine hydrochloride in solution, again 76% of the actual dose.

Data shown in Fig. 4 add proof to the validity of test methods employed here and previously (1) for studying the effects of adsorbents on the absorption of the model amine. While the assay method is indeed nonspecific and will determine only a portion of total drug metabolites excreted (1, 8), Fig. 4 shows that a direct relationship exists between the initial dose of promazine and the amount of excretion product collected over a 36-hr. period. While not presented here, similar plots of data for other collection periods are also linearly related to initial dose. Differences between the three doses with respect to the mean cumulative excretion products at each collection period were significant in every case at the $P = 0.05$ level as well. It is thus established that the differences between excretion curves found both here and previously (1) reflect differences in the amount of test drug absorbed.

[3] No attempt was made to distinguish between adsorbing and nonadsorbing solids in the antidiarrhea mixture. It is likely that only a portion of the total solids present after evaporating the mixture to dryness possess adsorbent power.

The decision to perform the experiments in replicate with one subject rather than by using a test panel of different subjects was based on several factors. Between subjects variability with respect to elimination of the test drug is quite high (1). This reduces the sensitivity of the experiment with respect to its ability to detect adsorbent effects. Replicate experiments in one individual should be subject to less variability. The data show that even with one subject, variability is high but it is not so great as was obtained previously (1). Data in the literature (9–12) point out the importance of urine flow rate and urine pH to the excretion rate of various amines. It was not considered to be desirable to attempt to control urine pH since this would involve administration of materials which might alter the local environment within the gastrointestinal tract at the time of administering the test dose. It was thought that by using a single test subject, patterns of diet and fluid intake would be less varied and would result in a more constant influence of urine pH and flow rate on promazine excretion. The difficulty of securing cooperation of test subjects in an experiment where many samples are collected and several tests are to be performed was another factor behind use of a single test subject. Others have also employed a single test subject in cases where precise and careful subject cooperation was required (13).

Of course it may be argued that the responses of one individual cannot be extrapolated to the general population of subjects. This would certainly be true in many cases and indeed one must proceed with caution in interpreting the results of this experiment. In this case, however, the human subject is used to provide a suitable environment to study uptake and release of the test drug from the adsorbent material. A more precise estimation of these effects may be obtained where the data are not subject to the additional variables arising from differences in the way individuals metabolize and excrete the drug once it is absorbed. In a sense the human subject provides an experimental instrument which no *in vitro* apparatus can be made sufficiently refined to do, *viz.*, to provide a system containing all of the variables usually met within the human gastrointestinal tract. It is also necessary to study the adsorption phenomenon where it is subject to the influence of the dynamic situation which is provided by constant removal of drug from solution in the gut through absorption. Obviously, there will be quantitative differences in the magnitude of adsorbent effects between different individuals. Between subject differences with respect to gastric and intestinal pH, gastrointestinal motility, ionic content of the gastrointestinal fluids, presence of food, the adsorbent dose, and even the disease state may all alter the magnitude of the adsorbent effect in a given individual. It is considered, however, that the results of this experiment amply demonstrate the potential effect of an adsorbent on drug absorption. In showing that this potential exists, the results should be applicable to the general population of subjects.

SUMMARY

It is apparent, on the basis of evidence presented, that the presence of the antidiarrhea mixture within the gastrointestinal tract is sufficient to retard absorption of the model amine drug. While availability was retarded in this case, the results still compare qualitatively with those obtained previously (1) for attapulgite-promazine adsorbates. Considering the overwhelming excess of adsorbent present, and its affinity for the drug *in vitro*, the degree of interference with absorption is more remarkable for the fact that it does not occur to a greater extent rather than for the fact that it does occur. It has already been shown *in vitro* (1) that desorption is facilitated in acidic media. It was also found for other clay-type adsorbents (14) that acidic media retard adsorption of various phenothiazine derivatives. In the gastrointestinal tract, therefore, the affinity between drug and the antidiarrhea mixture may be considerably less than found *in vitro*.

The results of this study have important clinical implications. While it has not been established whether adsorbent effects determined here and previously (1) are applicable to other amine-type drugs, it is probably true that effects would be similar for amines of similar basicity and adsorbability *in vivo*. If this is the case, a potential exists for interference with the absorption of many drugs when the gastrointestinal tract is "loaded" with an antidiarrhea mixture. In view of the somewhat questionable merits of these preparations as adjuncts to controlling diarrhea (15), their use in a given situation should be carefully weighed against potential harmful effects on the absorption of certain more therapeutically important drugs which the subject might be taking concurrently.

Further studies now in progress should provide more information concerning the general applicability of these results to other amine-type drugs. Also in progress are studies aimed at establishing correlations between *in vitro* adsorption studies and *in vivo* effects of adsorbents on drug absorption.

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Technical Articles

Microcrystalline Cellulose in Tableting

By GEORGE E. REIER* and RALPH F. SHANGRAW

The development of microcrystalline cellulose has made available to the pharmaceutical industry an extremely valuable tableting agent. It was found that tablets of plain microcrystalline cellulose will tend to soften and swell when exposed to humid conditions, but the effect is reversed upon the removal of increased humidities. Elevated temperatures have no effect on these tablets. Microcrystalline cellulose tablets will disintegrate very slowly in solvents of a relatively low polarity. It is postulated that tablets of this material are a special form of cellulose fibril in which the individual crystallites are held together largely by hydrogen bonding. Tablet disintegration is merely the breaking of the intercrystallite bonds by the disintegrating medium. No significant separation of components was found during the compression of a microcrystalline cellulose-containing formulation. The release of amphetamine sulfate and sodium phenobarbital from tablets containing microcrystalline cellulose is excellent. Determinations after 10 weeks at various environments indicate that no release problems exist. When the cellulosic compound was used as a dry binder–disintegrator in the direct compression of formulations of ephedrine hydrochloride, quinine sulfate, and a low melting steroid, tablets of outstanding quality were produced.

THE SUCCESSFUL application of direct compression as a tableting procedure is dependent upon the development of suitable materials which in themselves are both highly fluid and cohesive. Spray-dried lactose exhibits these characteristics and has enjoyed considerable success as a tableting agent in direct compression. However, it has the disadvantage of browning under certain conditions (1–3) and there is a limiting hardness to tablets produced from spray-dried lactose which, if exceeded, results in capping. This pressure sensitivity occurs at lower tablet hardnesses than usually encountered with granulations of conventional lactose.

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Presented to the Scientific Section, A.PH.A., New York meeting, August 1964.
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Another material which possesses the required properties for direct compression is microcrystalline cellulose.[1] This material is not a derivative of the parent compound, nor is it merely purified cellulose (4, 5).

A preliminary report pointed up the ability of microcrystalline cellulose to form extremely hard tablets that are not friable and yet possess unusually short disintegration times (6). The preparation and stability of glyceryl trinitrate tablets produced by direct compression of the drug in a microcrystalline cellulose matrix have been described (7). A comparison of the effect of water vapor pressure on the moisture sorption and stability characteristics of aspirin and ascorbic acid tablets containing various fillers including microcrystalline cellulose has been published (8). Microcrystalline cellulose has been included in

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TABLE I.—THE EFFECT OF ENVIRONMENT ON WEIGHT AND HARDNESS OF PLAIN MICROCRYSTALLINE CELLULOSE TABLETS COMPRESSED TO A CONSTANT THICKNESS

Batch	Initial			After 1 Wk.		
	Wt., Gm.	Hardness, S.C. units	Thickness, in.	Wt., Gm.	Hardness, S.C. units	Thickness, in.
			75% Relative Humidity			
1	0.149	13.1	0.1011	0.156	6.4	0.1094
2	0.144	11.5	0.0986	0.152	6.3	0.1067
3	0.132	8.3	0.0985	0.139	5.2	0.1058
4	0.128	7.6	0.0973	0.135	5.0	0.1045
5	0.123	5.7	0.1001	0.129	4.3	0.1072
6	0.114	4.3	0.1015	0.119	3.4	0.1074
7	0.108	3.6	0.1007	0.113	2.6	0.1059
			60° Temperature			
8	0.150	13.1	0.1020	0.146	11.6	0.1020
9	0.144	11.0	0.1013	0.141	10.5	0.1014
10	0.132	9.3	0.0990	0.129	7.2	0.0992
11	0.130	6.9	0.1021	0.127	6.3	0.1023
12	0.124	5.9	0.1020	0.121	5.4	0.1021
13	0.115	4.9	0.0973	0.113	5.1	0.0973
14	0.107	3.9	0.1005	0.105	4.1	0.1004

studies of disintegrants in direct compression systems (9, 10).

There can be no doubt that microcrystalline cellulose is a unique material and a more detailed study of the following appeared warranted: (*a*) the effect of elevated temperature and humidity on plain microcrystalline cellulose tablets; (*b*) the disintegration time in media of varying polarities of plain microcrystalline cellulose tablets; (*c*) the extent of hopper segregation occurring during tableting; (*d*) the extent of irreversible adsorption of drugs of different ionic character onto microcrystalline cellulose; and (*e*) the further investigation of microcrystalline cellulose as a dry binder.

EXPERIMENTAL

Throughout the investigation, tablets were evaluated with respect to weight, hardness, disintegration time, and friability. All hardnesses were measured by means of a hand operated Strong Cobb hardness tester. Disintegration times were determined by means of the U.S.P. disintegration apparatus, utilizing distilled water at 25° as the immersion fluid.

TABLE II.—THE EFFECT OF PROGRESSIVE CHANGES IN CONDITIONS ON WEIGHT AND HARDNESS OF PLAIN MICROCRYSTALLINE CELLULOSE TABLETS

Time, Days	Conditions, R. H. and Temp.	Wt., Gm.	Hardness (S.C. units)[a]
0	...	0.145	11.7
2	75% R. H.	0.153	7.2
4	60° C.	0.141	10.7
6	Ambient conditions[b]	0.146	11.6
8	60° C.	0.143	12.3
10	75% R. H.	0.151	8.0
12	Ambient conditions[b]	0.147	11.5

[a] Strong Cobb units. [b] Ambient conditions are approximately 25° and 40% relative humidity.

Effects of Temperature and Humidity.—A study of variations in hardness and weight caused by environment was conducted on tablets compressed to a constant thickness by means of a hand operated single punch Erweka tablet machine using $^{5}/_{16}$-in. dies and flat-faced punches. In order to vary hardness while keeping thickness constant, it was necessary to change the die fill and, consequently, the average tablet weight between batches. The tablets were stored at a high relative humidity (75%) or high temperature (60°) for 1 week and changes in weight, hardness, and thickness noted. These results can be seen in Table I. Tablets were also cycled through temperature and humidity changes. These results are summarized in Table II.

Influence of the Polarity of the Disintegration Medium on Tablet Disintegration.—Flat-faced $^{5}/_{16}$-in. plain microcrystalline cellulose tablets having an average weight of 0.15 Gm. and hardness of 6 were produced on a hand operated press. The effect of the polarity of the disintegration medium on the disintegration of these tablets was studied by determining the time for disintegration in media of different dielectric constants. One tube from the U.S.P. basket rack assembly was suspended in a 250-ml. graduated beaker containing the disintegration medium at room temperature. The tube was raised and lowered according to U.S.P. specifications but disks were not used. Table III shows the results of this study.

Determination of Component Separation During Compression.—A formula containing 30% red spray-dried lactose was chosen since it was thought that spherically shaped particles in such an amount could easily "settle through" the microcrystalline cellulose when the powder blend was subjected to vibration. The colored material was utilized so that the degree of separation could be estimated by extraction of the dye from the tablet. Amphetamine sulfate was also included in a concentration such that each tablet of the mixture would contain

TABLE III.—THE DISINTEGRATION TIME OF PLAIN MICROCRYSTALLINE CELLULOSE TABLETS IN MEDIA OF VARYING POLARITY

Solvent[a]	Disintegration Time, sec.	Dielectric Constant,[b] 25°
Water	20	78.5
25% Glycerin	30	...
50% Glycerin	57	...
75% Glycerin	232	...
Glycerin U.S.P.	>300[c]	42.5
25% Alcohol	38	...
50% Alcohol	65	...
75% Alcohol	180	...
Alcohol U.S.P.	>300	24.3
Acetone N.F.	>300	20.7
25% Isopropyl alcohol	75	...
50% Isopropyl alcohol	300	...
75% Isopropyl alcohol	>300[d]	...
Isopropyl alcohol N.F.	>300[e]	18.3

[a] Percentage strengths refer to aqueous solutions (v/v). [b] Reported in "Handbook of Chemistry and Physics," 39th ed., Chemical Rubber Publishing Co., Cleveland, Ohio, 1957, pp. 2331–2335. [c] Tablet softened, lateral cracks observed but no flaking off. [d] Some flaking occurred, tablet soft but not disintegrated. [e] A few small pieces flaked off.

5 mg. No lubricant was necessary in the compression of the formulation. The total composition was:

Amphetamine sulfate.....	75 Gm. (1.7%)
Red spray-dried lactose.....1350 Gm.	(30.0%)
Microcrystalline cellulose...3075 Gm.	(68.3%)

Tablets were produced on a Colton model 216 rotary press fitted with $^{7}/_{16}$-in. standard concave punches. Die fill and pressure were adjusted at the beginning of the run to produce tablets of about 300 mg. in weight and 6–7 Strong Cobb units in hardness. Three different machine speeds were used during the course of the study: 600, 860, and 1120 tablets/min.

Tablet samples were taken at intervals that varied with the speed of the machine. These tablets were assayed by a spectrophotographic method which allowed for the separate determination of amphetamine sulfate and red spray-dried lactose. The means of the assay results for each sample were calculated, and the per cent deviations of the sample means from the over-all mean are shown in Table IV. The disintegration times of all tablet samples were less than 30 sec. while friability ran from 0.1–0.25% weight lost.

Amphetamine Sulfate and Sodium Phenobarbital Release from Tablets of Microcrystalline Cellulose.—Since the acidity or basicity of the therapeutic agent must be taken into account, an ionic drug from each category was included in this study. The tablets were placed at three storage stations. The determination of drug release was carried out by placing the tablets, previously weighed, into 1-oz. dry square bottles and adding 25 ml. of distilled water prewarmed to 37°. The bottle and contents were placed in a 37° water bath and rotated end-over-end at 20 r.p.m. At the end of the desired time, the mixture was quickly filtered, and a sample collected for further dilution and spectrophotometric assay. The tablets were compressed using flat-faced $^{13}/_{32}$-in. tooling and the mg. per tablet formulas were as follows:

Amphetamine sulfate	15.0	...
Sodium phenobarbital	...	15.0
Spray-dried lactose	75.0	75.0
Microcrystalline cellulose	158.8	157.5
Magnesium stearate	1.2	2.5

The results of this experiment are shown in Tables V and VI.

Microcrystalline Cellulose as a Dry Binder–Disintegrator.—Ephedrine hydrochloride and quinine sulfate have long posed problems in regard to disintegration and until recently were allowed to possess unusually long disintegration times (11). For this reason, these materials were chosen for use in further evaluating the binding and disintegration properties of microcrystalline cellulose in direct compression.

An androstane-type steroid (under clinical investigation by the National Institutes of Health) was also included in this part of the work. The steroid melts at 60° and liquefies under pressure, thus microcrystalline cellulose was utilized as an adsorbent as well as a dry binder–disintegrator in order that tablets of a uniform surface appearance might be produced by direct compression.

In all cases it was necessary to slug the powder blend. The slugs were passed through a No. 16 screen and additional lubricant added before recompression on a rotary tablet machine. The mg. per tablet formulas were as follows:

Ephedrine Tablet	
Ephedrine hydrochloride.....	30.0
Spray-dried lactose.....	37.5
Microcrystalline cellulose.....	112.5
Calcium sulfate.....	187.5
Magnesium stearate.....	7.5

TABLE IV.—DETERMINATION OF COMPONENT SEPARATION DURING COMPRESSION

Sample	—% Deviation from Over-All Mean— Amphetamine Sulfate	Red Spray-Dried Lactose
1[a]	−3.48	+6.92
2	−3.48	−0.61
3	+0.79	−0.27
4	−0.43	−0.27
5	−1.53	+0.38
6	−0.86	+0.03
7	−1.89	+1.60
8	−1.71	+0.38
9	−0.43	−2.18
10	−0.79	+0.44
11	−0.61	+0.72
12	+0.79	+2.52
13[b]	+1.83	−0.85
14	+0.49	−2.08
15	−0.31	−0.99
16	+3.24	−1.09
17	−0.67	+1.02
18	+1.41	+0.17
19[c]	+1.89	−2.28
20	+2.50	−2.28
21	+2.19	−0.38
22	+2.20	−0.85

[a] Samples 1–12, machine speed of 600 tablets/min. [b] Samples 13–18, machine speed of 1120 tablets/min. [c] Samples 19–22, machine speed of 860 tablets/min.

TABLE V.—EFFECT OF 10-WEEK STORAGE ON CHARACTERISTICS OF DIRECTLY COMPRESSED AMPHETAMINE SULFATE–MICROCRYSTALLINE CELLULOSE TABLETS

Storage Conditions	Hardness, S. C. units[a]	Disintegration Time, sec.	% Drug Release, 3 min.	% Drug Release, 30 min.
Control before storage	10.1	39	98.8	...
Ambient conditions[b]	10.0	54	99.3	...
75% R. H.	4.7	42	95.5	102.8
60°	9.1	36	103.0	...

[a] Strong Cobb units. [b] Ambient conditions approximately 25° and 40% relative humidity.

TABLE VI.—EFFECT OF 10-WEEK STORAGE ON CHARACTERISTICS OF DIRECTLY COMPRESSED SODIUM PHENOBARBITAL–MICROCRYSTALLINE CELLULOSE TABLETS

Storage Conditions	Hardness, S.C. units[a]	Disintegration Time, sec.	% Drug Release, 3 min.	% Drug Release, 30 min.
Control before storage	8.2	31	100.1	...
Ambient conditions[b]	8.5	68	89.3	101.9
75% R. H.	5.1	42	96.9	99.2
60°	7.3	36	97.0	101.6

[a] Strong Cobb units. [b] Ambient conditions approximately 25° and 40% relative humidity.

Quinine Tablet	
Quinine sulfate	300.0
Spray-dried lactose	60.0
Microcrystalline cellulose	200.0
Calcium sulfate	28.0
Magnesium stearate	18.0

Steroid Tablet	
Androstane-type steroid	250.0
Spray-dried lactose	62.5
Microcrystalline cellulose	300.0
Magnesium stearate	12.5

Attempted recompression of the steroid product resulted in considerable "picking." However, freezing of the granulation by placing it in a container with dry ice for a short period of time immediately prior to compression eliminated this difficulty. The success of this procedure would suggest that further studies should be conducted on the effect of low temperatures on compaction. The characteristics of the tablets produced in this study are shown in Table VII.

DISCUSSION

Mechanism of Compaction and Disintegration.—As may be seen from Table I, plain microcrystalline cellulose tablets after storage at 75% relative humidity for 1 week exhibit an increase in weight and a decrease in hardness. The latter result is consistent with that noted in an earlier report (6). In addition, tablet thickness appears to increase. The mean thicknesses of the group before and after exposure to increased humidity were tested by means of Student t test (12). While the changes in thickness appear quite large, they are not statistically significant at the 95% confidence level. This lack of significance can be attributed to the relatively high variance found in the group. However, the consistent increase in thickness for the batches studied certainly is indicative of a swelling effect. Similar tablets stored at 60° for 1 week exhibit a uniform weight loss but no change in thickness. A slight decrease in hardness may be noted, but it was not large enough to even consider a test for significance.

The changes in plain microcrystalline cellulose tablets brought about by storage in various environmental conditions are not permanent as evidenced by the data in Table II. Loss of hardness of microcrystalline cellulose tablets at increased humidities may be explained by the adsorption of water onto the surfaces of the cellulose crystals. The adsorbed water molecules interrupt the bonding between particles thus causing a softening of the tablet and a tendency to swell. When humid conditions are removed, the equilibrium moisture content is lowered in the tablet, and it resumes its original hardness and thickness. At elevated temperatures, there is a slight weight loss due to the driving off of water, but this appears to have no appreciable effect on hardness.

As the mechanism of disintegration of microcrystalline cellulose tablets has been attributed to hydrogen bonding and its interruption (6), a study of disintegration times in media of variable polarities should and did give interesting results. Table III shows the variation of tablet disintegration time with the dielectric constant of the disintegrating medium. The dielectric constant is a measure of a solvent's polarity or ability to form dipoles and is,

TABLE VII.—PROPERTIES OF MICROCRYSTALLINE CELLULOSE-CONTAINING TABLETS

	Active Ingredient		
	Ephedrine Hydrochloride	Quinine Sulfate	Androstane-Type Steroid
Wt., Gm.	0.374	0.621	0.625
Punch, in.	13/32[a]	13/32[a]	7/16[a]
Wt. variation, %	1.18	1.55	...
Hardness, S.C. units	8.4	14.6	23.5
Disintegration time, sec.[b]	104	59	12
Friability, % wt. lost	0.37	0.10	...

[a] Standard concave. [b] With disks.

consequently, a measure of a compound's ability to hydrogen bond, since this phenomenon is a particular kind of dipole–dipole interaction. As the dielectric constant decreases, the medium becomes less able to hydrogen bond with the individual crystals of cellulose. The result is that the crystals remain bonded to each other and tablet disintegration does not occur.

Because of the extreme ease of compression of microcrystalline cellulose into hard tablets, apparently very little elastic deformation occurs within the microcrystals during tablet formation (13). In fact, it is even doubtful that significant amounts of plastic slip and crystal fusion take place. Undoubtedly, a much more important factor contributing to the compaction of this material is hydrogen bonding. A microcrystalline cellulose tablet may be visualized as a special form of cellulose fibril in which the crystals are compacted close enough together so that hydrogen bonding between them can occur.

Problems in Tableting.—Due to the small particle size of microcrystalline cellulose as well as its fluidity, hesitancy to assume a charge, and lack of aggregation, blending with other substances does not appear to offer difficulty. While one might expect these mixtures to separate when subjected to vibration, the results in Table IV do not so indicate even though the formulation studied was designed to point up segregation. The initial high reading for spray-dried lactose in the first sample can be ascribed to a small quantity of the higher density lactose separating from the mixture during the period of free fall when the hopper was loaded. The effect rapidly disappeared and deviation from the average appears relatively random until the end of the experiment where some increase in drug concentration did occur. However, none of the variations found can be considered serious.

Because of the large surface area of microcrystalline cellulose and its adsorptive capacity (5), tablets composed largely of this material might not exhibit complete release of the therapeutic agent contained therein. The data in Tables V and VI show the effects of various environmental conditions on the release of amphetamine sulfate and sodium phenobarbital from tablets containing microcrystalline cellulose and spray-dried lactose as the fillers. Immediately following manufacture, full drug release was achieved within 3 min. After 10 weeks storage at 75% relative humidity and room temperature, the release of the amphetamine sulfate product was slightly decreased. However, the full amount of drug was available after 30 min. No slowing of release was found at the other storage stations.

In the case of the sodium phenobarbital tablets, the same slight decrease in drug release was found in those tablets stored at 75% relative humidity and room temperature as well as at 60° and ambient humidity. Those tablets stored under ambient conditions showed a considerably greater reduction in the amount of drug released within 3 min. However, all sodium phenobarbital tablets exhibited complete drug release at the end of 30 min. thus indicating no drug–binder interaction.

While the usefulness of microcrystalline cellulose as a dry binder-disintegrator has previously been reported (6), the results in Table VII serve to emphasize the uniqueness of the material when used in such a manner. The properties of the ephedrine hydrochloride and quinine sulfate products illustrate the outstanding tablet quality routinely available through the use of microcrystalline cellulose as a dry binder–disintegrator. It would certainly appear that microcrystalline cellulose should be given serious consideration by tablet formulators as a dry binder, particularly in direct compression.

SUMMARY AND CONCLUSIONS

1. It was found that increased humidities caused a softening and a tendency to swell of plain microcrystalline cellulose tablets. Neither tablet change is permanent since they disappear upon removal of the humid conditions. Elevated temperatures do not affect tablets of this material.

2. The disintegration time of such tablets is markedly influenced by the polarity of the disintegrating medium. From the environment and disintegration observations, it was postulated that a plain microcrystalline cellulose tablet is a special form of cellulose fibril which is held together largely by hydrogen bonding.

3. Hopper segregation was studied. The results of the experiment indicate that no significant separation need be anticipated.

4. Amphetamine sulfate and sodium phenobarbital tablets prepared utilizing a blending of spray-dried lactose and microcrystalline cellulose as a filler exhibited no problems in respect to dissolution of active ingredients.

5. The characteristics of tablets produced with microcrystalline cellulose as a dry binder–disintegrator were outstanding.

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Effect of Physical Activity on the Absorption Rates of Procaine Penicillin G Implants

By BERTON E. BALLARD

The pellet implantation technique was used to estimate quantitatively the effect of rat body movement on solid drug absorption rate. Animals placed in a rodent activity cage and rotated at 2.81 and 3.83 r.p.m. showed significantly greater mean absorption rates per mean area for procaine penicillin G pellets than nonrotated controls.

To DATE no study has appeared in the literature which shows the possible quantitative relationship that may exist between drug pellet absorption rate and degree of animal physical activity (1). Such a study would have theoretical as well as practical significance, because the intensity of the pharmacological, toxicological, and therapeutic response to an implanted solid drug may be directly related to the magnitude of the absorption rate of the drug.

MATERIAL AND METHODS

Implants.—Commercially available procaine penicillin G was the model drug used in these studies. After it was recrystallized from water and dried in a desiccator for 48 or more hr., it was compressed at 2.81×10^3 Kg./cm.^{2} on a Carver laboratory press designed to allow use of standard tableting machine punches and dies. The mean diameter of the disks was 0.639 cm. and their mean initial weight was 66.9 mg. (range 51.8 to 86.9 mg.). The mean apparent density of the disks was 1.167 Gm./cm.^{3}. No binders, excipients, diluents, or lubricants were added.

Implantation.—Female Sprague-Dawley rats having a mean weight of 228 Gm. (range 182 to 298 Gm.) were used in these tests. Animal weight was not rigidly controlled, because it was obvious by inspection that no correlation could be found between disk absorption rate and animal weight for any given rotational velocity. The animals were anesthetized with ethyl ether, and a ventral midline incision of about 1-cm. length was made in the abdominal skin in an anatomical region that has been defined before (2). The subcutaneous connective tissue lateral to the incision was teased apart to provide sites for the implantation of 2 preweighed disks, one to either side of the midline incision. The incision was sutured closed following the implantation of the disks. After the "rest" and exercise periods, the animals were reanesthetized and the implants removed. The disks were briefly washed with distilled water and placed to air dry for 24 hr. or more on pieces of filter paper. The disks were reweighed and the mean absorption rate per mean area, $\bar{R}/\bar{A}$, was calculated by methods previously described (3), except that no correction for the proteinaceous "ghost" weight was made, because of the short implantation times used.

Activity.—After implantation of the 2 disks, the animals were allowed to recover ("rest") from effects of anesthesia in their cages for about 2 hr. They then were placed in a rodent activity cage[1] for varying periods of time, depending in part upon the rotational velocity of the cage. The cage has a circumference of 1.13 M., and was rotated at a constant angular velocity by means of a dual shaft electronic controlled mixer[2] fitted with a small pulley having an outside diameter of 2.1 cm. A piece of laboratory rubber tubing about 140 cm. long and 8 mm. in outside diameter with the ends stapled together was wound around part of the circumferences of the pulley and cage. The revolutions per minute (r.p.m.) shown in Table I were read at the end of each exercise period from a counter attached to the wheel.

Calculation.—Because periods of rest (r) and exercise (e) varied from animal to animal and from one rotational velocity to another, Eq. 1 was used to account for these variables.

$$(\bar{R}/\bar{A})_e = \frac{[(\bar{R}/\bar{A})_t \cdot T_t] - [(\bar{R}/\bar{A})_r \cdot T_r]}{T_e} \quad \text{(Eq. 1)}$$

where $(\bar{R}/\bar{A})_e$ is the mean absorption rate per mean area attributed to the exercise period in the activity cage; $(\bar{R}/\bar{A})_t$ is the mean absorption rate per mean area for the total implantation time, T_t; the term $(\bar{R}/\bar{A})_r$ is the mean absorption rate per mean area for the rest period; T_r is the total time an implanted animal was "resting" or outside the activity cage and is the sum of the times after implantation and before exercise and after exercise up to the time of implant removal; and T_e is the time that the implanted animal was "exercising" in the activity cage.

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[1] Rodent activity cage (AC-66F), Acme Animal Care Equipment, Chicago, Ill.

[2] Cole-Palmer Instrument and Equipment Co. (No. 4650), Chicago, Ill.

TABLE I.—INTENSITY OF PHYSICAL ACTIVITY AND PROCAINE PENICILLIN G ABSORPTION DATA

r.p.m.[a]	T_e[b] (hr.)	T_r[c] (hr.)	Distance Traveled, M.	$(\bar{R}/\bar{A})_e$[d] (± 95% Confidence Limits) × 10^4 Gm./hr./cm.^{2}	p[e]
0 (12)	0	6.27	...	1.93[d] (0.29)	...
1.04 (6)	3.79	3.23	267	2.18 (0.00)	$0.20 < p < 0.25$
2.05 (8)	3.19	3.15	443	1.97 (0.00)	$0.8 < p < 0.9$
2.81 (12)	2.98	3.08	567	2.34 (0.04)	$0.001 < p < 0.005$
3.83 (7)	2.00	3.26	519	2.57 (0.03)	$0.001 < p < 0.005$

[a] Weighted mean revolutions of activity cage per minute. Number of animals is in parentheses. [b] Mean time for exercise in activity case. [c] Mean time an implanted animal was "resting" or outside the activity cage. [d] Mean absorption rate per mean area attributed to the exercise period in the activity cage as calculated by Eq. 1. The value for zero r.p.m., included for comparison purposes, is actually $(\bar{R}/A)_r$. The 95% confidence limits appear in parentheses. [e] The p value from the 2-tail t test when comparing the mean absorption rate per mean area for zero r.p.m. with rates at another r.p.m. The values at 1.04 and 2.05 r.p.m. should be considered as not significant.

RESULTS AND DISCUSSION

The results of this experiment summarized in Table I showed that when the animals' activity was substantially increased over normal values there was also a significant increase in the magnitude of the mean disk absorption rate per mean area. The present findings tend to substantiate the unverified suggestion made by Kearns (4) that the absorption rate of an implanted pellet (a steroid) could be increased if the patient would massage daily the skin area over the drug.

The results also show that the degree of dispersion about the mean values of $\bar{R}/\bar{A}$ (as reflected by the 95% confidence limits) is much broader for the animals "resting" than it is for those moving at a constant velocity on the activity wheel. The broader dispersion seen in the mean values for the "resting" animals might be expected because there was a wide variation in the intensity of body movement brought on by the use of ether as the anesthetic.

Some clinicians (5, 6) have studied the correlation between physical activity and the magnitude and duration of penicillin serum levels following an intramuscular injection of procaine or benzathine penicillin G. Elevations in penicillin serum levels observed by these investigators after vigorous exercise could be explained by the results of this experiment. If human physical activity increased the dissolution rate of the depot penicillin salt crystals, then elevations in the drug serum level should logically follow, assuming that drug elimination mechanisms remained unchanged before and after exercise.

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Cholinesterase Activity and Sulfonamide Absorption in Rats

By VERNON A. GREEN, THOMAS M. GLENN, SAMUEL J. STRADA, and GONZALO MEDINA

Rats administered, orally, a combination of neostigmine methylsulfate and sulfonamide showed an increased 4-hr. blood sulfonamide level as compared to animals administered only sulfonamide. Blood cholinesterase activity, 4 hr. after treatment, was determined and compared with that of control animals.

OVER A half-century ago Barbour and Abel (1) found that physostigmine increased the rate of diffusion of acid fuchsin in frogs. Lewis (2), in 1916, reported the same to be true for trypan red in dogs. Twenty-two years later Cole and Curtis (3) showed that acetylcholine increased the permeability of the marine organism *Nitella*. In the last decade there have been reports of the potentiation of the action of morphine in cats (4), streptomycin in rats (5), and barbital in mice (6) by pretreating with cholinesterase inhibitors. Other reported changes in drug activity brought about by anticholinesterases include the potentiation of the anesthetic activity of a given dose of phenobarbital and pentothal (7) and a lengthening of the duration of the anesthesia.

The increased permeability of *Nitella* in the presence of acetylcholine and the like phenomena seen in *P. vulgarus*, *S. typhosa*, and *P. aeruginosa* with

Received April 2, 1965, from the School of Pharmacy, University of Missouri at Kansas City, Kansas City.
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TABLE I.—INTENSITY OF PHYSICAL ACTIVITY AND PROCAINE PENICILLIN G ABSORPTION DATA

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TABLE I.—RAT BLOOD CHOLINESTERASE ACTIVITY[a]

Group	Animals, No.	Units of Activity[b]	S.D.	% Depression
Controls	20	0.61	0.07	...
Neostigmine methylsulfate	20	0.49	0.04	20

[a] Neostigmine dose 6 mcg./Kg. [b] Mean value for 20 animals.

Table II.—4-hr. BLOOD SULFONAMIDE[a] LEVELS IN RATS

Group	Animals, No.	mcg./ml.	S.E.
Sulfacetamide	16	1.29	0.1
Sulfacetamide Neostigmine methylsulfate[b]	14	2.83	0.38
Sulfanilamide	16	1.45	0.12
Sulfanilamide Neostigmine methylsulfate	14	4.10	0.37
Sulfaguanidine	16	6.38	0.08
Sulfaguanidine Neostigmine methylsulfate	12	7.35	0.23

[a] All sulfonamides given orally 400 mg./Kg. [b] Neostigmine methylsulfate given orally 6 mcg./Kg.

eserine (8) would indicate a possible relationship between cholinesterase or acetylcholine and membrane permeability in these species.

Cholinesterase and acetylcholine are found throughout the body of higher animals. Alteration of either could affect the tissue permeability which would influence drug absorption. Neostigmine methylsulfate-induced increases of absorption of sulfisoxazole from everted sacs of guinea pig ileum (9) are indicative of the above.

In this work the *in vivo* absorption of sulfonamides in the presence of an anticholinesterase was studied and a comparison made with the sulfonamide absorption in animals with sulfonamide alone. Blood cholinesterase activity was determined 4 hr. after treatment to ascertain the anticholinesterase activity of the neostigmine methylsulfate.

EXPERIMENTAL

Cholinesterase Depression.—In order to correlate any change in sulfonamide absorption with the depression of the esterase, the activity of the enzyme was determined in untreated rats and rats receiving 6 mcg./Kg. of neostigmine methylsulfate 4 hr. prior to withdrawal of blood sample. Blood was removed by cardiac puncture; the enzyme determination was made immediately and expressed in units.[1] The depression was determined to be approximately 20% as shown in Table I.

Sulfonamide Determination.—Sulfacetamide, sulfanilamide, and sulfaguanidine were administered, 400 mg./Kg. orally, to randomly sexed Sprague-Dawley rats weighing 225–275 Gm. Twenty animals were used in each group. A similar number of animals was used for the same sulfonamides administered at the same dose but in combination with 6 mcg./Kg. of neostigmine methylsulfate.

Four hours after the administration of the sulfonamide, 2 ml. of blood to be used for the total sulfonamide determination was removed by cardiac puncture. Samples that clotted before being mixed with the buffer or that may have been diluted with tissue fluid were discarded.

The method used to determine the sulfonamide concentration was the colorimetric method for measuring free and total sulfonamides in tissue used by Bratton and Marshall (10).

Neostigmine methylsulfate increased the blood levels with all three sulfonamides. (Table II.)

DISCUSSION

From the data obtained in this study, it appears that neostigmine methylsulfate increased the absorption of the sulfonamides from the gastrointestinal tract. The observed increase in blood sulfonamide is in agreement with that reported earlier for sulfisoxazole in guinea pigs (9).

It appears probable that the inhibition of cholinesterase, by some mechanism, alters the permeability of the intestinal tract to allow for greater absorption of the drug. It is possible that the anticholinesterase by some combination with the enzyme as a part of cell membrane may change the characteristics of the membrane to allow for greater permeability. Furthermore, it may be that the acetylcholine accumulates in sufficient quantities to change the permeability as it does in *Nitella*. If vasodilation due to the accumulation of acetylcholine is the basic factor in the increased permeability, it should be blocked by atropine. The failure of atropine to block this phenomenon has been shown earlier by Green (7). It appears that cholinesterase–inhibitor combination is the most likely explanation for the observed increases in absorption.

SUMMARY

In this study, rats receiving neostigmine–sulfonamide combinations, orally, showed higher blood levels of the sulfonamides than animals receiving sulfonamide alone. The neostigmine methylsulfate-treated animals showed a 20% depression of blood cholinesterase activity.

Numerous drug agents have anticholinesterase activity. If esterase activity affects absorption, increased absorption of the inhibitor-drug or other drugs used in combination could be a factor in creating undesirable responses. The investigators feel that these findings warrant further investigation.

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[1] A unit of cholinesterase, as used in this study, is defined as the number of milliliters of 0.1 *M* acetic acid derived from 3 ml. of 0.1 *M* acetylcholine bromide solution by the cholinesterase activity in 1 ml. of blood at pH 8.

Repeated Test of Mice in the Photocell Activity Cage After Different Time Intervals

By NATHAN WATZMAN, HERBERT BARRY, III, WILLIAM J. KINNARD, JR., and JOSEPH P. BUCKLEY

A total of 384 mice, given 2-hr. tests twice in the photocell activity cage under the same conditions, showed lower activity scores (with square root transformation) in the second test. This behavioral carry-over effect was greater for subgroups given the second test at an interval of 1 or 3, instead of 7 or 14 days, after the first. Chlorpromazine produced a greater decrement in activity in the first 0.5 hr. of the second test than of the first. In both tests, aggregations of five, rather than single animals, showed a greater drug decrement in the first hour and some recovery from the drug effect in the last 0.5 hr. The most stable data were obtained in the first 0.5 hr. A high correlation between tests, especially with a 1-day interval, indicated that a repeated-test design can provide a sensitive measure of drug effects.

IT IS HIGHLY desirable to give repeated tests to the same animals when evaluating the effects of psychotropic agents on behavior. Not only is it an economic advantage, but a more sensitive test is obtained, because the consistency of individual performances usually found in repeated tests means a small variation in scores against which the effects of drugs can be measured.

The disadvantage of repeated tests on the same animals is that both drug and behavioral carry-over effects may occur from one test to the next, especially when there is a short time interval between sessions. In order to assess this disadvantage, it is necessary to measure the degree of this carry-over effect at different intersession intervals, with the experimental conditions held constant in both sessions. This was the purpose of the study reported in the present paper.

The photocell activity cage, which has been used extensively to study the depressant properties of chlorpromazine (1–3), was used as the test apparatus.

METHOD

Subjects.—The subjects were 384 male, Swiss-Webster mice (Taconic Farms, N. Y.) weighing approximately 18–20 Gm. Housing and testing were in separate rooms, both with the temperature controlled between 73 and 75°F. Food and water were available *ad libitum*.

Apparatus.—The experimental work was performed in four 6-beam photocell activity cages, circular in shape (Actophotometers, Metro Industries, Inc.). The electronic circuits of each cage are designed so that a single digital counter (General Controls, Des Plaines, Ill.) is activated whenever an animal blocks, or moves through any beam, regardless of whether any of the other beams are interrupted. Therefore, an animal continuously blocking a single beam could not preclude the registration of counts emanating from the other beams. The experimental room was sound attenuated, and was effectively shielded from the noise of the counters which were placed in an adjoining room.

Experimental Design.—Each animal, or group of animals, was tested in 2 sessions, both 2 hr. in duration. Several experimental conditions were varied in a complete factorial design, so that all levels of each variable were equally represented as follows.

Intersession Interval.—1, 3, 7, and 14 days.

Drug Condition.—Chlorpromazine (4 mg./Kg.) or placebo (saline, 0.1 ml./10 Gm. body weight), given orally 30 min. before the start of the session. Each animal was assigned to the same condition (drug or placebo) in both tests.

Test Aggregation.—Singly, or in groups of five mice. They were housed during the intersession interval under the same aggregation conditions as during testing.

Different Units.—Four activity cages operated simultaneously.

Replication.—The complete design was repeated once.

This experimental design resulted in 128 different combinations of conditions, divided equally between single animals and groups of five.

Statistical Treatment of Data.—Normalization of skewed frequency distributions by means of data transformation is an acceptable statistical technique in research. Extremely high raw scores are likely to lead to a misinterpretation of relative magnitudes, if they are farther from the median values than the lowest scores. Logarithmic, or square root, transformation of data may provide a nonskewed, normal distribution of scores by reducing the magnitude of the high scores more than the low ones. The parametric tests of statistical significance, including the analysis of variance, depend on the assumption that the frequency distribution of scores is nonskewed.

A comparison of the raw, square root, and logarithmic forms of these experimental data indicated that the raw scores were skewed in a positive direction, with the extreme high scores being much farther from the median than were the extreme low scores, whereas the logarithmic scores were skewed in the opposite, negative direction. The square root scores were skewed to the least degree (positive direction), especially in the first hour of the 2-hr. session. Also, activity appeared to be the most consistent and correlations between the two sessions highest for the scores in square root form. Irwin (4) has used square root transformation of data on locomotor activity in the treadwheel.

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The computer analysis was done at the University of Pittsburgh Computation and Data Processing Center, supported by grant G-11309 from the National Science Foundation, Washington, D. C.

Mr. Jerome V. Lisovich provided the programing of the data analysis for the IBM 7090 computer.

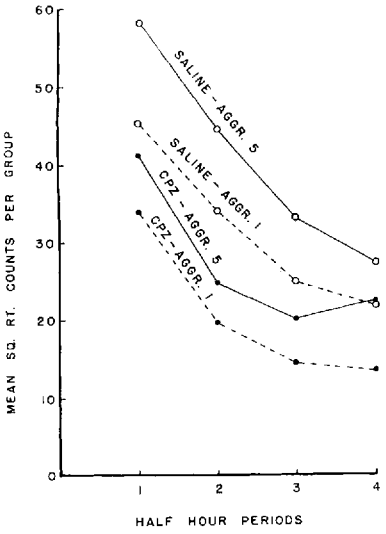

Fig. 1.—The effects of chlorpromazine and aggregation on the spontaneous activity of mice.

In the present study, the number of counts for each 0.5 hr., hour, and the 2-hr. total in both sessions were punched on IBM cards, converted to square root scores by the IBM 7090 computer, and tested for statistical significance by the BMD 02V analysis of variance program on the same computer. Each fixed factor (drug, aggregation, intersession interval) was tested for statistical significance in relation to the pooled interactions, which included the interaction of the randomly selected variables (test units with replication) and either or both variables with one or more of the fixed variables (5). Since each animal was tested in both sessions, the pooled interaction term for testing the difference between the sessions, and interactions of any other variables with sessions, included the session variable. Correlations between the first and second session for all the animals, and for each level of the selected variables, were computed by the BMD 03D program. Stability of performance within the same session was computed according to the split-half method (6), for 0.5-hr. intervals, utilizing a specialized program written for the IBM 7090 computer. This method correlates the performance of an animal, or aggregation of animals, in the even-time segments (second and fourth 0.5-hr. periods) with the scores recorded in the odd-time segments (first and third 0.5-hr. periods). The test of statistical significance for the difference between correlation coefficients was that described by Edwards (7).

RESULTS

Figure 1 shows the effects of two variables (drug and aggregation) on activity in each 0.5-hr. period, with scores for the other experimental variables averaged together. In the first 0.5 hr., there was a highly significant difference between the chlorpromazine and saline conditions ($F = 174$, $df = 1,117$, $p < 0.01$) and between animals tested singly and in groups of five ($F = 90$, $df = 1,117$, $p < 0.01$). There was also a significant interaction between drug and aggregation, indicating that chlorpromazine had a greater effect on the activity of aggregated than single mice for the first 0.5 hr. ($F = 5.76$, $df = 1,117$, $p < 0.05$). This interaction was also significant for the second 0.5 hr. ($F = 5.31$, $df = 1,117$, $p < 0.05$), but not for the last two 0.5-hr. periods. These findings confirm results reported previously by Watzman *et al.* (2), utilizing raw scores.

In the last 0.5 hr. of the session, the animals tested in groups of five showed some recovery from the depressant effects of the drug. Out of 32 groups of aggregated animals under chlorpromazine, 25 showed higher activity in the fourth than in the third 0.5 hr., and only seven groups were lower. This recovery from the depressant effects of the drug was statistically significant ($\chi^2 = 9.0$, $df = 1$, $p < 0.01$) and is in contrast with the lower activity in the fourth than third 0.5-hr. period found with each of the other three conditions shown in Fig. 1. In general, all of these experimental effects decreased in statistical significance in the later 0.5-hr. periods, due to a decrease in stability of the scores. However, the difference between animals tested singly and in groups of five continued to be highly significant, even in the last 0.5-hr. period ($F = 25$, $df = 1,117$, $p < 0.01$).

The replication showed no consistently significant difference from the original experiment. There was a significant difference among the four test units ($F = 9$, $df = 3,117$, $p < 0.01$) for the entire 2 hr., but no consistently significant interaction between this variable and any of the others.

The main subject of interest in the present experiment is the comparison between the first and second test session, shown in Fig. 2. The activity counts were preponderantly lower in the second session ($F = 48$, $df = 1,112$, $p < 0.01$ for the total 2 hr.). This decrement in second-session performance was greater for the groups tested at shorter time intervals (1 and 3 days). This difference, generally, persisted throughout the second session, but was statistically significant for the first 0.5 hr. only, measured by the interaction of sessions with the four intervals ($F = 3.06$, $df = 3,112$, $p < 0.05$). When the test for linearity of the scores is applied, with the assumption that the 1, 3, 7, and 14-day intervals represent a progressive function, the interaction between tests and intervals is statistically significant

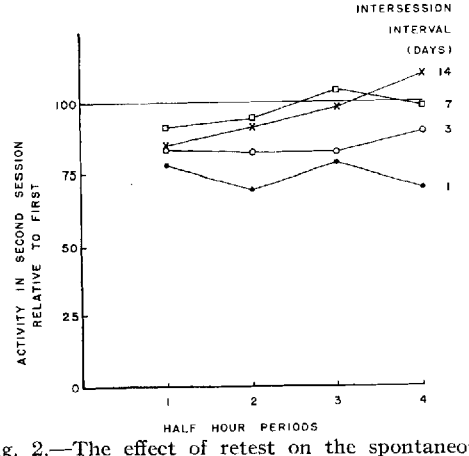

Fig. 2.—The effect of retest on the spontaneous activity of mice at 1, 3, 7, and 14-day intervals.

($p < 0.05$) in each of the 0.5-hr. periods, except the third ($F = 4.6$, 6.0, 3.7, and 6.5, respectively, $df = 1,112$).

In the first 0.5 hr., chlorpromazine produced a 23% decrement in activity in the first session and a 32% decrement in the second session. This difference was statistically significant, as indicated by the interaction between sessions and drug conditions ($F = 4.68$, $df = 1,112$, $p < 0.05$). This difference was approximately equal for the groups given the two tests at different intervals ($F < 1$ for the three-way interaction among drug conditions, sessions, and intervals). In the last three 0.5-hr. periods, the drug-induced decrement in activity was approximately equal in the two sessions, as indicated by the absence of any significant interaction between sessions and drug conditions. The other variables did not interact significantly with the two sessions, and therefore showed similar patterns in both tests.

There was a high degree of stability of performance within each session, with the split-half correlation being 0.75 for session *1* and 0.74 for session *2*. The over-all correlation between the two sessions, pooling all of the other parameters together, was 0.74 for the total 2 hr. As would be expected, the correlation between sessions was greater for the shorter intersession intervals, with the stability scores for the total 2 hr. being 0.84, 0.78, 0.80, and 0.61, respectively, for the groups given the second session 1, 3, 7, and 14 days after the first. The correlation between sessions was highest in the first 0.5 hr. of the session (0.73) and lowest in the fourth 0.5 hr. (0.35), and this difference was statistically significant at the 1% level. For the total 2 hr., this stability score showed little difference between saline (0.62) and drug treatment (0.57), but in the first 0.5 hr., the saline scores were significantly more stable, with correlations between the sessions of 0.68 for saline and 0.46 for chlorpromazine ($p < 0.05$). In the first 0.5 hr., the correlation between sessions was higher for the animals tested in groups of five (0.74) than those tested singly (0.60), but this difference was not statistically significant.

DISCUSSION

The present experiment clearly shows a behavioral carry-over effect from one session to the next for both saline and chlorpromazine animals. After an interval of 1 or 3 days between tests, activity in the second session was substantially lower than in the first, in all four 0.5-hr. periods. Even at the 7 and 14-day intervals, the recovery of the original activity level was not complete, as shown by significantly lower activity in the first 0.5 hr. for these two groups pooled together ($F = 20$, $df = 156$, $p < 0.01$.)

The dosage of chlorpromazine used (4 mg./Kg., orally) greatly decreased the activity of mice under all the experimental conditions tested. However, the magnitude of this drug effect was somewhat altered by certain experimental conditions. The greater decrement produced by chlorpromazine in the first two 0.5-hr. periods, for animals tested in groups of five rather than singly, confirms findings previously reported (2). Since the present experiment used square root transformations which produced normally distributed scores, this interaction between drug condition and aggregation appears to be a general phenomenon, not depending on the skewed distribution of the raw scores used in the prior study. The present experiment also showed that the aggregated condition, which produced a greater decrement under the drug in the first hour, produced an earlier recovery from the drug effect, indicated by the statistically significant increase in activity from the third to the fourth 0.5 hr. The stimulating aggregation situation, in which activity was decreased to a greater degree by chlorpromazine, apparently caused a more rapid recovery from the depressant effects of the drug.

The present experiment also demonstrated a greater depressant effect of chlorpromazine on activity in the first 0.5 hr. of the second session than of the first session. Adler (8), also utilizing the photocell activity cage, found a greater depressant effect of tetrabenazine on the motor activity of rats in the second of two tests with a 1-week intertrial period. Rushton *et al.* (9) found different magnitudes of drug effect on exploratory activity of rats, depending on whether the drug test was on their second rather than first exposure to the test situation, and also depending on whether the drug test was preceded by a drug or placebo test in the same situation. The stimulating effect of an amphetamine–barbiturate mixture was apparently greater in the second test, when the same drug was given in both tests. In the present experiment, and in Adler's study (8), the animals were given the same drug, or placebo, condition in both tests, so that it is not possible to determine whether the greater depressant drug effect in the second test was due solely to a greater familiarity with the test situation, or to the prior administration of the drug associated with this test situation. However, this effect was clearly not due to accumulation of the drug from the first session, because the greater drug effect was found in the second session after the 14-day interval, as well as after the 1-day interval.

The behavioral and drug carry-over effects, from the first to the second session, indicate that the experimenter should be careful when using the same animals more than once in tests of drug effects. It is obviously necessary to counterbalance the sequence, giving placebo first to half the animals and the drug first to the other half. On the other hand, the high over-all correlation in activity between the first and second sessions, especially in the early part of the sessions, shows that the use of repeated tests on the same animal can increase the sensitivity of the test of drugs, or other conditions which are experimentally varied in the different test sessions. With the factorial design as used in the present experiment, the advantages of repeated testing can be fully obtained, even when there is a decrement in performance in the second session and a different magnitude of drug effect in the two sessions. It should be emphasized that, in the present experiment, these differential effects in the two sessions were fairly small, relative to the magnitude of the over-all drug effects and to the effects of other conditions, such as aggregation and 0.5-hr. intervals. The same general pattern of depressant drug effects was found in both sessions. Therefore, the effects of the drug could be tested adequately with a greatly reduced number of animals by the use of the repeated test design, giving chlorpromazine in one session and placebo in the other, at least for this dosage of this compound and under these conditions.

The most stable data were obtained in the first

0.5 hr. of the test, and with the shortest interval between sessions. These conclusions are based on the higher correlations between the two tests for the first 0.5 hr. and for the 1-day interval. The disadvantage of the greater decrement in activity after the shortest intersession interval is offset by the generally higher correlations between the first and second sessions found for the 1-day group, as well as by the practical advantage of using a shorter interval. These findings may be of use to experimenters in selecting the optimum conditions for testing the effect of chlorpromazine on the activity of mice in the photocell cage.

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Preliminary Investigations of *Heracleum mantegazzianum*

By EUGENE C. LEE, PHILIP CATALFOMO, and LEO A. SCIUCHETTI

Preliminary investigations of the air-dried roots of *Heracleum mantegazzianum* grown under greenhouse conditions were conducted. Seed germination requires moist cold treatment. The germination rate appears to be directly related to the length of the moist cold period. Gibberellin seed treatment did not substitute for the cold requirement. Thin-layer chromatography revealed the presence of 6 coumarins which were tentatively identified as bergapten, isobergapten, pimpinellen, isopimpinellen, sphondin, and umbelliferone. Results also indicate that the plant can cause photosensitization.

EXCELLENT reviews concerning the distribution, chemistry, or pharmacological properties of the naturally occurring coumarins are available (1–5); included are phytochemical studies revealing the presence of coumarins in a number of *Heracleum* species. A notable aspect is the involvement of furocoumarins in certain cases of phytophotodermatitis; several of them occur in *Heracleum* species. The distribution of photodynamically active furocoumarins were recently reviewed by Pathak *et al.* (6). Although phytochemical investigations of the coumarins of *Heracleum* species has been extensive, the species *Heracleum mantegazzianum* Somm. et Lev. is a noteworthy exception. However, during the course of this investigation, a report by Beyrich (7) revealed the presence of phellopterin and other coumarins in this species, but the results are not entirely consistent with those reported in this investigation. Since *H. mantegazzianum* has been reported to evoke phytodermatitis, a preliminary investigation was undertaken to determine the presence of photosensitizing coumarins and related compounds.

To obtain sufficient plant material for this and subsequent studies the plant was propagated under greenhouse conditions. Seeds of the *Umbelliferae* have been noted for germination difficulties, and germination standards for cultivated members of this order have been set much lower than those of other plants (8). The seeds of some *Heracleum* species have a requirement for after ripening in moist cold (9). Attempts have also been made to obviate the cold requirement in the dormancy of certain seeds by chemical means, especially with the gibberellins (10). Since no report could be found in the literature concerning the cold requirement of the effects of gibberellic acid on seed germination of *H. mantegazzianum*, preliminary germination studies were also conducted.

EXPERIMENTAL AND RESULTS

Germination Studies.—Preliminary studies were designed to compare the effect of cold treatment *versus* treatment with gibberellic acid on the germination rate of the seeds. Three groups of seeds (39 per group) were planted in flats containing a mixture of 1 part sand and 2 parts sandy loam plus 50 Gm. of complete fertilizer.[1] Group *A* was pretreated by storage at 2–5° for 74 days; group *B* was soaked for 20 hr. in a solution of gibberellic acid (100 p.p.m.); and group *C*, soaked in distilled water, was considered the control group. The flats were maintained under normal greenhouse conditions and germination was allowed to occur at a temperature range of 18–27° for 38 days. Ger-

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Abstracted in part from a thesis submitted by Eugene C. Lee to the Graduate School, Oregon State University, Corvallis, in partial fulfillment of the Master of Science degree requirements.
Presented to Section Np, A.A.A.S., Berkeley meeting, December 1965.

[1] Organic Morcrop, Chas. Lilly Co., Seattle, Wash. (Analysis: 5% total nitrogen, 3% available phosphate, 2% available potash.)

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mination did not occur in group *B* or group *C;* however, 4 seeds in group *A* germinated. Although this is a low germination rate (10.3%), it was apparent that cold treatment is required for germination. Based on these results, 1000 selected seeds were soaked for 36 hr. and refrigerated at 2–5° for 294 days. This treatment resulted in a 55% germination.

Preparation of Root Material.—To obtain root material for subsequent investigations, seeds were cold treated and germinated on moist blotting paper in Petri dishes. After germination occurred, they were transplanted in peat pots containing the soil mixture previously described. After 92 days the plants (average height 33.5 cm.) were harvested, the roots washed free of soil, and separated from the plant. The roots were allowed to dry at room temperature for 8 weeks. The dried material was milled to a No. 20 powder in a Wiley mill and the powder placed in air-tight colored glass containers until utilized in subsequent analyses.

Extraction of Coumarins.—Approximately 13.5 Gm. of dried powdered root was placed in a Soxhlet-type apparatus and extracted to exhaustion with 100 ml. of ethyl ether. The extractive was filtered and the filtrate evaporated on a steam bath to a syrupy residue. The residue was washed 3 times with 2-ml. portions of petroleum ether and redissolved in a mixture containing equal parts of ethyl ether and alcohol to a total volume of 10 ml. in a volumetric flask. The extract was stored in a refrigerator until subjected to chromatographic analysis.

Chromatographic Analysis.—Tentative identification of the coumarins was accomplished by employing two-dimensional thin-layer chromatography on Silica Gel G. The extract was applied in 20-μl. portions to thin-layer plates, 200 mm. on each side. The plates were developed in ethyl acetate–xylene (1:1) in the first direction and hexane–ethyl acetate (2:1) in the second direction. The solvent front was allowed to proceed a distance of 100 mm. in each direction. Solutions of known coumarins which were chromatographed singly, in mixture, and in combination with extracts, were compared to chromatoplates of extracts without additions. The coumarins were detected by examining the plates under ultraviolet light. This procedure revealed the presence of 7 principal spots which were tentatively identified as bergapten, isobergapten, pimpinellin, isopimpinellin, sphondin, and umbelliferone. The seventh spot, which fluoresced blue, was not identified, but co-chromatography indicated that it was not imperatorin.

DISCUSSION

The germination studies indicated that seeds of *H. mantegazzianum* will germinate only after extended cold treatment since no seeds germinated in the group where this procedure was omitted. Seeds exposed to moist cold for 74 days showed a germination rate of 10.3%. When the cold period was extended to 294 days, the germination rate increased to 55%. It appears, therefore, that the germination rate may be directly related to the period of moist cold treatment. An attempt to overcome this requirement by soaking in a solution of gibberellic acid met with no success. Similar attempts by Stuart and Cathey (10), but with sweet cherry and peach, indicated that gibberellic acid only partially substituted for cold treatment. Although gibberellins have been credited with substituting for other germination conditions, *i.e.*, light requirement, they do not adequately substitute for the cold treatment (10).

Thin-layer chromatographic analysis of the ether extract of air-dried root revealed the probable presence of 6 coumarins: bergapten, isobergapten, pimpinellin, isopimpinellin, sphondin, and umbelliferone, the last being the only nonfurocoumarin. A seventh spot, which fluoresced blue, was not imperatorin. A preliminary report by Beyrich (7), who investigated the fruits of *H. mantegazzianum*, showed the presence of pimpinellin and isopimpinellin in addition to the following furocoumarins: angelicin, xanthotoxin, imperatorin, and phellopterin. Since he examined a different plant part, direct comparison of results is precluded.

The plant under investigation has been shown to evoke phytophotodermatitis (6). The furocoumarins, especially bergapten and xanthotoxin, cause dermatitis. Since these compounds in all probability appear to exist in *H. mantegazzianum*, as well as others possibly having similar biological activity, this plant should be considered a photosensitizing species.

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Stability of the Folin Phenol Reagent

By ROBERT C. PETERSON

A comprehensive study has been made of the stability of the Folin phenol reagent as the initial acidic concentrate. Even though refrigerated, an apparent continuing and linear decomposition of about 2 per cent per month occurs which should be considered in the determination of mcg. amounts of protein.

DURING work in which it was attempted to determine the specific activity of trypsin, the protein content of electrophoretically fractionated zones was determined with the Lowry (1) modification of the Folin-Ciocalteu (2) reagent.

A satisfactory standard curve could be prepared for protein concentration, but when a known sample was referred to it at a later time, the sample value was always low. It was found that the low values were produced by a slow but consistent deterioration of the Folin reagent whether it was from commercial sources[1] or prepared in our laboratory according to Folin-Ciocalteu (2). This deterioration occurred even though the reagent was refrigerated (6°) immediately after preparation and removed only when portions were desired for dilution in the protein tests.

The Folin phenol reagent is known to be rapidly destroyed by an alkaline solution (1), but this writer is not aware of any comments in the literature regarding its instability as the initially prepared acid.

Since this reagent is continually used, it seemed worthwhile to acquire more knowledge concerning its stability. It was felt that reagent prepared in the laboratory and used shortly thereafter would give the greatest response, and so a preparation was made as mentioned above and immediately refrigerated. The reagent titrated as 2.5 *N* acid with 0.5 *N* NaOH in the presence of phenol red; Fisher reagent gave the same value under these conditions. The laboratory reagent was tested approximately once a month for 6 to 7 months.

METHODS AND MATERIALS

The standard protein was N.F. Trypsin Crystallized Reference Standard, lot 6040 (3). A new solution, 5 mg. in 100 ml. of water, was made up for each test. A second standard was L-tyrosine.[2] A new solution, 3 mg. in 100 ml. of water, was made up for each test.

The phenol reagent was tested for optimal response according to Oyama and Eagle (4) and their Lowry reagent modifications were used. Reagent *A* consisted of 20 Gm. of Na_2CO_3, 4 Gm. of NaOH, and 0.2 Gm. of $K_2C_4H_4O_6 \cdot 1/2\ H_2O$ made to 1 L. with water. Reagent *B* was 0.5 Gm. of $CuSO_4 \cdot 5H_2O$ made to 100 ml. with water. Reagent *C* was made freshly before each test series from reagents *A* and *B*:50 to 1 by volume. For each test 0.5 ml. of each standard was diluted to 1 ml. with water and this solution was reacted with 2.5 ml. of reagent *C* for 10 min. Folin reagent, 0.25 ml., at various dilutions with water (4) was jetted in and mixed thoroughly. The absorbance was determined at 750 mμ every 30 min. for 2 hr. with a Beckman model DU spectrophotometer. For each test the optimum response of the Folin reagent was considered to be the absorbance value from the phenol reagent dilution which gave the greatest stability of color, *i.e.*, the absorbance values produced a plateau. These absorbance values, determined at approximately 1-month intervals, gave an indication of the stability of the phenol reagent.

With L-tyrosine, 15 mcg. per test, the curve in Fig. 1 was obtained when absorbance was plotted against time elapsed since preparation of the reagent. Figure 2 presents similar data for 25 mcg. of trypsin per test.

DISCUSSION

In all cases it was found that the 1 to 2 dilution of phenol reagent gave optimum response.

The continual deterioration of the phenol reagent is obvious. With trypsin this is a decrease in absorbance of 0.0044 every 30 days. With L-tyrosine, the decrease is greater.

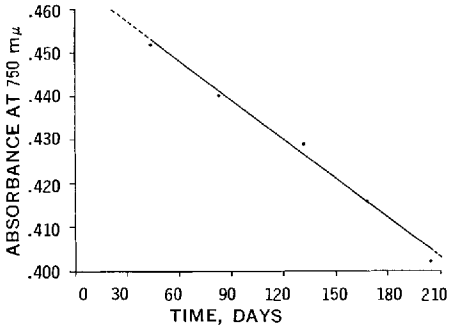

Fig. 1.—Lowry test with L-tyrosine as related to age of phenol reagent.

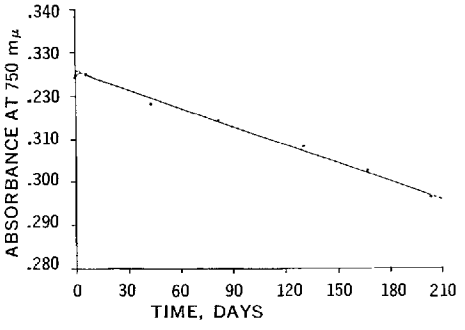

Fig. 2.—Lowry test with trypsin as related to age of phenol reagent.

Received February 7, 1966, from the Analytical Research Department, Eli Lilly and Co., Indianapolis, Ind.
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The author expresses his gratitude for the assistance of Mr. Robert Beck of these laboratories.

[1] Fisher Scientific Co.; Hartman-Leddon Co.
[2] M. A., lot MJ999, Mann Research Laboratories.

For accurate quantitative use of the Folin phenol reagent, either its response as related to a standard decomposition curve must be considered or its activity must be correlated with a known amount of standard protein or amino acid.

It follows that to get sensitivity with the reagent with microgram quantities of protein, the state of decay of the reagent must be known. If deterioration is sufficiently great, either fresh reagent must be used or the quantity must be increased.

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Nuclear Magnetic Resonance Spectra of Amines II. Identification of *N*-Phenyl Amines

By R. J. WARREN, W. E. THOMPSON, J. E. ZAREMBO, and I. B. EISDORFER

The effects of a strongly acidic solvent on the chemical shift and spin-spin splitting of the phenyl protons in *N*-phenyl amines are characteristic for this functional group. These effects provide the basis for the identification of the *N*-phenyl group in primary, secondary, and tertiary amines.

THE EFFECT of protonation of amines containing *N*-methyl groups has been recently reported by these laboratories (1). The authors wish to report on the *N*-phenyl group as characteristically identified by examination of the NMR spectrum of free base in deuterated chloroform and the spectrum in trifluoroacetic acid.

EXPERIMENTAL

All NMR spectra were recorded on a Varian A-60 spectrometer using Varian sample tubes. Deuterated chloroform and trifluoroacetic acid were used as solvents. Spectra were obtained on samples at room temperature at a concentration of 50 mg./ml.

The *N*-phenyl amines used were Eastman organic chemicals as purchased from Distillation Products Industries, Rochester, N. Y., or K and K chemicals as purchased from the K and K Laboratories, Plainview, N. Y.

RESULTS AND DISCUSSION

The pronounced change in the aromatic proton pattern of an *N*-phenyl amine free base on conversion to the amine cation is illustrated in Fig. 1, curve A, diphenylamine in deuterated chloroform, and curve B, diphenylamine in trifluoroacetic acid. This phenomenon, the collapse of a complex A_2B_2C aromatic pattern into a simple peak (or narrow band of peaks), is general for any protonatable *N*-phenyl group with no other substituents on the *N*-phenyl ring. The collapse of this pattern can be attributed to equalization of the chemical shifts of the phenyl protons. The principal cause of inequality of chemical shifts for the phenyl protons in an amine free base is conjugation of the amine group with the phenyl ring. Protonation of the amine blocks this conjugation and results in nearly uniform chemical shifts for the protons on the benzene ring. The reduction of conjugation of an *N*-phenyl amine on formation of the amine cation is well established in the theory of ultraviolet spectra for anilines (2).

The authors have found the collapse of the A_2B_2C spectral pattern of the *N*-phenyl group useful for determining whether one or more *N*-phenyl groups in an unknown compound have other substituents on the *N*-phenyl ring. For example 3-chloro-*N*-phenyl aniline shows 2 species of protons in trifluoroacetic acid due to the *meta* substituted benzene ring.

The NMR data for 5 representative *N*-phenyl reference compounds are listed in Table I. It should be noted that acidic solvents, such as aqueous hydrochloric and sulfuric acids, all influence the NMR absorption pattern of the *N*-phenyl group in the same fashion, regardless of whether the amine is primary, secondary, or tertiary.

If the *N*-phenyl group is close to another aromatic ring, the asymmetric magnetic field generated by the second aromatic ring may prevent observation of collapse of the A_2B_2C pattern on cation formation. For example, the NMR spectra for *N*-methyl-*N*-phenyl-benzylamine (Fig. 2) show a more complex pattern for the ion (Fig. 2, B) than for the free base (Fig. 2, A). The authors have observed a similar

TABLE I.—NMR CHEMICAL SHIFTS FOR *N*-PHENYL GROUPS IN ANILINE AND *N*-SUBSTITUTED ANILINES

Compd.	Appropriate Range of Complex A_2B_2C Aromatic Pattern in $CDCl_3$, p.p.m.	Chemical Shift Downfield from Tetramethylsilane for Single Aromatic *N*-Phenyl Peak in CF_3COOH, p.p.m.
Aniline	6.7–7.4	7.52
Diphenylamine	6.5–7.5	7.60
N,N-Dimethyl-aniline	6.3–7.4	7.62
N-Methylaniline	6.6–7.4	7.55
N-Methyldi-phenylamine	6.7–7.4	7.65

Received November 3, 1965, from Smith Kline & French Laboratories, Philadelphia, Pa.
Accepted for publication January 4, 1966.

For accurate quantitative use of the Folin phenol reagent, either its response as related to a standard decomposition curve must be considered or its activity must be correlated with a known amount of standard protein or amino acid.

It follows that to get sensitivity with the reagent with microgram quantities of protein, the state of decay of the reagent must be known. If deterioration is sufficiently great, either fresh reagent must be used or the quantity must be increased.

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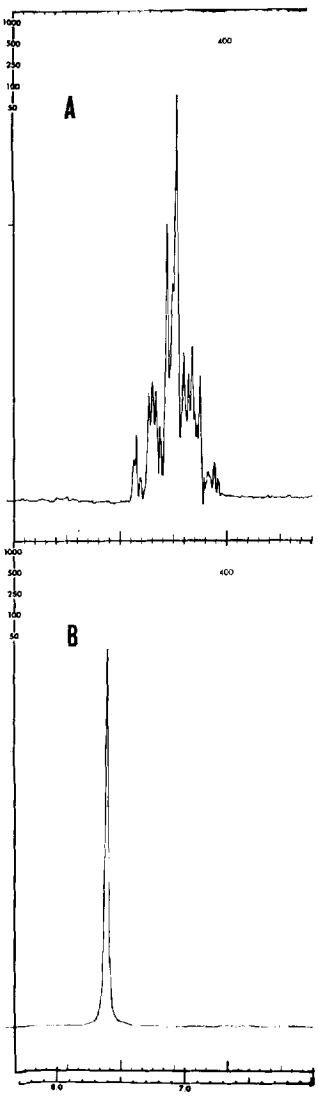

Fig. 1.—NMR spectra of diphenylamine. Key: A, solvent, deuterated chloroform; B, solvent, trifluoroacetic acid.

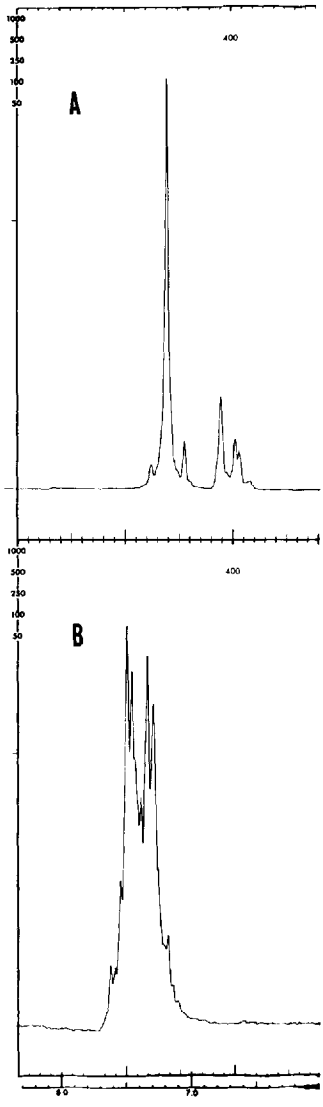

Fig. 2.—NMR spectra of *N*-methyl-*N*-phenlybenzylamine. Key: A, solvent, deuterated chloroform; B, solvent, trifluoroacetic acid.

lack of collapse for the *N*-phenyl protons of *N*-phenylbenzylamine.

CONCLUSION

The difference between the NMR spectrum of the *N*-phenyl amine free base and that of the *N*-phenyl amine ion is a useful diagnostic tool for structure determinations of these compounds. This difference makes it possible to establish the presence of the *N*-phenyl structures in primary, secondary, and tertiary amines. Interference with identification of the *N*-phenyl group may occur from other aromatic rings in the molecule. It is conceivable that long-range effects of any functional group possessing magnetic anisotropy could cause some interference; however, no examples of this effect were noted in this study.

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Interaction Between Pharmaceuticals and Sodium Polyethylenesulfonate

By G. LEONARDI, M. SOLINAS, and C. BOTRÉ

Several drugs, mainly in the field of local anesthetics, were studied with a synthetic polyelectrolyte, sodium polyethylenesulfonate, by means of membrane equilibrium dialysis measurements. Data on equilibrium constants of the association between the drugs considered, as hydrochlorides, and the polyelectrolyte are reported.

THE STUDY of interactions between drugs and macromolecules has received increasing interest recently (1–5).

The result of such interaction, in general, is the formation of association complexes between the drug and the macromolecule, which may lead to unusual features, both from physicochemical and biological points of view.

In some cases, for instance, the pharmacological activity of the complex is improved with respect to the single drug, either in the sense of the intensity and/or the duration of action.

This paper reports data about the interaction observed between several local anesthetics and a synthetic polyelectrolyte, *i.e.*, sodium polyethylenesulfonate.

This study was performed mainly by means of membrane equilibrium dialysis according to a procedure proposed by Klotz *et al.* (6).

The equilibrium constants of associations between drugs and the polyelectrolyte are reported.

EXPERIMENTAL

The sample of sodium polyethylenesulfonate (NaPES) was prepared according to Breslow and Kutner (7). Its molecular weight was about 7,000. The dissociation degree (α) of the NaPES was determined potentiometrically in an aqueous solution by using a cationic glass electrode (Beckman catalog No. 39728) and saturated calomel electrodes connected to the solution by means of an agar bridge. The procedure and the apparatus were extensively described in a previous paper (8). The experimental value of α was 0.25, in good agreement with the theoretical value calculated according to Oosawa (9).

All the pharmaceuticals were used as hydrochlorides. They are listed in Table I together with the wavelength corresponding to a maximum of absorbance. Table I also reports association constants and pKa values at 25° (10).

Conductivity measurements were performed in a thermostatic bath at 25 ± 0.02° using a WTW bridge model LF 3.

The dialysis experiments were carried out with cellophane bags (HMC type). These were previously treated with boiling water for about 5 min., washed with cold water, wiped, filled with a known volume of the NaPES solution, and then dipped into a known volume of drug solution.

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The polymer concentration was 10^{-3} *M;* the drug concentration was 10^{-4} *M*. The equilibrium across the membrane was reached at room temperature after about 48 hr. No change in volume inside or outside the bag was observed.

The concentration of the drug in solution was determined with a grating spectrophotometer (Hitachi, Perkin-Elmer, model 139). The measurements of the absorbance were performed in each case at the wavelength corresponding to the maximum value of the absorption of the drug. Quartz cells, 1 mm. optical path, were employed.

At each concentration a blank test was set up. In this case, water was placed inside the bag instead of polyelectrolyte solution. The amount of drug bound to the polyelectrolyte was determined by measuring the concentration of the solution outside the bag containing the polyelectrolyte as well as the outside concentration of the drug in the blank.

RESULTS AND DISCUSSION

In order to prove that in the range of concentration considered no aggregation takes place in solution of the drugs considered, specific conductivity was measured at different concentrations. In each case the plots of specific conductivity *versus* the concentration gave straight lines. On the other hand, it was shown that when aggregation of drug molecules in solution occurs, a break in the plot of specific conductivity *versus* concentration may be detected (2).

The data obtained from the membrane equilibrium dialysis were treated according to the method of Klotz *et al.* (6). Such treatment, in fact, may be applied to a general association equilibrium between a polyelectrolyte (P) and a drug (D), for which

$$P + D \rightleftharpoons PD \qquad \text{(Eq. 1)}$$

The equilibrium constant for the ith association, K_i, is:

$$K_i = \frac{n - (i - 1)}{i} k \qquad \text{(Eq. 2)}$$

When $i = 1$, first equilibrium constant, Eq. 2 reduces to

$$K = nk \qquad \text{(Eq. 3)}$$

where n = number of sites available along the polymer
K = equilibrium constant
k = constant which depends on the nature of the anion

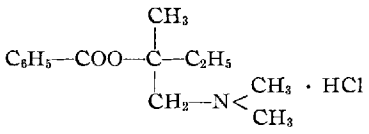
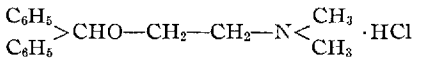
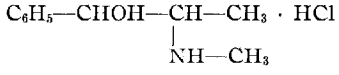
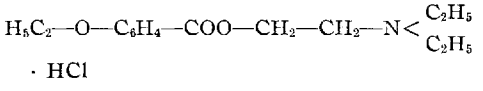
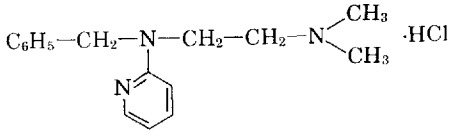
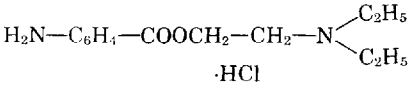
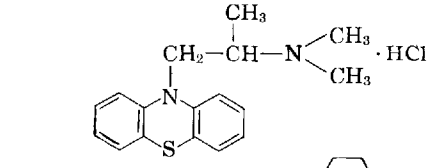
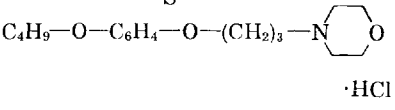
The relationship which correlates the number of sites available along the polymer to the interaction with the molecules of the drug can be written as follows:

$$\frac{1}{r} = \frac{1}{n\,k\,D} + \frac{1}{n} \qquad \text{(Eq. 4)}$$

where r is the ratio between the number of moles of bound drug and total moles of polymer.

By plotting $1/r$ *versus* the reciprocal of nonbound

TABLE I.—ASSOCIATION CONSTANTS FOR DRUG–POLYELECTROLYTE SYSTEM

Hydrochlorides of	Formula	λ mμ	Association Constant $K \times 10^{-4}$	pK_a at 25°
Amylocaine		232	7.94	8.40
Benzocaine	$H_2N—C_6H_4—COOC_2H_5 \cdot HCl$	235	...	2.43
Diphenhydramine		250	6.52	9.00
d-Ephedrine		210	25.00	9.55
Parethoxycaine		260	6.25	...
Tripelennamine		305	7.60	8.96
Procaine		290	3.33	8.95
Promethazine		248	16.95	8.65
Pramoxine		224	5.91	...

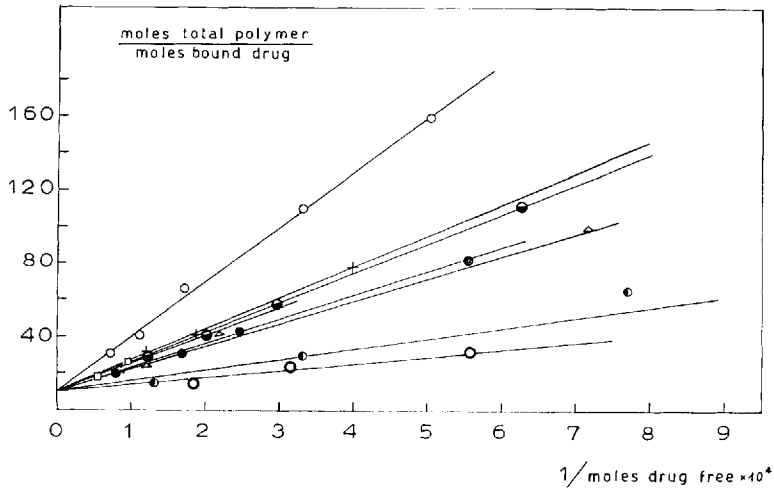


Fig. 1.—Ratio between total moles of polymer and moles of bound drug *vs.* the reciprocal of free drug molecules. Key: ○, procaine; ◐, promethazine; +, pramoxine; ○, *d*-ephedrine; ●, tripelennamine; ◒, parethoxycaine; △, amylocaine; □, diphenhydramine.

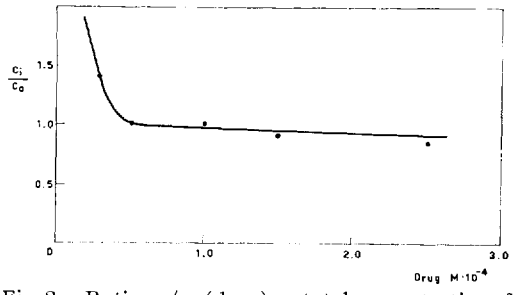


Fig. 2.—Ratio, c_i/c_0, (drug) *vs.* total concentration of the drug.

By solving the system one obtains,

$$(0.5\,a_0 - a_i)x_0^2 + (Da_i - Pa_i + a_0a_i - 0.5\,a_0^2)x_0 + (DPa_i - Da_0a_i) = 0 \quad \text{(Eq. 5)}$$

By substituting in Eq. 5 the values of D, P, a_0, and a_i, respectively, the values of x_0 and x_i are obtained.

In Fig. 2, the ratio between inside molar concentration of the drug, c_i, and outside molar concentration, c_0, is plotted against the total concentration of the drug. Figure 3 reports the ratio, c_i/c_0, respective to the chloride ions as a function of the total concentration of the drug.

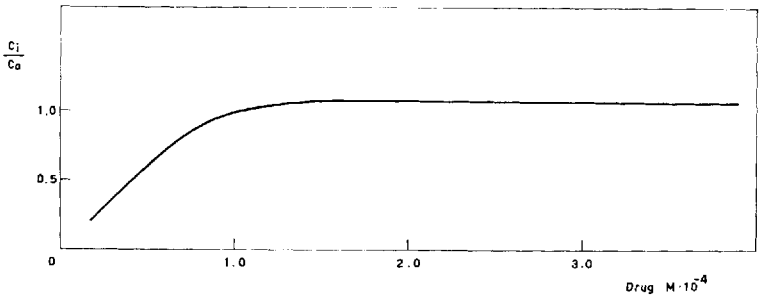


Fig. 3.—Ratio, c_i/c_0 (chloride ion) *vs.* total concentration of the drug.

moles of drug (Fig. 1), straight lines are obtained, showing that the electrostatic effect can be neglected with respect to the statistical effect. This is reasonable since the concentration of the solution is very low. The number of free sites of the polymer was, under our conditions, roughly 10 times the concentration of the drugs considered.

Under the experimental conditions of this work, the Donnan effect may be neglected, since we have

$$\begin{cases} \dfrac{x_i}{v_i} \cdot \dfrac{a_i}{v_i} \cdot \dfrac{y_i}{v_i} = \dfrac{x_0}{v_0} \cdot \dfrac{a_0}{v_0} \cdot \dfrac{y_0}{v_0} \cdot \\ \dfrac{y_0}{v_0} = \dfrac{1}{v_0}(a_0 - x_0) \end{cases}$$

and

$$\begin{cases} \dfrac{D - x_0}{v_i} \cdot \dfrac{a_i}{v_i} \cdot \dfrac{P - y_0}{v_i} = \dfrac{x_0}{v_0} \cdot \dfrac{a_0}{v_0} \cdot \dfrac{y_0}{v_0} \\ y_0 = a_0 - x_0 \end{cases}$$

where D = mmoles of total drug
P = mmoles of polymer (inside)
a_0 = mmoles of chloride outside
a_i = mmoles of chloride inside [this value was obtained by Kennon *et al.* (11)]
x_0 = mmoles of drug outside
x_i = mmoles of drug inside
v_0 = outside volume of solution (20 ml.)
v_i = inside volume of solution (10 ml.)
v_i/v_0 = 0.5
y_0 = mmoles of sodium outside
y_i = mmoles of sodium inside

The trend of the values, c_i/c_0 *versus* the concentration of the drug, shows that in the considered range of concentrations, they may be considered roughly constant and near unity. Therefore, under the above-mentioned conditions the Donnan effect can be neglected.

The association constants between the drugs and polyelectrolyte reported in Table I are approximately higher for monomethyl derivatives (*d*-ephedrine), followed by dimethyl and diethyl derivatives, while no significant differences can be observed in their respective pKa values.

Further studies are in progress to evaluate the role of the steric hindrance on such interactions.

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Evaluation of *N*-Haloacyl Analogs of α,α-Diphenyl-4-piperidinemethanol Against Ehrlich Ascites Carcinoma in Mice

By WILLIAM D. ROLL

The antitumor activity of 8 haloamide derivatives of α,α-diphenyl-4-piperidinemethanol was studied in C3H agouti mice against the Ehrlich ascites carcinoma. The preliminary screening studies would seem to indicate that the chemotherapeutic effect of these compounds is potentially significant. There seems to be a definite correlation between structure and activity within the propionamide and acetamide analogs in that within each group of compounds the iodoamides have the highest order of activity; the bromoamides have intermediate activity; and the chloroamides have the least activity. The haloacetamide analogs seem to be more toxic than the corresponding halopropionamide derivatives.

In a previous paper (1) the synthesis and potential antitumor activity of a series of *N*-haloacyl derivatives of α,α-diphenyl-4-piperidinemethanol were reported. A continuation of the antitumor screening study shows that the compounds (Table I) have significant activity against Ehrlich ascites tumor in C3H mice.

EXPERIMENTAL[1]

Experiments were performed on 5–10-week-old C3H agouti mice purchased from the R. B. Jackson Memorial Laboratory, Bar Harbor, Maine. The testing procedure used was similar to that described by Linder (2) and Hauschka *et al.* (3).

Transplantation of the tumor was carried out by aseptically withdrawing ascites fluid from a donor mouse bearing a 7-day ascites tumor. Each experimental animal was inoculated i.p. with 2×10^6 ascites cells. Ten animals were used as controls and 10 animals for treatment. Mice were distributed into groups of comparable weight and treatment was begun 24 hr. after tumor implantation. The compounds were administered in peanut oil (1%) and control animals received corresponding volumes of peanut oil alone. Change in body weight was noted as a measure of the accumulation of tumor cells and ascitic fluid. Cell counts were used as a measure of the inhibition of tumor cell growth. The dosages recorded in Table II were administered in single intraperitoneal injections per day commencing 24 hr. after transplantation of the tumor and continuing for 3 days. The results recorded in Table II were determined on the sixth day after intraperitoneal transplantation of the tumor.

RESULTS

The chemotherapeutic activity of the halopropionamide analogs (I, IV, VII) against ascites tumor showed that the iodopropionyl derivative (VII) exhibited the greatest inhibition, and the relative activity within this group of compounds was VII > IV > I. The same order of activity was noted with regard to the haloacetamide analogs: iodoacetamide > bromoacetamide > chloroacetamide. The toxicity of haloacetyl derivatives was greater than that of the halopropionyl analogs. (See *Footnote c*, Table II.)

Table I.—Derivatives of α,α-Diphenyl-4-piperidinemethanol

Compd.
1-(3-Chloropropionyl)-α,α-diphenyl-4-piperidinemethanol (I)
1-(2-Chloropropionyl)-α,α-diphenyl-4-piperidinemethanol (II)
1-Chloroacetyl-α,α-diphenyl-4-piperidinemethanol (III)
1-(3-Bromopropionyl)-α,α-diphenyl-4-piperidinemethanol (IV)
1-(2-Bromopropionyl)-α,α-diphenyl-4-piperidinemethanol (V)
1-Bromoacetyl-α,α-diphenyl-4-piperidinemethanol (VI)
1-(3-Iodopropionyl)-α,α-diphenyl-4-piperidinemethanol (VII)
1-Iodoacetyl-α,α-diphenyl-4-piperidinemethanol (VIII)

Table II.—Results of Screening Tests *Versus* the Ehrlich Ascites Carcinoma[a]

Compd.	Dosage, mg./Kg./Day[c]	Mortality Treated Group	Av. Wt. Change, *T/C*. Gm.	Growth Inhibition Ratio[b]
I	20	0/10	1.9/4.5	5:1
II	20	0/10	0.5/4.4	2:1
III	10	0/10	−1.7/3.5	3:1
IV	20	0/10	3.2/5.3	20:1
V	20	0/10	−1.6/4.5	13:1
VI	10	2/10	−3.6/4.1	32:1
VII	20	0/10	−0.6/4.5	75:1
VIII	10	0/10	−2.1/4.2	27:1

[a] *T* = treated group; *C* = control group. [b] Ratio of the total number of tumor cells in control mice to number in treated animals. [c] Represents one-half the LD_{50} as determined in C3H mice.

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Accepted for publication January 13, 1966.
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[1] The author wishes to thank Dr. Robert J. Schlembach and members of the staff of Maumee Valley Hospital, Toledo, Ohio, for their assistance in the testing of these compounds; and Dr. Dante Scarpelli, Ohio State University, for providing us with the original donor animals.

Saccharin Derivatives VIII. Hypotensive Agents

By GLENN H. HAMOR

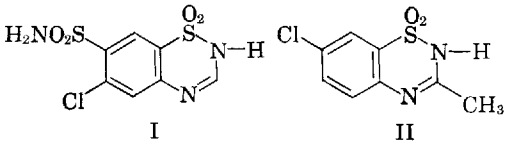
Saccharin analogs of chlorothiazide have been reported to possess diuretic activity. This paper relates the synthesis of 6-chloro-2-methylsaccharin, which is structurally similar to 7-chloro-3-methyl-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide), a non-diuretic, hypotensive benzothiadiazine. The 6-chloro-2-methylsaccharin along with some 9 related saccharin derivatives, which had been synthesized earlier in a continuing study of saccharin chemistry, were screened for pharmacological activity. Preliminary results indicate hypotensive effects in cats and dogs, with little diuretic response. In fact, 2-ethyl-4-nitrosaccharin showed antidiuretic properties. In addition to producing a moderate transient hypotension in the anesthetized dog, 6-chloro-2-methylsaccharin produced CNS stimulation in rats.

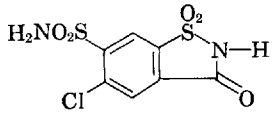
THE DIURETIC activity of compounds related to 6-chloro-7-sulfamoyl-1,2,4-benzothiadiazine 1,1-dioxide (chlorothiazide) (I) is well known (1). Certain of them also show hypotensive activity and are used clinically in the treatment of mild hypertension (2). Several recent papers describe closely related substances lacking the sulfamoyl group, exemplified by 7-chloro-3-methyl-1,2,4-benzothiadiazine 1,1-dioxide (diazoxide) (II), as possessing antihypertensive but not diuretic activity (3–6).

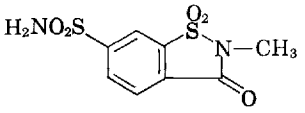
The saccharin analogs, 5-chloro-6-sulfamoylsaccharin (III) (7) and 2-methyl-6-sulfamoylsaccharin (IV) (8), are reported to exhibit diuretic activity.[1] This paper relates the results of preliminary pharmacological testing of some 9 saccharins corresponding to the hypotensive 1,2,4-benzothiadiazines in not containing an extranuclear sulfamoyl group. The synthesis of 6-chloro-2-methylsaccharin (V) is described. The remaining 8 saccharin derivatives had been prepared earlier in a continuing study of saccharin chemistry (9–11).

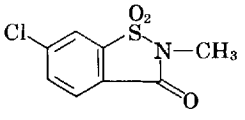
Because Topliss *et al.* (4) had reported most of the hypotensive activity of the benzothiadiazines to be retained if the chlorine at position 7 were replaced by nitro, various nitrosaccharins and related compounds were selected for testing (Table I). The 6-chloro-2-methylsaccharin was synthesized by chlorosulfonation of 4-chlorotoluene, followed by treatment with aqueous ammonia, which gave 4-chlorotoluene-2-sulfonamide. Oxidation of this compound succeeded by a Williamson reaction of the resulting 6-chlorosaccharin with methyl iodide gave the desired 6-chloro-2-methylsaccharin.

Preliminary pharmacological results[2] indicate these compounds to possess hypotensive effects, on intravenous injection in cats and dogs, of primarily a transient nature. The 4-nitro-2-*n*-propylsaccharin (compound *4*) shows some evidence of autonomic ganglionic blocking. These substances possess little diuretic response. In fact, 2-ethyl-4-nitrosaccharin (compound *3*) showed antidiuretic properties. In contrast, 2-methyl-6-sulfamoylsaccharin (IV), the diuretic compound, produced transient pressor effects in the cat after doses of 0.5–10.0 mg./Kg. intravenously. In addition to producing a moderate transient hypotension following administration of 5 and 10 mg./Kg. i.v. in the dog, 6-chloro-2-methylsaccharin (V) caused CNS stimulation in rats orally at 300 mg./Kg. It may be of interest to note that this saccharin compound (V) is tasteless, as have been other *N*-substituted saccharins in our experience.









EXPERIMENTAL

6-Chloro-2-methylsaccharin (V).[3]—In a 200-ml. round-bottom flask fitted with a reflux condenser and heating mantle were placed 5.0 Gm. (0.023 mole) of 6-chlorosaccharin,[4] m.p. 217° [reported m.p. 218° (12)], 1.3 Gm. (0.012 mole) of sodium carbonate, and 100 ml. of dimethylformamide. To this was added 7.3 Gm. (0.051 mole) of methyl iodide. The solution was refluxed for 1 hr. and then poured into approximtely 700 ml. of ice water. The mixture was cooled for 30 min., then filtered and washed with 2 portions of cold water to give 3.2 Gm. (60%) of white solid, m.p. 174–175.5°.[5] Recrystallization

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Presented to the (Pharmaceutical Sciences) Section (Np), American Association for the Advancement of Science, Berkeley, Calif., meeting, December 1965.
Previous paper: Hamor, G. H., *J. Pharm. Sci.*, **52**, 603 (1963).

[1] However, deStevens reports III to be inactive as a diuretic. (See *Reference 1*, p. 113.)

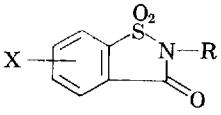
[2] The author is indebted to Smith Kline & French Laboratories, Philadelphia, Pa., for pharmacological testing.

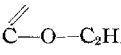
[3] *Chemical Abstracts* nomenclature, 6-chloro-2-methyl-1,2-benzisothiazolin-3-one 1,1-dioxide.

[4] The author thanks Bernard L. Reavlin for synthesis of the sample of 6-chlorosaccharin.

[5] Melting points were determined by the open capillary tube method and are uncorrected.

TABLE 1.—SACCHARIN DERIVATIVES



Compd.	X	R	M.p., °C.[a]	Ref.
IV	6-SO_2NH_2	CH_3	230–232	(8)
V	6-Cl	CH_3	180–181	This paper
1	6-NO_2	*n*-C_3H_7	120–121	(9)
2	6-NO_2	*i*-C_3H_7	148	(14)
3	4-NO_2	C_2H_5	184.5–185.5	(10)
4	4-NO_2	*n*-C_3H_7	138–139.5	(10)
5	H	*i*-C_3H_7	62–64	(14, 15)
6	H		136	(11, 13)
7	H	$OCH(CH_3)_2$	136–137	(14, 15)
8	H	$N(C_2H_5)_2$	206–207	(11)

[a] Melting points were determined either with a Fisher-Johns melting point apparatus or by the capillary tube method and are uncorrected.

from acetone–water gave white needle crystals, m.p. 180–181°.

Anal.[6]—Calcd. for $C_8H_6ClNO_3S$: C, 41.50; H, 2.61. Found: C, 41.60; H, 2.73.

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[6] Analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif.

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(14) Hamor, G. H., unpublished data.
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Thin-Layer Chromatography of Cardiac Glycosides

By EUGENE J. JOHNSTON and ALLEN L. JACOBS

A rapid thin-layer chromatographic procedure for the separation and identification of the common cardiac glycosides is presented. A benzene–ethanol solvent is used for development and a perchloric acid spray for visualization.

THIN-LAYER chromatography has proven to be more rapid and sensitive than paper chromatography for the identification and purity determination of many drugs. A number of solvent systems and sprays useful for cardiac glycosides have been published (1–4). This paper reports the development of a relatively simple technique which has certain advantages over these approaches.

Received January 10, 1966, from the Analytical Research Department, Sandoz Pharmaceuticals, Hanover, N. J.
Accepted for publication February 21, 1966.

EXPERIMENTAL

Thin-Layer Plates.—A 0.25-mm. layer of Silica Gel G (E. Merck, Darmstadt) is applied to the plates. The plates are air dried for 10 min., then heated in an oven for 45 min. at 120°. The plates are stored in a desiccator and used without further activation.

Solvent System.—Benzene–95% ethanol (7:3 v/v).

Spray Reagent.—Fifteen milliliters of 70% perchloric acid added to 100 ml. of water.

Preparation of Samples.—The substances are dissolved in a suitable solvent, usually methanol. For purity studies, 100 mcg. of substance is spotted on

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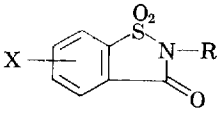

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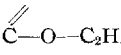
6	H		136	(11, 13)
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Preparation of Samples.—The substances are dissolved in a suitable solvent, usually methanol. For purity studies, 100 mcg. of substance is spotted on

TABLE I.—CHROMATOGRAPHIC DATA

Substance	R_f	Fluorescence
Acetyl digitoxin	0.82	Red
Digitoxin	0.72	Red
Digoxin	0.62	Blue
Lanatoside A	0.52	Red
Lanatoside B	0.41	Red
Lanatoside C	0.36	Blue
Desacetyl lanatoside C	0.27	Blue
Ouabain	0.09	Yellow-green

the plate, although for simple detection 1 mcg. is sufficient.

Visualization.—The plates are sprayed, then placed in a 100° oven for a few minutes. The perchloric acid produces a charring effect and a fluorescence which is visible under long-wave ultraviolet light (366 mμ.).

DISCUSSION

The sensitivity of detection by fluorescence is augmented by the visualization of the spots in ordinary light. Good separation of the common cardiac glycosides can be obtained (Table I). The presence of certain contaminants is easily detected. The system has the advantages of simplicity, speed (approximately 0.5 hr.), efficiency of separation, and avoids the use of hazardous spray reagents, such as antimony trichloride.

Good results can also be obtained using a spray consisting of 0.5 ml. of *p*-anisaldehyde in 50 ml. of glacial acetic acid and 1 ml. of concentrated sulfuric acid (5). After heating the plate for a few minutes at 100°, the glycosides appear as blue spots with the exception of ouabain which appears as a yellow spot.

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Determination of Organically-Bound Iodine in Pharmaceuticals

By GERALD J. YAKATAN* and MURRAY M. TUCKERMAN

Eight official iodinated compounds with iodine contents from 50 to 66 per cent were assayed by the methods official in U.S.P. XVI and N.F. XI, by reduction with zinc in alkali, and by a standard procedure using oxygen flask combustion. An additional four compounds, formerly official, with iodine content as low as 23 per cent were also examined. The results indicate that the suggested standard procedure is equal or superior to the other methods in recovery of iodine and generally equivalent in reproducibility. The alkaline zinc reduction method is suggested for those compounds having an electronegative substituent *ortho* or *para* to the iodine atoms on the aromatic ring. The oxygen flask combustion is suggested for all other iodinated compounds and as a general method. The suggested standard procedure consists of combustion by the oxygen flask method, absorption of the combustion products in an alkaline sulfite solution, and titration of the acidified solution of iodide with standard silver nitrate using a silver-calomel electrode pair for potentiometric determination of the end point.

THE QUANTITATIVE determination of iodine in organic compounds has long proved a source of difficulty for the analytical chemist. This is reflected in the numerous proposals for the determination of iodine in organic substances. An assay procedure for the determination of iodine in an organic compound actually involves two problems. First, the organic compound must be decomposed to liberate the iodine, and then the iodine must be quantitatively determined. By far the more difficult of these problems is finding an efficient method for the decomposition of the organic compound which will not result in the loss of any of the iodine present.

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Abstracted in part from a thesis submitted by Gerald J. Yakatan to the Graduate School, Temple University, Philadelphia, Pa., in partial fulfillment of Master of Science degree requirements.
The authors thank Ciba Pharmaceutical Co., Mallinckrodt Pharmaceuticals, Metalsalts Corp., Schering Corp., G. D. Searle and Co., E. R. Squibb and Sons, and Winthrop Pharmaceuticals for supplying the materials used in this study.
* National Science Foundation Summer Fellow, 1964. Present address: College of Pharmacy, University of Florida, Gainesville.

EXPERIMENTAL

Reagents.—All reagents employed were reagent grade chemicals. All standard solutions employed were prepared and standardized according to the official compendia.

Methods.—*A. Parr Bomb Method* (*1–3*).—The organic matter is oxidized by fusion with sodium peroxide in a bomb and the halide present is converted to sodium halide.

B. Alkaline Permanganate Method (*4*, *5*).—This assay is based on the conversion of organically-bound iodine to iodide by the action of permanganate in alkali and reduction with bisulfite.

C. Zinc–Sodium Hydroxide Method (*6*).—In this method the organic iodine is replaced with hydrogen generated in the nascent form by the reaction of zinc and sodium hydroxide.

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DISCUSSION

The sensitivity of detection by fluorescence is augmented by the visualization of the spots in ordinary light. Good separation of the common cardiac glycosides can be obtained (Table I). The presence of certain contaminants is easily detected. The system has the advantages of simplicity, speed (approximately 0.5 hr.), efficiency of separation, and avoids the use of hazardous spray reagents, such as antimony trichloride.

Good results can also be obtained using a spray consisting of 0.5 ml. of *p*-anisaldehyde in 50 ml. of glacial acetic acid and 1 ml. of concentrated sulfuric acid (5). After heating the plate for a few minutes at 100°, the glycosides appear as blue spots with the exception of ouabain which appears as a yellow spot.

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Determination of Organically-Bound Iodine in Pharmaceuticals

By GERALD J. YAKATAN* and MURRAY M. TUCKERMAN

Eight official iodinated compounds with iodine contents from 50 to 66 per cent were assayed by the methods official in U.S.P. XVI and N.F. XI, by reduction with zinc in alkali, and by a standard procedure using oxygen flask combustion. An additional four compounds, formerly official, with iodine content as low as 23 per cent were also examined. The results indicate that the suggested standard procedure is equal or superior to the other methods in recovery of iodine and generally equivalent in reproducibility. The alkaline zinc reduction method is suggested for those compounds having an electronegative substituent *ortho* or *para* to the iodine atoms on the aromatic ring. The oxygen flask combustion is suggested for all other iodinated compounds and as a general method. The suggested standard procedure consists of combustion by the oxygen flask method, absorption of the combustion products in an alkaline sulfite solution, and titration of the acidified solution of iodide with standard silver nitrate using a silver-calomel electrode pair for potentiometric determination of the end point.

THE QUANTITATIVE determination of iodine in organic compounds has long proved a source of difficulty for the analytical chemist. This is reflected in the numerous proposals for the determination of iodine in organic substances. An assay procedure for the determination of iodine in an organic compound actually involves two problems. First, the organic compound must be decomposed to liberate the iodine, and then the iodine must be quantitatively determined. By far the more difficult of these problems is finding an efficient method for the decomposition of the organic compound which will not result in the loss of any of the iodine present.

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EXPERIMENTAL

Reagents.—All reagents employed were reagent grade chemicals. All standard solutions employed were prepared and standardized according to the official compendia.

Methods.—*A. Parr Bomb Method* (*1–3*).—The organic matter is oxidized by fusion with sodium peroxide in a bomb and the halide present is converted to sodium halide.

B. Alkaline Permanganate Method (*4*, *5*).—This assay is based on the conversion of organically-bound iodine to iodide by the action of permanganate in alkali and reduction with bisulfite.

C. Zinc–Sodium Hydroxide Method (*6*).—In this method the organic iodine is replaced with hydrogen generated in the nascent form by the reaction of zinc and sodium hydroxide.

TABLE I.—IODINE CONTENT DETERMINED BY VARIOUS METHODS OF ANALYSIS

Compd.[a]	Theoretical % I	Official Methods[b] A Results % I	A No. of Detn.	B Results % I	B No. of Detn.	C Results % I	C No. of Detn.	Proposed Method[c] D Results % I	D No. of Detn.
Chiniofon	26.5–29[d]	...	...	23.6 ± .4	3	23.9 ± .3	3	23.8 ± .1	3
Diiodohydroxyquin U.S.P.	63.9	...	...	59.2 ± .8	6	...[e]	...	63.6 ± .3	5
Iodipamide U.S.P.	66.8	...	...	...	...	65.4 ± .1	3	66.3 ± .5	4
Iodoalphionic acid N.F.	51.4	...	...	50.7 ± .5	3	49.8 ± .6	3	51.7 ± .2	4
Iodochlorhydroxyquin U.S.P.	41.5[f]	40.6 ± .8	3	...	...	...[e]	...	40.7 ± .2	4
Iodophthalein	61.8	...	...	60.3 ± .2	3	57.0 ± 1.0	4	60.6 ± .6	4
Iodopyracet N.F.	49.8	...	...	47.1 ± .1	3	46.9 ± .1	4	48.4 ± .4	4
Iopanoic acid U.S.P.	66.7	66.5 ± .5	3	...	...	64.5 ± .7	3	66.6 ± .6	4
Sodium methiodal N.F.	52.0	...	...	49.5 ± .1[g]	3	50.4 ± .6[g]	6	50.6 ± .3[g]	5
Diatrizoic acid[h]	62.0	...	...	...	...	57.3 ± .4	6	58.4 ± .3	3
Sodium acetrizoate[i]	65.8	...	...	60.3 ± .7	3	60.7 ± .3	3	61.6 ± .3	4
Sodium diatrizoate[j]	59.9	...	...	...	...	53.7 ± .5	5	54.2 ± .5	4
Potassium iodide	76.5	...	...	...	...	...	...	76.5 ± .2	7

[a] All assays done on samples as received. [b] The standard deviation was calculated by the Dean and Dixon method (10). [c] Sample weight chosen to yield about 10 mg. of iodine. [d] N.F. XI specifications are given since chiniofon is a mixture. [e] Method will not produce quantitative dehalogenation. [f] U.S.P. specifications are 40–42% I. [g] Loss on drying was 3.25%. [h] Used to prepare meglumine diatrizoate injection U.S.P. [i] Used to prepare sodium acetrizoate injection U.S.P. XVI. [j] Used to prepare sodium diatrizoate injection U.S.P.

D. Oxygen Flask Combustion Method (7–9).—This is an extremely rapid, simple, and inexpensive technique for combustion analysis of organic materials in oxygen. The procedure converts organic materials into soluble combustion products which are then analyzed.

Methods *A*, *B*, and *C* were official in U.S.P. XVI and N.F. XI, and the complete procedures may be found in the compendia in the monographs for the various compounds used in this study.

Preliminary testing indicated that, of the official methods, method *C* offered the best possibility of a general assay method. All compounds used in this study were assayed by the official method, by method *C*, and by the proposed general method, method *D*, which is given in detail under *Proposed General Method*. Table I shows a comparison of the four methods.

Proposed General Method

Apparatus.—Five-hundred-milliliter oxygen combustion flask (Arthur H. Thomas Co., Philadelphia, Pa.), 10-ml. buret, pH meter, silver and calomel electrodes, magnetic stirrer.

Reagents.—Sodium hydroxide solution (1 in 100), sodium bisulfite solution (1 in 100), approximately 1 *N* nitric acid solution, oxygen, 0.01 *M* $AgNO_3$ solution prepared by weighing as primary standard, low-ash filter paper (Whatman 42).

Combustion Method.—Weigh the sample accurately on a piece of the filter paper and fold the paper to enclose the sample. Place the sample, together with a filter paper fuse-strip, in the platinum wire loop fused into the flask stopper. Place 5 ml. of the sodium hydroxide solution and 2 ml. of the sodium bisulfite solution in the flask. Flush the air from the flask with a stream of rapidly flowing oxygen, moisten the stopper joint with water, and ignite the fuse strip. Immediately plunge the sample holder into the flask, and hold the stopper firmly in place. After combustion is complete, shake the flask vigorously, inverting and rotating the flask to absorb all of the iodine vapor, and allow the flask to stand for 3 min.

Completion Method.—Place a few drops of water around the stopper joint and twist the stopper to loosen it. Rinse the stopper and sample holder with water and transfer the flask contents to a 250-ml. beaker, washing the flask several times with a total of about 70 ml. of water. Add 2 ml. of the nitric acid solution, and if the solution turns a pale yellow, add 1 or 2 drops of the bisulfite solution to decolorize. Titrate potentiometrically with 0.01 *M* silver nitrate using a silver indicator electrode and a calomel reference, adding titrant in 1-ml. increments until the potential is about −100 mv. Now add titrant dropwise, taking a reading after each addition until the meter shows a change of 150–200 mv. This maximum potential change occurs at about 0 mv. and is evidenced by a slow drift in the meter pointer. The volume of titrant is recorded. The meter pointer will stabilize in a few minutes and the addition of 1 drop of excess titrant will result in a potential change of about 40 mv. The end point is further shown by the coagulation of the silver iodide particles. The silver electrode may be cleaned by immersing it in a 50% nitric acid solution until the electrode gases freely.

DISCUSSION

Some of the assay methods for iodinated compounds in the U.S.P. XVI and N.F. XI can produce low results and be quite time-consuming. Because the zinc–sodium hydroxide method requires no elaborate equipment and utilizes a simple procedure, it was hoped at the onset of this work that this method might be applicable to all of the compounds studied. In proving this, all of the compounds used in this study were analyzed by this procedure. The data reveal that the method is acceptable for those compounds which have a carboxyl or sulfonic acid group attached directly to the ring structure with the iodine atoms being *ortho* or *para* to the acid groups. In those compounds in which the acid function is separated from the ring structure by one or more carbons, the results with this method are a few per cent lower than those obtained with other methods. In compounds without an acid group present, the zinc–sodium hydroxide method will not work.

These observations can be explained on the basis of electron densities in the ring systems. The carboxyl group, a *meta* director, decreases the electron densities in the *ortho* and *para* positions, thus decreasing the strength of the carbon-iodine bonds in these positions and allowing dehalogenation to take place readily. When the carboxyl function is sep-

arated from the ring, a decrease in electron density is effected to a lesser extent, possibly explaining the lower results obtained with compounds of this type. In the hydroxyquinoline compounds, the hydroxyl group, an *ortho-para* director, increases the electron density at the *ortho* and *para* positions, strengthening the C-I bond, thus resulting in the failure of the zinc–sodium hydroxide method.

Tetrabromophenolphthalein ethyl ester (0.1% in glacial acetic acid) is the adsorption indicator used in the official compendia for the titration procedure following decomposition by zinc–sodium hydroxide. In the course of this work, it was noticed that the indicator was stable only for a period of 3–4 weeks. This is believed due to hydrolysis of the ester linkage, since refrigeration of the indicator prolonged the stability by several weeks. The freshly prepared indicator produces a sharp color change at the end point, but the sharpness of this change decreases as the indicator solution ages. It is important, therefore, to follow the U.S.P. XVII requirement that the indicator be freshly prepared.

The oxygen flask procedure produced values closer to the theoretical values than were obtained with any of the other methods used except the longer, more involved Parr bomb procedure. No difficulty was encountered from loss due to the transfer of the absorbing medium to another container prior to the titration procedure. Many of the compounds used in the study contained over 60% I and not a single case of incomplete combustion was enountered, probably because of the small sample size.

The potentiometric titration technique was employed because of the difficulties mentioned with tetrabromophenolphthalein ethyl ester and because of the difficulty in visual detection of the equivalence point at low iodide concentration. The validity of the titration method was shown by the recovery of 100.0% of potassium iodide from seven different samples having iodide concentrations equivalent to that obtained from the combustion procedure.

The suggested technique differs from that official in U.S.P. XVII and N.F. XII in that the iodine is converted into iodide and titrated with silver nitrate as is required for iodochlorhydroxyquin U.S.P. The official method converts iodine to iodide, then to iodate by oxidation with bromine in an acetate-buffered medium, removal of excess bromine by formic acid, removal of oxygen by displacement with nitrogen, conversion of iodate to iodine by treatment with iodide solution, and titration of the liberated iodine with standard sodium thiosulfate.

The official procedure has the advantage of producing six equivalents of iodine for each atom of iodine originally present, and is not subject to interference by chloride. The sample size allows titration volumes of about 30 ml. of 0.02 *N* thiosulfate.

The suggested procedure eliminates the need for further chemical treatment after combustion, but produces only one equivalent of iodide for each atom of iodine originally present. The sample size requires about 9 ml. of 0.01 *N* silver nitrate. The potentiometric end point prevents interference by chloride.

The choice between the two methods is certainly open to debate. The official method requires more handling, more reagents, a cylinder of nitrogen, use of a hazardous bromine solution, storage of standard sodium thiosulfate whose normality must be frequently checked and which is a secondary standard, and use of starch T.S. indicator which must be freshly prepared. In contrast, the suggested method uses the solution from the combustion and a stable solution of primary standard for titration, but does require the less common 10-ml. buret and silver electrode as well as a pH meter capable of use as a millivoltmeter. The official method might be best for a laboratory which routinely uses 0.02 *N* thiosulfate for iodine determinations, whereas the suggested method might be better for laboratories with a low volume of iodine titrations but with adequate instrumentation.

In several instances the data obtained by all methods is at least 4% lower than the theoretical values for the compound. In all cases where this is true, the compound is not official as the powdered material supplied to the authors for this investigation, but as the injection. The data obtained do not reflect on the validity of the assay methods; in many cases it is the manufacturers' policy to assay the powdered material for iodine content and then prepare the injection to meet the standards of the official compendia.

CONCLUSIONS

The proposed method utilizing the oxygen flask combustion is in all cases superior to or equal to other methods in recovery of iodine and generally equivalent in reproducibility. With the procedure suggested, an analyst with a minimum of practice can complete an analysis in 20 min., far faster than any of the presently official methods. The method is so rapid and simple that a technician could obtain excellent results in routine laboratory work.

Despite the general applicability of the suggested method, it is recommended that the alkaline zinc method be retained for appropriate compounds because of its case, absence of hazard, use of common equipment, and an indicator end point.

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Quantitation of the PVA–Borate–Iodine Complex

By CARL A. PANNUTI, VINCENT DEPAUL LYNCH, ANTHONY J. MONTE-BOVI, and JOHN J. SCIARRA

While the PVA–borate–iodine complex is shown to be suitable for the qualitative determination of boric acid, attempts to adapt this to a colorimetric procedure were of no avail. A possible explanation is suggested as a dilution phenomenon.

IN A PREVIOUS publication (1), it was reported that the PVA–borate–iodine reaction was suitable for the detection of boric acid in urine. At the same time it was inferred that this test might be adaptable to a quantitative determination of boric acid, on the basis of the fact that the authors were able to detect as little as 0.3 mg. of that substance. The fact that this reaction involved the development of a color as an end point might also lead one to believe that variation in the color produced by different concentrations of boric acid could be used as the basis for a colorimetric assay.

In a series of experiments conducted along those lines in these laboratories, the following facts were ascertained.

(*a*) Simple dilution with water caused the typical blue-black color to be discharged. The resultant color was either yellow or red-brown. The intensity of the dilute color could not be related to differences in the concentration of any of the components of the reaction mixture. Colorimetric determinations were carried out using a Spectronic 20 colorimeter.

(*b*) The discharge of color could not be attributed to alteration in pH. It was determined that an optimum pH of 4.0 was necessary for initial color development, but that subsequent dilution caused the color to discharge even though the optimum pH was maintained throughout the series of dilutions.

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(*c*) A variety of water-miscible, organic solvents was tested to determine whether the discharge of color could be caused by the available excess hydroxyl or hydronium ions. A spectrum of glycols and alcohols caused color discharge. In addition, color loss was also noted with the addition of dioxane, acetone, and ethyl acetate. This seems to indicate that the change in color from blue to yellow is a dilution phenomenon not related to either excess hydroxyl or hydronium ions.

(*d*) In the case of dilution with absolute ethyl alcohol, it was noted that the yellow color which developed could be related to the concentration of boric acid. Using a Beckman model DB recording spectrophotometer, a linear relationship was established for concentrations of boric acid down to 10.0 mg. Below this concentration, only erratic readings were obtained. The developed color was discharged on standing. Decoloration was hastened when heat was applied. It was shown subsequently that the yellow color represented an excess of iodine.

(*e*) The above observations lead us to believe that this test, in all probability, will have to remain qualitative.

It must be concluded that the color developed in the PVA–borate–iodine reaction is dependent upon the optimum concentration of reactants in an optimum volume of solvent. The nature of the solvent is important to the extent that all reactants must be mutually soluble in it. The initial color is probably not due to a production of a color pigment in the complex formation, but rather to the ability of that specific concentration of substances to exhibit spectral absorbance within the blue range.

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Preparation of Some Cyanoalkylpiperidines

By MORRIS FREIFELDER

Selective conversion of the pyridine ring was accomplished without attack of the nitrile group when some alkyl cyanoalkylpyridinium halides were hydrogenated in the presence of platinum oxide or rhodium on carbon catalyst.

CATALYTIC hydrogenation of a ring system is more difficult to achieve than reduction of a functional group. In the pyridine series the difficulty is due to the poisoning effect of the ring nitrogen or more likely to the effect of the resultant more basic piperidine nitrogen atom. Nevertheless, a number of examples have been cited where the ring was preferentially attacked when a methylene bridge separated it from a ketone group (1). In general this took place when the ring was quaternized. There are only a few examples of selective conversion when the carbonyl group is adjacent to the ring. Lyle and Warner converted 3-benzoylpyridine hydrochloride and methyl 3-benzoylpyridinium iodide to the corresponding benzoylpiperidines in 35–40% yield (2); Freifelder obtained 70% of 3-acetyl-1,4,5,6-tetrahydropyridine and 7% of 3-acetylpiperidine

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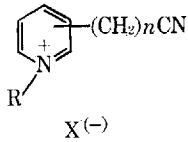
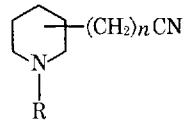
TABLE I.—QUATERNARY SALTS



Compd.	R	n	Position of Side Chain	X	Yield, %	M.p., °C.	Formulas	Anal., % Calcd.	Found
I	CH_3	1	3	I	84.5	119–120	$C_8H_9IN_2$	C, 36.44	36.56
								H, 3.48	3.52
								N, 10.77	10.45
II	$C_6H_5CH_2$[a]	1	3	Cl	74	154–157	$C_{14}H_{13}ClN_2$	C, 68.70	68.69
								H, 5.30	5.20
								N, 11.45	11.58
III	CH_3[b]	2	2	I	61[c]	138–139.5	$C_9H_{11}IN_2$	C, 39.43	39.45
								H, 4.04	3.96
								N, 10.22	10.38

[a] Reaction to form quaternary compound carried out at 100° for 18 hr. [b] Reaction run at 125° for 16 hr. [c] The crystalline mass was admixed with tar. Pure material was obtained by continuous extraction with refluxing isopropyl alcohol.

TABLE II.—HYDROGENATION PRODUCTS



Compd.	R	n	Ring Position of Side Chain	Yield,[a] %	Constants B.p., mm. °C.		n_D^{25}	M.p. Salt	Formulas	Anal., % Calcd.	Found
IV	CH_3	1	3	59	106	13	1.4654	...	$C_8H_{14}N_2$	C, 69.51	68.91
										H, 10.21	10.21
										N, 20.27	20.30
		...	...	...	...	...	...	120–122[b]	$C_8H_{15}ClN_2 \cdot 1/2H_2O$	C, 52.31	52.47
										H, 8.78	8.62
										N, 15.26	15.28
		...	...	...	...	...	...	93.5–94.5[c]	$C_{12}H_{20}N_2O_4$	C, 56.23	55.94
										H, 7.86	8.00
										N, 10.92	10.71
V	$C_6H_5CH_2$	1	3	56	169	3.8	1.5301	...	$C_{14}H_{18}N_2$	C, 78.46	78.74
										H, 8.46	8.70
										N, 13.07	13.14
VI	CH_3	2	2	54	135	32	1.4710	...	$C_9H_{16}N_2$	C, 71.00	70.96
										H, 10.59	10.81
										N, 18.41	18.51

[a] The bases are easily soluble in water. The yields might be substantially increased by continuous extraction. [b] Hydrochloride salt. [c] Succinate salt.

from the hydrogenation of 3-acetylpyridine (3). Other investigators report selective conversion of the carbonyl group or concurrent reduction in such instances.

1-Methyl-3-cyanomethylpiperidine was obtained from the reduction of methyl 3-cyanomethylpyridinium iodide in the presence of platinum oxide or rhodium on carbon. The instability of 2- and 4-cyanomethylpyridines prevented any work on subsequent quaternization and reduction to obtain the isomeric 2- and 4-cyanomethylpiperidines. 1-Benzyl-3-cyanomethylpiperidine was also prepared and subjected to hydrogenolysis to yield 3-cyanomethylpiperidine.

Attempts were made to reduce 3-cyanomethylpyridine to the piperidine selectively in aqueous alcoholic hydrochloric acid or in glacial acetic acid. In all cases the nitrile group was perferentially attacked.

It had been postulated that the selective conversion of the ring which occurs when a quaternary compound is reduced is due to the existence of a single species incapable of reversibility as compared to the equilibrium existing between the base and the ionic form of an acid salt (1). The unsuccessful conversions in aqueous acidic media were probably due to water competing for protonation. In glacial acetic acid protonation apparently was not complete enough for selective ring reduction. When the hydrogenation of 2-(2-cyanoethyl)pyridine was carried out in trifluoroacetic acid selective conversion of the ring did not take place. On the other hand

reduction of the methiodide in 50% aqueous alcohol yielded 1-methyl-2-(2-cyanoethyl)piperidine in fairly good yield.

EXPERIMENTAL[1]

The following procedure was used to prepare the quaternary salts listed in Table I.

Methyl 3-Cyanomethylpyridinium Iodide (I).—A mixture of 11.8 Gm. (0.1 mole) of 3-cyanomethylpyridine and 21.3 Gm. (0.15 mole) of methyl iodide in 60 ml. of dry benzene was heated in a 183-ml. stainless steel rocker type bomb for 12 hr. at 100°. After cooling, the solid product in the reactor was filtered, washed with anhydrous ether, and dried.

Hydrogenation of the quaternary compounds was carried out in the following manner.

1-Methyl-3-cyanomethylpiperidine (IV).—A solution of 20.8 Gm. (0.08 mole) of I in 200 ml. of 90% aqueous alcohol was hydrogenated (3 Atm.) in the presence of 0.6 Gm. of platinum oxide.[2] Uptake for 0.24 mole was complete in 4 hr. The catalyst was removed by filtration. It was washed with 25 ml. of water and the combined filtrate and washings concentrated to dryness under reduced pressure. The residue was dissolved in a small amount of water and the solution, kept below 15°, was made strongly basic with 40–50% sodium hydroxide solution. The mixture was extracted thoroughly with benzene and the extract dried over anhydrous magnesium sulfate. The solution was then concentrated and the residue distilled. Gas-liquid chromatography of the distilled product as well as the other amines (V and VI) showed each to be a single component. Infrared examination gave no evidence of the presence of primary or secondary amine but did show the characteristic C≡N band at 4.46–4.48 μ. It was difficult to get good carbon, hydrogen, and nitrogen values unless the bases were analyzed immediately after distillation because they absorbed carbon dioxide so rapidly. Some were additionally characterized as salts. (See Table II.)

3-Cyanomethylpiperidine (VII).—A solution of 32.8 Gm. (0.153 mole) of V in 100 ml. of 95% ethanol containing 0.153 mole of dry hydrogen chloride was hydrogenated in the presence of 5.0 Gm. of 5% palladium on carbon at room temperature and 2 Atm. pressure. Hydrogen uptake was interrupted at 90% of theory to insure selectivity. After removal of catalyst, the solution was concentrated to dryness under reduced pressure. The residue was dissolved in 50–75 ml. of water and cooled to about 10–15° and kept cold while adding an excess of 50% sodium hydroxide solution. The oily layer was extracted with benzene, dried over anhydrous magnesium sulfate, and concentrated after filtration from the drying agent. The residue was distilled, b.p. 125° (15 mm.); n_D^{25} 1.4791; yield, 53%.

Anal.—Calcd. for $C_7H_{12}N_2$: C, 67.69; H, 9.74. Found: C, 67.79; H, 9.95.

The base picks up carbon dioxide rapidly and must be analyzed immediately. The found nitrogen value continued to drop after each analysis.

A hydrochloride melted at 140–142°. It was found to be a hemihydrate.

Anal.—Calcd. for $C_7H_{13}ClN_2 \cdot 0.5H_2O$: C, 49.55; H, 8.35; N, 16.52. Found: C, 49.39; H, 8.00; N, 16.58.

On further drying at 100° under reduced pressure for 8 hr. the salt appeared to stabilize at 0.25 H_2O.

Anal.—Calcd. for $C_7H_{13}ClN_2 \cdot 0.25H_2O$: C, 50.90, H, 8.24; Cl, 21.45; N, 16.96; O, 2.45. Found: C, 50.75; H, 8.35; Cl, 20.79; N, 16.98; O, 1.87.

Attempts to dry the salt at 130° *in vacuo* caused loss of hydrogen chloride.

Hydrogenation of 3-Cyanomethylpyridine in Acidic Media.—A solution of 11.8 Gm. (0.1 mole) of 3-cyanomethylpyridine in 50–75 ml. of water containing 1.0 to 1.2 moles of hydrogen chloride was hydrogenated in the presence of 0.350 Gm. of platinum oxide. Only 2 molar equivalents of H were absorbed. After removal of catalyst, concentration of the filtrate and basification, as in previous experiments, the crude product on infrared examination showed that the C≡N band was no longer present. The presence of the pyridine ring C=N, and the presence of primary or secondary amine was noted. On distillation about a 10% yield of 3-(2-aminoethyl)pyridine was obtained. The residue was a tarry formation.

In a reduction in glacial acetic acid containing 0.1% of concentrated sulfuric acid, 30% of the same amine was obtained. We never were able to show more than trace amounts of VII by gas-liquid chromatography in these or other experiments in various acidic media.

[1] Microanalyses were carried out by O. F. Kolsto and his group, infrared examination by A. Kammer and W. Washburn, gas-liquid chromatography by Mrs. Taimi Anderson. The author is grateful to these people for their assistance and to Miss Evelyn Schuber for the preparation of 2-(2-cyanoethyl)pyridine.

[2] A 15–20% ratio of 5% rhodium on carbon to substrate may be substituted. Both catalysts are available from Engelhard Industries, Newark, N. J.

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In Vitro Determination of Defoaming Inactivation of Silicone Antacid Tablets

By MORTON REZAK

A simple and inexpensive *in vitro* test was utilized to evaluate the defoaming activity of a silicone defoamer in tablet combination with a number of commonly used antacid materials. The results covered the complete range of defoaming action.

THE USE OF silicone defoamers with routine antacid therapy has come into common practice where gas formation has been implicated as a difficulty in effective antacid therapy (1), and where gaseous distention is a factor in patient distress (2–4).

A study conducted in this laboratory indicated that the silicone defoaming action in an antacid-defoamer tablet was not stable, and seemed to reduce with aging. The objectives of this study were to (*a*) compare the defoaming activity of a silicone defoamer in tablet combination with commonly used antacids, (*b*) determine the stability of the above products.

EXPERIMENTAL

Selection of Antacids.—The antacids selected as representative of the antacids in use today were calcium carbonate, sodium bicarbonate, sodium citrate, aluminum hydroxide, magnesium trisilicate, magnesium hydroxide, magnesium carbonate, glycine, magnesium carbonate–aluminum hydroxide coprecipitate, magnesium peroxide, and bismuth subcarbonate. The silicone used is simethicone (Antifoam A, Dow Chemical Co.), an activated silicone.

Composition and Manufacture of the Tablets.—The following ingredients were used (mg./tablet): antacid, 400; cornstarch, 30; mannitol, 100; simethicone, 25; polyoxyl 40 stearate,[1] 3; lactose, 50; PVP, 80; microcrystalline cellulose,[2] 35; and magnesium stearate, 7; totaling 730.

Procedure.—The antacid, starch, and mannitol are blended. The simethicone and polyoxyl 40 stearate are melted together and added to the lactose. All the above materials are blended and granulated with an aqueous solution of the PVP. The granulation is wet and dry milled, blended with the cellulose and stearate, and compressed.

Both granulation and tablet were placed on stability at 45°.

PROCEDURES

Materials to be Tested.—The tablet to be tested was passed through a No. 20 screen. The granulation was tested as a granulation.

Foam.—The foam was generated by dissolving 200 mg. of a commercial laundry detergent in 100 ml. of purified water at 37°. The solution was placed in a 250-ml. graduated cylinder, and shaken vigorously until 150 ml. of foam was produced.

Defoaming Rate.—The material to be tested was placed into the graduated cylinder; the cylinder was then stoppered and inverted quickly 5 times to place the granules in contact with both foam and liquid. The clock was started and at each 15-sec. interval, the volume of remaining foam was noted, and the cylinder inverted once again. The test was considered complete after 2 min. or when all the foam disappeared, leaving the original 100 ml. of water in the cylinder.

Prolonged Defoaming Rate.—A defoaming rate was run as above, extending the time to 5 min. Tablets and pure simethicone were tested to obtain some estimate of free simethicone remaining in the tablets. The simethicone was spread out on micro-

TABLE I.—DEFOAMING TIME OF ANTACID-SILICONE TABLETS

Antacid Material	Days on Stability at 45°			
	0	2	7	14
Calcium carbonate	15 sec.	30 sec.	30 sec.	30 sec.
Sodium bicarbonate	15	15	15	15
Sodium citrate	30	15	15	15
Aluminum hydroxide	<120	<120	<120	<120
Magnesium trisilicate	15	15	90	75
Magnesium hydroxide	15	30	30	30
Magnesium carbonate	<120	<120	<120	<120
Glycine	15	15	15	15
Magnesium carbonate–aluminum hydroxide co-ppt.	90	<120	<120	<120
Magnesium peroxide	40	40	70	90
Bismuth subcarbonate	<120	<120	<120	<120

TABLE II.—DEFOAMING TIME OF TABLETS AFTER PROLONGED STORAGE

Antacid	Storage at 45°, Days	Defoaming Time, sec.
Calcium carbonate	57	30
Sodium bicarbonate	44	<120
Sodium citrate	44	15
Magnesium trisilicate	60	90
Magnesium hydroxide	49	30
Glycine	49	15
Magnesium peroxide	44	105

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[1] Marketed as Myrj 52 by the Atlas Powder Co., Wilmington, Del.

[2] Marketed as Avicel by the American Viscose Co., Marcus Hook, Pa.

TABLE III.—DEFOAMING TIME OF TABLET GRANULATIONS

Aluminum hydroxide	<120 sec.
Magnesium carbonate	<120
Magnesium carbonate–aluminum hydroxide co-ppt.	90
Bismuth subcarbonate	<120

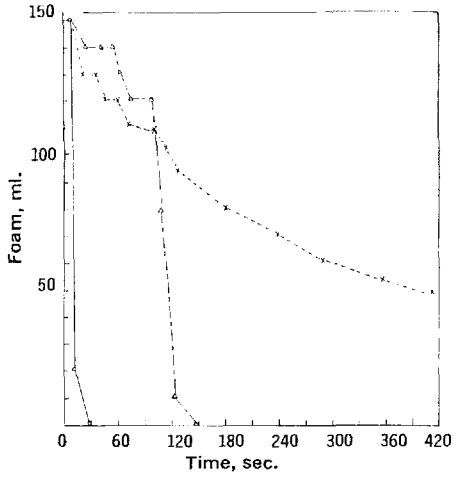

Fig. 1.—Foam depression of antacid-silicone tablets. Key: O, calcium carbonate tablet; ×, aluminum hydroxide; Δ, magnesium carbonate–aluminum hydroxide coprecipitate.

crystalline cellulose at a concentration of 25 mg./Gm. to facilitate testing.

RESULTS AND DISCUSSION

The speed of action of a gastrointestinal defoaming agent may be an indication of its efficiency in dispersing gas *in vivo* (5). This can be demonstrated by its defoaming action. The results listed in Table I indicate that there is a wide variation of defoaming action depending upon the antacid used in a silicone defoamer-antacid tablet.

There is also an apparent aging effect on these tablets, as some of the tablets exhibited a decrease in defoaming activity with storage at 45°. These test results can be seen in Table II.

It is significant that those antacids which most inactivate the simethicone (Antifoam A) did so independently of whether or not the material was compressed. In Table III it is evident that the simethicone inactivation is just as pronounced in these granulations as in the compressed tablets.

An attempt was made to correlate the defoaming activity of the tablets with the defoaming activity of the pure simethicone. In Figs. 1 and 2, the foam reduction as a function of time is shown.

The complete defoaming range of the tablets is shown in Fig. 1. The calcium carbonate tablet releases the simethicone almost immediately, so that

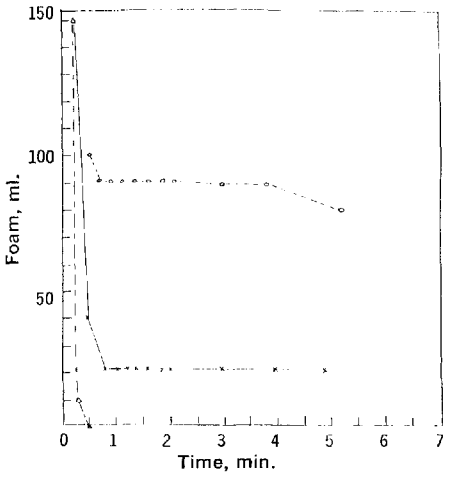

Fig. 2.—Foam depression of simethicone. Key: O, 5 mg.; ×, 10 mg.; Δ, 15 mg.

the foam disappears in 30 sec. The magnesium carbonate–aluminum hydroxide coprecipitate retards the simethicone, so that the foam drops after a 90-sec. delay. The aluminum hydroxide adsorbs the simethicone to a significant degree, so that a very slow reduction in foam height takes place.

In Fig. 2, a 15-mg. dose of plain simethicone approximates the calcium carbonate tablet curve in Fig. 1. The calcium carbonate tablet is at least equivalent to 15 mg. of free simethicone. Smaller doses of the simethicone quickly reduce smaller amounts of the foam, and do not approximate the simethicone-antacid tablet curves of Fig. 1.

There appears to be a gross correlation of the amount of foam reduction with the amount of free simethicone. In Fig. 2, we can see that 5 mg. of simethicone will reduce 60 ml. of foam, 10 mg. of simethicone will reduce 130 ml. of foam, and 15 mg. of simethicone will reduce at least 150 ml. of foam. Therefore, after 5 min., the aluminum hydroxide tablet had released the equivalent of approximately 7.5 mg. of free simethicone.

SUMMARY

An *in vitro* evaluation of the defoaming ability of antacid-silicone tablets was conducted. It was demonstrated that there are marked differences among the antacids tested with regard to their effect of the activity of the silicone defoamer.

The role of the amount of silicone defoamer was studied and correlated with foam depression. Stability studies indicate that some antacids will adsorb the silicone over a period of time.

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REVIEWS

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The first part (Chapters 1–5) deals with fundamental studies in simple systems and the classification of antioxidants by their mechanisms. Modes of breakdown of peroxides are considered in relation to the autoxidative process. Data from kinetic studies are used to give information on reaction intermediates of the oxidative free radical chain mechanism. Effective use is made of illustrative thermo-chemical calculations in discussing reactivity of hydrocarbons toward oxygen.

The various mechanisms by which antioxidants interrupt the autoxidative chain are discussed on the basis of chemical and kinetic evidence. The author distinguishes two mechanistically distinct classes of antioxidants: (*a*) radical chain-breakers that remove species in the propagation step; (*b*) preventive agents that remove sources of free radicals (peroxide decomposers). Synergistic effects obtained by combining both kinds of antioxidants in a system are considered.

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This excellent book is remarkably free of errors. It is recommended highly to all who wish to learn more about atmospheric oxidation and antioxidants.

Reviewed by Louis C. Schroeter
The Upjohn Company
Kalamazoo, Mich.

The Mast Cells. By HANS SELYE. Butterworth, Inc., 7235 Wisconsin Ave., Washington, D. C. 20014, 1965. xxix + 498 pp. 17.5 × 25 25 cm. Price $19.75.

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Review Article

Kinetic Methods of Analysis

By J. GORDON HANNA and SIDNEY SIGGIA

KINETIC CONSIDERATIONS always have been of prime importance to the analytical chemist in the development and use of his procedures. Because he can be satisfied with no less than quantitative recoveries, he must utilize optimum experimental conditions for his reactions. He uses these conditions not only to produce the desired stoichiometric reaction in the minimum time but also to obtain a specific reaction with the least interference from side or parallel reactions. Analytical chemists have gone a step further and are applying the kineticists' reaction rate techniques directly to the solution of analytical problems. This has yielded methods which give quantitative results based on reactions which are not necessarily complete. In fact, some methods require the occurrence of only a small fraction of the reaction. Also, procedures have been developed for the determination of traces of materials based on their catalytic effect on the rates of suitable reactions. Another result is the production of methods for the differential determination of two or more compounds in a mixture which are reacting concurrently but at different rates with a common reagent. These developments in the use of reaction rate approaches are considered in this review.

KINETIC METHODS FOR A SINGLE REACTIVE SPECIES

The application of kinetic principles to analysis renders possible the use of reactions which normally do not produce quantitative conversions because of the establishment of unfavorable equilibria or because of the necessity for impractically long reaction times. The concentration of a single reactive species can be determined in some cases based on an accurate knowledge of the rate constant and the measurement of the rate of reaction of the sample. A near quantitative conversion is not required because a measurement of the fraction of the material which has reacted during a specified time interval can be related directly to the original concentration. For example, if the reaction is first order, the integral form of the rate expression is

$$kt = 2.303 \log \frac{a}{(a - x)} \qquad \text{(Eq. 1)}$$

Then, if the reaction is run under carefully controlled conditions, and if x, which represents the amount of the initial concentration, a, which has reacted in time, t, is measured, a can be calculated using Eq. 1. The same technique can be used for second-order reactions, in which case the integral form of the rate expression is

$$kt = \frac{2.303}{(b - a)} \log \frac{a(b - x)}{b(a - x)} \qquad \text{(Eq. 2)}$$

Here b is the initial concentration of the reagent which must be known. If $a = b$, the integral form is then

$$kt = \frac{1}{(a - x)} - \frac{1}{a} \qquad \text{(Eq. 3)}$$

In practice, it sometimes is more convenient to

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refer the rate found for the sample to a calibration curve prepared from data obtained using known amounts of the pure compound.

Blaedel and Petitjean (3) investigated the possibility of developing kinetic methods based upon measurements of the rate of a reaction at several times during the early stages of the reaction. They carried out a preliminary study of the utility of the approach using the alkaline hydrolysis of ethyl acetate. The reaction vessel was part of a tuned circuit of a high frequency oscillator and conductance changes in the solution as the reaction proceeded caused changes in the frequency of the loaded oscillator. The amount of organic compound in the sample being analyzed was then determined by referring the measured rates of frequency change, f/t, to a working curve plotted from data obtained with known amounts of the substance being determined. The results obtained with ethyl acetate indicated that the method yielded highly precise determinations of single constituents in samples free of interferences. They then applied the developed method successfully to acetylacetone determinations by measurement of the reaction rate of the ketone with hydroxylamine hydrochloride. This particular oximation reaction does not reach substantial completion in a practical period of time and, therefore, presents problems in the usual straightforward procedures. These investigators concluded that data obtained during the first 5 or 10 min. of a reaction are capable of yielding results accurate to 0.3% for simple systems.

The same principle has been used to determine glucose in serum plasma or blood, based on the enzyme-catalyzed oxidation of the sugar to gluconic acid and hydrogen peroxide. Guilbault and co-workers (27) followed the rate of peroxide production by recording the potential difference between two platinum indicator electrodes polarized with constant current. The potential changes are proportional to the glucose concentration. Malmstadt and Piepmeier (47) used a pH stat to maintain the pH stationary at a preselected value with continuous neutralization of the gluconic acid formed during the reaction. The instrument which they described provides direct digital readout of initial rate data that are proportional to the concentration of glucose present. Results reproducible within 1–2% in the 50–250 p.p.m. range were obtained. Use also has been made of the color produced by the action of the peroxide formed on *o*-tolidine (2, 68) or on *o*-dianisidine (45). The elapsed time for the production of a definite absorbance change is proportional to the initial rate and to the concentration of glucose. The peroxide produced during the reaction has been determined also by its rapid molybdate-catalyzed oxidation of iodide and the glucose oxidation followed by measuring the iodine formed potentiometrically (46, 58), spectrophotometrically (2, 41), and amperometrically (56).

Pardue and Frings (60) used autometric amperometric control equipment to provide a direct readout of the time required for a definite amount of iodine to be produced from iodide as the result of the enzyme-catalyzed oxidation of galactose to peroxide. This amperometric method was further extended to furnish digital readout in concentration units (61). Frings and Pardue (23) also used an automatic spectrophotometric measurement of the peroxide produced by the enzymatic reaction by measuring the color produced by the oxidation of *o*-dianisidine. A similar arrangement was used to obtain automatic direct readout of p.p.m. cystine based on potentiometric measurement of the rate of an azide–iodine reaction (57, 59, 62).

Malmstadt and Hadjiioannou (42) reported a similar technique for the determination of alcohol in blood. The time required for a small fixed change in absorbance as a result of the selective enzyme-catalyzed oxidation of the alcohol was measured in the early stages of the reaction, and this value related directly to the alcohol concentration. The method was used to determine alcohol in the range 0.015–0.300 Gm./100 ml. of blood with a relative error of 2–3%. Sample sizes ranged from 0.1–0.25 ml. and the time of measurement ranged from a few seconds to 2 min.

Malmstadt and Hadjiioannou (43) determined some L-amino acids by an automatic spectrophotometric reaction rate method based on the coupled enzyme reaction in which oxidative deamination is specifically catalyzed by L-amino acid oxidase to form hydrogen peroxide. The time required, from a few seconds to about 2 min., for the reaction to produce a small fixed amount of colored product as a result of the reaction of the resultant peroxide with *o*-dianisidine was related to the initial amino acid concentration. The concentration range determined was 4–50 p.p.m. with a relative error of about 2%.

A pH stat with digital readout of data collected early in the hydrolysis of urea in the presence of urease was used by Malmstadt and Piepmeier (47). The pH was maintained constant by adding dilute hydrochloric acid to neutralize the ammonia formed. The increments of acid added during a preset time interval, about 2.5 min., was counted. The count was directly proportional to the urea concentration. The procedure was

tested in the 2- to 10-p.p.m. range, and the results were reproducible within 1–2%.

The relationship between the concentration of the catalyst and the rate of the catalyzed reaction often is used to quantitatively estimate the catalyst. The approach is not new, but new and revised procedures based on it continue to appear because, where applicable, it is one of the most sensitive techniques devised to determine minute traces of materials. Some of these methods are cited here as illustrations.

The catalytic effect of copper on the oxidation of cysteine to cystine and the subsequent measurement of oxygen evolved in a specified time has been used to determine copper in blood (82) and in milk (9). The reduction of ceric ion by arsenite in an acid medium is catalyzed by soluble iodides. Sandell and Kolthoff (71) measured the time required for this reaction to go to completion and found a linear relationship between the reciprocal of that time and the quantity of iodine present. The reduction of the yellow ceric to colorless cerous ion was followed colorimetrically. The change in absorbance was recorded continuously, and the slope of the recorded curve was related to the iodine concentration by Chaney (14). Lein and Schwartz (39) used the first-order velocity constant as a function of the iodine concentration. Malmstadt and Hadjiioannou (44) noted the time, 10–100 sec., required for the reaction to consume a small fixed amount of ceric ions, and, therefore, the absorbance to decrease by a preselected amount, measured automatically, and related this directly to the iodine concentration. The latter investigators reported the determination of iodine in the range of 0.015–0.45 mcg. with a relative error of about 1–2%. Hadjiioannou (28) used this procedure to determine iodine in common salt and in natural waters. This same reaction system was used for the determination of labile organic-bound iodine by Zak and Baginski (86). Yatsimirskii and co-workers (84) determined micro amounts of iodides by their catalytic action on the oxidation of thiocyanate with ferric iron and with nitrate. Changes in the extinction of the thiocyanate complex of iron were evaluated. The sensitivity of this method was reported to be 0.001 mcg./ml. and the error of a single determination was about 2%.

Underwood, Burrill, and Rogers (81) determined submicrogram quantities of silver using the catalytic action of silver on the persulfate oxidation of manganous ion to permanganate. Spectrophotometric measurement at 525 mμ after a specified time was related to a calibration curve prepared using standard silver solutions. The practical lower limit of the method was reported to be about a millimicrogram of silver.

Vanadium operates as a catalyst in the oxidation of *p*-phenetidine citrate by potassium chlorate in the presence of phenol as an activator yielding a colored product. Bontschev (10) determined 0.1 mcg. of vanadium by means of the linear relationship between the extinction at 510 mμ and the time of heating. Fishman and Skongstad (19) used the catalytic effect of vanadium on the rate of oxidation of gallic acid by persulfate in acid solution to determine vanadium in water. The absorbance at 415 mμ was determined and compared with standard solutions treated in an identical manner. The procedure is applicable for concentrations of vanadium in the range 0.1–8.0 mcg./L. and shows a standard deviation of 0.2 or less.

Alizarin is oxidized only slowly by hydrogen peroxide but in the presence of cobalt chloride in alkaline solution, the rate is increased in proportion to the concentration of alizarin and the cobalt. The color of the alizarin is destroyed by the oxidizing action of the peroxide, and then if the extinction of the solution is measured at intervals after the addition of the cobalt solution, the rate of reaction can be determined. A calibration curve was used (64) to determine cobalt in concentrations ranging from 0.001–0.0066 mcg./ml. Bognar and Jellinek (5) determined as little as 0.002 mcg. of cobalt with an accuracy of ±10% by measuring the rate of reaction of diphenylcarbazone and hydrogen peroxide in the presence of iron. They measured the time interval to the attainment of a specific extinction by the reaction mixture.

Yatsimirskii and Raizman (85) utilized the catalytic effect of zirconium and hafnium on the oxidation of iodide by hydrogen peroxide to determine the concentrations of these catalysts.

Bulgakova and Zalubovskaya (13) followed amperometrically the rate of iodide oxidation with hydrogen peroxide as a method to determine molybdenum in cadmium sulfide and in lithium fluoride crystals.

Pantaler (52) determined tungsten and molybdenum based on the catalytic oxidation of rubeanic acid by hydrogen peroxide in 0.1 *N* hydrochloric acid. He plotted absorbance against time and obtained the amount of tungsten or molybdenum by comparing the slope of the line with slopes given by standard solutions. He reported the method satisfactory for 0.1 mcg. of either metal in 25 ml. of reaction mixture.

Babkin (1) followed the rate of the manganese-catalyzed permanganate–oxalate reaction to determine concentrations of manganese in the

0.01–0.6 mg. range. The results were obtained from a calibration curve constructed for the relationship between reaction time and amount of manganese. Fernandez, Sobel, and Jacobs (18) used the oxidation of leucomalachite green with periodate in the presence of manganese for the determination of submicrogram amounts of manganese in human serum. The progress of the reaction was followed photometrically, and the slope of the rate curve was compared with the slope for a standard curve to calculate the manganese content.

Bognar and Sarosi (6) investigated the effect of osmium tetroxide on the kinetics of oxidation of hydroquinone, orcinol, and 1,3-dihydroxynaphthalene by hydrogen peroxide and found they could determine as little as 0.001 mcg. of osmium tetroxide in 5 ml. of solution. (Ethylenedinitrilo) tetraacetic acid (EDTA) does not interfere with the catalytic reaction so that this compound could be used to increase the selectivity of the method. The same workers (7) also determined osmium tetroxide based on its catalytic effect on the oxidation of 3,3′-dimethylnaphthidine by potassium chlorate. The formation of the violet-red colored product was followed spectrophotometrically.

Michalski and Wtorkowska (50) established the optimum conditions for the determination of sulfide and thiosulfate (51) from the catalytic effects on the iodine–azide reaction. The rate was determined from the changes in iodine concentration which was followed amperometrically with a rotating platinum indicator electrode. The value of the potential which should be applied to provide the limiting current, proportional to concentration, was found from polarographic measurements. Sulfide was determined in the range 10^{-15} to 10^{-18} Gm./ml. with a deviation from the mean of less than 10%.

The catalytic activities of acids and bases in selected reactions can be used for the determination of the concentrations of these species. For example, Clibbens and Francis (15) and Francis and Geake (22) found the decomposition of nitrosotriacetonamine into nitrogen and phorone to be a function of the catalytic activity of hydroxyl ion. The original concentration of nitrosotriacetonamine was known and the rate of decomposition found by measuring the nitrogen produced as a function of time. A relationship between the first-order velocity constant for the reaction and the hydroxyl ion was determined and used. Duboux (17) used a proportionality between the hydrogen ion concentration and the velocity constant for the acid-catalyzed inversion of sugar to determine acidity.

The general enzymatic reaction rate method can be used also to determine enzyme activity. Hadjiioannou and Santos (29) determined serum lactic dehydrogenase from the effect of this enzyme on the rate of oxidation of lactic acid in the presence of diphosphopyridine nucleotide to form a species absorbing in the ultraviolet. An automatic potentiometric method for the determination of glucose oxidase activity similar to the procedure used to determine glucose has been demonstrated (63). The direct electrochemical measurement of peroxide produced has been used also to determine glucose oxidase (27). The same electrochemical technique has been used to determine cholinesterase and acetylcholinesterase based on the rate of enzymatic hydrolysis of thiocholine ester by the enzyme sample (36). The method has been extended to the analysis of some highly toxic organophosphorous compounds which act as anticholinesterase compounds where the decrease in rate of the cholinesterase–butyrylthiocholine ester hydrolysis is linearly related to concentration of the organophosphorous compound (26).

Magerum and Steinhaus (40) introduced an interesting kinetic method for the determination of ultratrace concentrations of metals. They used a coordination chain reaction involving the exchange of triethylenetetramine–nickel II (trien–Ni II) and (ethylenedinitrilo)tetraacetocuprate II (EDTA–Cu II). The presence of free ligand, which can originate from the pure complexes or can be added separately, greatly accelerates the reaction. The bright blue color of trien–Cu II produced is used to follow the progress of the reaction. A metal at 5×10^{-8} *M* concentration can react with free ligand (EDTA) reducing the exchange rate. The metal ion detected in this manner need have no connection with the reactant metals, but only be capable of reacting with EDTA. The reaction is first order with respect to the EDTA concentration and was followed for at least half-life, normally 10–15 min. A calibration curve was prepared of the rate constant against concentration of EDTA used. Experimental results were given down to 10^{-8} *M*.

DIFFERENTIAL REACTION RATES TO ANALYZE MIXTURES

The differential reaction rate approach is effective for the analysis of mixtures of two or more compounds, each of which reacts at a different rate with a common reagent, resulting in products which are either identical or are indistinguishable by the analytical method used.

The rate of disappearance of the reagent or the appearance of a product is monitored to obtain data for the total reaction of the components. Usually a graphical analysis or an equivalent mathematical treatment of the data based on the appropriate rate law is then applied to quantitatively resolve the mixture. The process normally requires a prior knowledge of the reaction order. The applicable graphical plot for a mixture of two reactive components shows a linear portion arising from the reaction of the slower component after substantially complete consumption of the faster component has occurred. The extrapolation of this linear portion to zero time provides data which can be used to calculate the original composition of the mixture.

Thus, for a first-order reaction log $(a - x)$ is plotted against t, based on Eq. 1. In this case, a represents the total concentration of the reactive components of the mixture and is determined by an independent method or by permitting the rate reaction to go to completion. For a two-component mixture, $a = a_1 + a_2$, if a_1 and a_2 are the concentrations of the faster and slower reacting constituents, respectively. When the final linear portion of the plot is extrapolated to $t = 0$, $x = a_1$ and $a - x = a - a_1 = a_2$, and sufficient data are then available to calculate the composition of the mixture. This process is illustrated in Fig. 1. If P is the value at the intercept $t = 0$, then from Eq. 1

$$\log a_2 = P \qquad \text{(Eq. 4)}$$

The concentration of the remaining component, a_1, is obtained by difference from a.

In some cases, it may be more convenient to transform a second-order reaction, one that is first order with respect to the reactant to be determined and first order with respect to the reagent, into one that is pseudo first order. To achieve this, the reagent is used in sufficient excess that its concentration can be considered constant. In this event, the data are treated according to the normal first-order rate laws. However, in most situations, if a large excess of reagent is

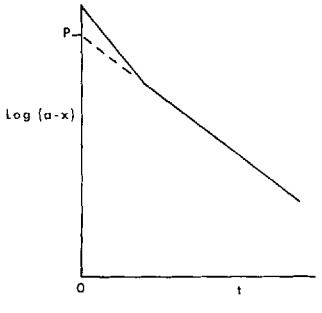

Fig. 1.—Graphical extrapolation procedure for a first-order reaction.

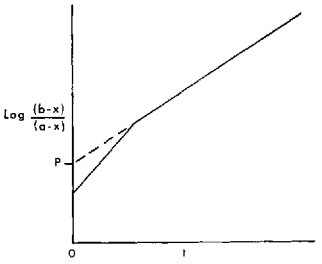

Fig. 2.—Graphical extrapolation procedure for a second-order reaction.

present, the reaction becomes so rapid that it is not manageable.

The procedure is based on Eq. 2 for a second-order reaction if the total concentration of the reactants and the concentration of the reagent are unequal. The initial concentration of the reagent, b, and the total initial concentration of reactants must be known and log $[(b - x)/(a - x)]$ is plotted against time. Extrapolation of the final linear portion is performed as described for the first-order reactions and is illustrated in Fig. 2. For the second-order case, at the intercept, $t = 0$,

$$\log [(b - a_1)/(a - a_1)] = P \qquad \text{(Eq. 5)}$$

Eq. 5 is solved for the concentration of the faster reacting constituent, a_1, and a_2 is determined by difference from a.

A plot of $1/(a - x)$ against t is used for the special second-order rate situation where $a = b$ (Eq. 3). At the intercept of the extrapolated final portion of the plot with the zero time axis

$$1/a_2 = P \qquad \text{(Eq. 6)}$$

Eq. 6 can then be solved for a_2, and a_1 obtained by difference. This procedure based on the equivalency of the total concentration of the components of the mixture determined and the reagent concentration is more convenient in some cases and simplifies the computations. If there is not a precise match of a and b, however, a small error in the slope of the linear portion can result in a large error in the calculation of a_2, particularly if a_1 is relatively large. Also, if the imbalance is quite significant, the latter portion of the curve will not be linear. Reilley and Papa (66) rearranged Eq. 3 and obtained the form indicated in Eq. 7.

$$x = k_2 a_2 (a - x)\, t + a_1 \qquad \text{(Eq. 7)}$$

Here k_2 represents the rate constant for the slower reacting component. When x is plotted against $(a - x)t$, a straight line is obtained after substantially all a_1 has reacted. The slope of this straight line portion is then $k_2 a_2$, and a_1 is the intercept at $t = 0$.

A scheme has been presented for differential rate analysis for second-order reactions applicable to mixtures of organic compounds which react with differing stoichiometry (8). It involves the introduction into Eq. 2 of a term indicating the number of moles of a polyfunctional compound which reacts with the reagent. It is illustrated by application to the hydrolysis of mixtures of cyclotrimethylenenitramine and cyclotetramethylenenitramine by sodium hydroxide. However, such systems usually can be handled easily on the equivalency basis rather than on the mole basis without resorting to special equations.

ANALYSES BASED ON SINGLE AND DOUBLE POINTS

It is possible to calculate the concentration of each component in a binary mixture based on a single determination after a measured reaction time if the rate constant for the reaction of each component and the total original concentration of the components are known. If measurements are made at two separate times, it is also possible to calculate the original composition without the use of rate constants.

Lee and Kolthoff (38) expressed the first-order rate law as shown in Eq. 8.

$$a - x = a_1 e^{-k_1 t} + a_2 e^{-k_2 t} \qquad \text{(Eq. 8)}$$

The rate constants for the two components are k_1 and k_2. Therefore, if $(a - x)$ is measured and k_1, k_2, and t are known, then the values of a_1 and a_2 can be found from a calibration curve prepared from the analysis of known mixtures or from calculations based on Eq. 8. The calibration curve is the linear plot obtained from values of $(a - x)/a$ *versus* a_1/a. Garmon and Reilley (24) proposed a double point method also based on Eq. 8. They determined the amount of reaction at two different time intervals, substituted the known and measured values into Eq. 8, and solved the two equations simultaneously to obtain values for a_1 and a_2.

Reilley and Papa (66) based a single point method for second-order reactions on Eq. 3. To make this equation applicable they arranged the concentration of the reagent equal to the total concentration of the components in the mixture being determined, *i.e.*, they used the conditions that $a = b$. In addition to the value of x measured at a single time, the rate constant must be known. The rate constants need not be known if x is measured at two different times and the resulting two equations are solved simultaneously.

The single and double point methods have been extended so that it is not necessary to have the condition of equivalency of the reagent concentration and the total sample concentrations (54). In this case, Eq. 2 is used with the requirement that the value for x is obtained after complete reaction of a_1, and the rate constant for the reaction of the slower component of the mixture is known. Again this process can be expanded to a double point method obviating the need for the rate constant if the process of solving simultaneous equations is used.

Roberts and Regan (67) proposed a somewhat different approach for the analysis of mixtures. They changed a normally second-order reaction to pseudo first order by having the concentration of the sample mixture in large excess relative to the reagent. Then they represented the first-order rate constant, k, by

$$k = k_1 a_1 + k_2 a_2 \qquad \text{(Eq. 9)}$$

Because $a_1 = a - a_2$, Eq. 9 can be rewritten to obtain

$$a_1 = \frac{k - k_2 a}{k_1 - k_2} \qquad \text{(Eq. 10)}$$

If k_1 and k_2 are separately determined for pure compounds, a is determined and k is measured for experimental mixture, then Eq. 10 can be solved for a_1, and a_2 can be obtained by difference from a.

Greinke and Mark (25) used the Roberts and Regan approach but followed the reaction in two different solvent mediums and obtained two different over-all pseudo first-order rate constants. Then by proper substitution in Eq. 9 and simultaneous solution of the two resulting equations, they were able to resolve binary mixtures.

APPLICATIONS OF DIFFERENTIAL KINETIC ANALYSIS

The differential kinetic approach has proven quite general and has been applied to the analyses of most of the organic functional groups. Siggia and Hanna (73) used the second-order graphical method involving extrapolation to the zero time intercept to analyze mixtures of hydroxyl-containing compounds. They monitored the reaction with acetic anhydride and determined primary alcohols in the presence of secondary alcohols, primary hydroxyl groups in polyhydric alcohols also containing secondary hydroxyl groups, and an alcohol in the presence of its next higher homolog as well as in the presence of one further separated in the series. Reilley and Papa (66) applied the graphical approach based on Eq. 7 and also the single and double point methods with the condition that $a = b$ to analyze mixtures of *n*-butanol and *sec*-butanol. Hanna and Siggia

(32) determined the primary hydroxyl group content of polyglycol ethers by graphical extrapolation of the kinetic data obtained by reaction of the hydroxyls with both acetic anhydride and phenyl isocyanate. Budd (12) substituted phthalic anhydride as the reagent for the same system when aldehydes or phenols were present. Hendrickson (33) used an infrared method to determine primary hydroxyl groups in polyglycol ethers. He used trityl chloride as the reagent, followed the disappearance of the hydroxyl band, and performed a graphical analysis of the kinetic data. Willeboordse and Critchfield (83) followed the disappearance of the NCO band in the infrared spectrum as the hydroxyl groups of mixtures of alcohols including polyglycol ethers reacted with phenyl isocyanate. They showed the applicability of the graphical approach and the single and double point methods to their process. They also analyzed ternary mixtures of alcohols based on the equations worked out by Schmalz and Geiseler (72). These equations require two data points and the rate constants, but become very involved and impractical for more than three component mixtures. Mark (48) published data showing that enzyme catalyzed reactions can be applied to the analyses of mixtures of ethanol and *n*-propanol by a kinetic method involving double points. He used alcohol dehydrogenase to catalyze the oxidation of the alcohols at different rates and followed the reactions spectrophotometrically.

Garmon and Reilley (24) analyzed mixtures of glycolic and diglycolic acids by measuring the color produced with 2,7-dihydroxynaphthalene at two different reaction times and used the double point technique to calculate the concentrations. Roberts and Regan (67) analyzed mixtures of carboxylic acids based on the rates of reaction with diphenyldiazomethane. They applied their technique of having the concentration of the carboxylic acid mixture in large excess relative to the concentration of the reagent and based the calculations on Eq. 10.

Lee and Kolthoff (38) analyzed mixtures of esters by comparing the amount of saponification after a selected time interval with a calibration curve prepared using known concentration mixtures.

Lee and Kolthoff (38) also analyzed mixtures of carbonyl compounds in the same manner based on the rates of decomposition of the bisulfite addition products. Only aldehydes and a few reactive ketones can be determined with this reaction. Fowler, Kline, and Mitchell (20, 21) determined aromatic aldehydes in mixtures with aromatic ketones by their rates of reaction with hydroxylamine hydrochloride. They used calibration curves to relate the amount of reaction after a specified time to the original concentration. Siggia and Hanna (73) applied the second-order graphical approach to the oximation reaction and analyzed mixtures of an aldehyde and a ketone, and mixtures of two aldehydes and mixtures of two ketones. They followed the reaction by maintaining the pH constant by titrating the hydrochloric acid as it was liberated from the reagent. Papa and co-workers (55) followed the course of the oximation reaction with a direct reading conductance meter. They made the reaction pseudo first order by adding the reagent in large excess and then compared the time interval for the reaction of a preselected fraction of the mixture and a predetermined calibration curve. Greinke and Mark (25) employed this same indicating technique but used two different alcohol–water concentration mediums to obtain data for their evaluation of mixtures of ketones based on Eq. 9. Sihtola, Neimo, and Sumiala (80) applied the second-order graphical approach to analyze the carbonyl groups in cellulose and were able to classify six different carbonyl groups present according to reactivity. They determined the slowest component first from the intersection of its extrapolated line with the zero time axis as usual. Then they subtracted the contribution of the slowest component from *a* and *b*, made a new plot, and performed the extrapolation again to obtain the value for the concentration of the next slowest component. They repeated this procedure until all components were evaluated. Papa, Mark, and Reilley (53) applied both single and double point methods to analyze fructose and glucose in aqueous media and in blood. They measured the rate of formation of molybdenum blue from Mo VI and also analyzed mixtures of sucrose and fructose and of sucrose and glucose. Mark and co-workers (49) used 2,3,5-triphenyl-2H-tetrazolium chloride as a reagent for the determination of mixtures of reducing sugars. A red formazan was formed which was measured spectrophotometrically to follow the reaction. The double point method and the solution of simultaneous equations were used in the calculations. Krivis and Supp (37) demonstrated the use of polarography combined with a graphical handling of the data to evaluate mixtures of Δ^4-3-ketosteroids and $\Delta^{1,4}$-3-ketosteroids. The mixtures were reacted with semicarbazide and hydrocortisone–prednisolone and cortisone–prednisone mixtures were differentiated.

Hanna and Siggia (30) adapted the reaction of amines with phenyl isothiocyanate to the differential kinetic analysis of mixtures of primary and

secondary amines, mixtures of homologous primary and of secondary amines, and mixtures of aliphatic and aromatic amines. The reaction to form thiourea is a second-order process and the graphical extrapolation technique was applied.

Siggia, Hanna, and Serencha (78) measured the rate of evolution of ammonia as a result of alkaline hydrolysis to analyze mixtures containing both a nitrile and an amide as well as binary mixtures of each of these compounds. Standard first-order rate plots were made from the data obtained and the linear portion extrapolated to zero time to calculate the composition of the mixture.

Siggia, Hanna, and Serencha (75) used both bromination with free bromine and catalytic hydrogenation with hydrogen gas followed by graphical extrapolation of the rate curves to resolve qualitatively mixtures of unsaturated compounds. The decrease in bromine content of the bromination reaction mixture was followed colorimetrically with time and showed a second-order rate process. In the hydrogenation process, the hydrogen was present in a large excess causing the reaction to proceed in a pseudo first-order pattern. Polgár and Jungnickel (65) described a method to measure the amount of substitution realized when bromine was added to unsaturated linkages. Several additional determinations were made at times in excess of that needed for complete saturation of the compound. They plotted the results against time and extrapolated the final linear portion to zero time to obtain the correct value for the addition reaction. Kolthoff and Lee (35) and Saffer and Johnson (69) applied the rates of reaction of perbenzoic acid with olefinic double bonds as a basis for differential analysis. They found the reaction valuable to distinguish between the amounts of internal and external double bonds in polymers. Reference of the amount of reaction after a specified time was related to calibration curves to determine the compositions of mixtures. Schmalz and Geiseler (72) also used the perbenzoic acid reaction to analyze mixtures of unsaturated compounds. They determined the rate constants for the components based on a single reaction of a mixture. Their calculations were based either on the differential equations, using graphically determined values of the derivatives, or on the integrated rate laws, using the area under the experimentally determined reaction rate curves. The derivated rate constants were then used to calculate the composition of the sample mixtures. Connor and Wright (16) used the difference in rates of the addition of mercuric acetate to double bonds to determine the *cis–trans* concentration ratio in isomer mixtures, specifically for the analysis of mixtures of ethyl oleate and ethyl elaidinate.

Salzman and Gilbert (70) differentiated natural smog oxidants into components by following the rate of oxidation of potassium iodide colorimetrically. The reaction showed a pseudo first-order rate process because the iodide was present in a large excess. They applied the graphical method to resolve the mixtures.

Hass and Weber (34) found a linear relationship between the second-order rate constants for the reaction of mixtures of the monochlorides of *n*-pentane and isopentane and the composition. Brown and Fletcher (11) used the slope and zero time intercept of the first-order rate plot of the hydrolysis of mixtures of isomers of diethyl-*tert*-butylcarbinyl chloride for analysis.

Siggia, Hanna, and Serencha (77) measured the rate of liberation of nitrogen from diazonium compounds in the presence of cuprous chloride to analyze mixtures of these compounds. They found the decomposition followed a first-order pattern and applied the graphical extrapolation procedure.

Block, Morgan, and Siggia (4) combined the graphical differential reaction rate technique with the micro-Dumas method to analyze mixtures of nitrogen-containing compounds. The reactions studied were first order with respect to the nitrogen-containing compound.

Special approaches available to monitor rapid reactions should provide the mechanics for the performance of the differential rate analyses of mixtures of inorganic compounds. Hanna and Siggia (31) demonstrated that continuous flow techniques can be applied to the analysis of mixtures of iodate and bromate, periodate and bromate, and dichromate and bromate. These analyses involved the observance of the point of appearance of the starch–iodide color in the flow tube as a result of the reaction with potassium iodide. Various measurement techniques can probably be applied for other reactions; therefore, the scope of the flow systems for differential analysis is potentially wide.

Siggia, Hanna, and Serencha (76, 79) demonstrated that for analytical purposes, data obtained from the rates of distillation of mixtures, the rates of effusion of mixtures of gases through a small orifice, and the dialysis of mixtures under specific conditions could be treated in a manner similar to the treatment of data from chemical reactions. The distillation procedure was applied to mixtures of organic acids, mixtures of alcohols, mixtures of carbonyl compounds, and mixtures of aromatic compounds. The effusion method was applied to mixtures of both inorganic and organic gases. The dialysis method was found

applicable to mixtures of inorganic salts, of salts of organic acids, of inorganic acids, of organic acids, of amines, of sugars, and of amino acids.

Siggia and Hanna (74) have pointed out that in the case of some binary mixtures the effect of the presence of the faster reacting component is to decrease the rate of reaction of the slower component. When the graphical approach is used, this effect works to an advantage because the experimental differences between the slopes are more pronounced than are expected from theoretical plots. Methods that rely on rate constants obtained using pure materials for the final calculations, however, can give significant errors. The effect was noted particularly for mixtures of some ketones determined by the oximation procedure and mixtures of oleates determined both by bromination and hydrogenation.

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Kinetics of Reactions Involved in Penicillin Allergy I

Mechanism of Reaction of Penicillins and 6-Aminopenicillanic Acid with Glycine in Alkaline Solution

By MICHAEL A. SCHWARTZ and GAY-MAY WU

The principal antigenic determinant of penicillin allergy, penicilloyl-protein, may be formed either by direct reaction of penicillin with protein, or through penicillenic acid, a rearrangement product of penicillin. As part of a study of the mechanism of the direct reaction, the kinetics of reaction of glycine with a series of penicillins and 6-aminopenicillanic acid has been investigated. The aminolysis of penicillins by glycine is general base catalyzed, while the reaction with 6-APA is uncatalyzed. The latter is probably due to intramolecular general-base catalysis. The rates of reaction with glycine of all the penicillins studied are of the same order of magnitude. This is in contrast to the rates of penicillenic acid formation which are highly dependent upon the nature of the penicillin side chain. The pertinence of these results to the mechanism of formation of penicillin antigen is discussed.

It is generally agreed that the principal antigenic determinant of penicillin allergy is the penicilloyl group covalently bound to body protein, probably through ϵ-amino groups of lysine residues (1, 2). As shown in Scheme I, there are two pathways by which the antigen may form. One involves the known rearrangement of penicillin to penicillenic acid (II) which is known to react rapidly with amines. The other is a direct reaction of protein with penicillin. The latter will produce the α-diastereoisomer of the penicilloamide while the penicillenic acid route requires isomerization of the asymmetric carbons (marked with asterisk in Scheme I) of the β-lactam ring to produce a mixture of diastereoisomers.

The evidence for the penicillenic acid route was recently summarized by Levine (3). (*a*) Penicillenic acid has been found in penicillin preparations (4); (*b*) while penicillin does react rapidly with ϵ-aminocaproic acid at pH 11.5, this reaction would be slow at physiological pH; (*c*) penicillenic acid is a very reactive compound chemically (5); (*d*) studies on the nature of antibody to penicillin G indicated specificity toward the mixture of diastereoisomers of the benzylpenicilloyl haptenic group rather than for the α-diastereoisomer alone (6).

On the other hand, evidence has been accumulated to show that the direct reaction of penicillin with protein may play a more important role than previously thought. Thiel, Mitchell, and Parker (7) found that the sera of most of 114 patients allergic to penicillin contained antibody specific for the α-diastereoisomer of penicilloamide. This is in direct contrast to Levine's results cited above (6). More recently two groups (8, 9) have found that penicillins can indeed combine with protein at neutral pH at 37° *in vitro*. These included penicillins which were known to produce penicillenic acid at relatively low rates. Also included was 6-aminopenicillanic acid (6-APA), the nucleus common to all penicillins, which cannot form a penicillenic acid. It is known that 6-APA is antigenic in rabbits, and it has been suggested as an agent causing penicillin allergy (10).

The present work was undertaken to investigate the rates and mechanisms of reactions of penicillin which may be involved in penicillin allergy. Rather than a complex protein, glycine was selected as a model amine for study because of its ready availability in high purity, and its high solubility. The rate of penicillenic acid formation as a function of pH has also been measured for several penicillins.

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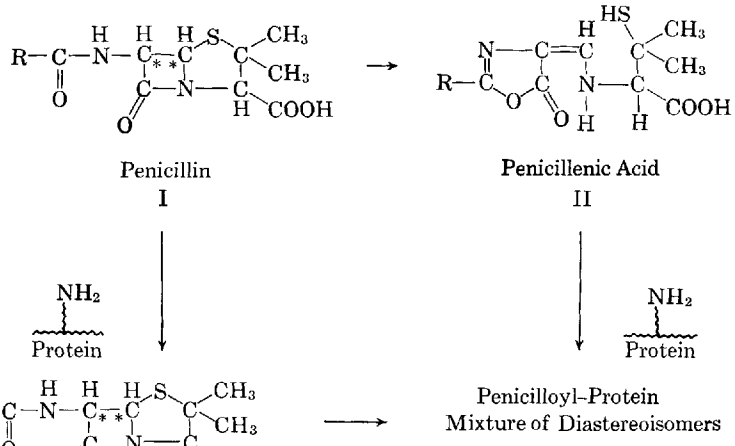


Formation of Penicillin Antigen

Scheme I

EXPERIMENTAL

The penicillins were all commercial products.[1] Glycine and mercuric chloride were Fisher certified reagent, as were the other reagents used.

The rates of formation of penicillenic acid from penicillin were measured by following the increase in absorbance at 322 mμ as a function of time. This wavelength is characteristic of the penicillenic acid chromophore. The rates were determined in a series of buffers extending over the pH range 1–4 at 34° on a Beckman DB recording spectrophotometer with thermostated cell compartment. Mercuric chloride was added to the buffer in a concentration equal to the initial penicillin concentration. It was known that mercuric chloride stabilizes the penicillenic acid (11). The authors' preliminary studies showed that mercuric chloride had no effect, however, upon the *rate* of penicillenic acid formation.

The rates of reaction of the penicillins with glycine were determined by following the changes in optical rotation accompanying opening of the β-lactam ring of the penicillin (12) with a Perkin-Elmer model 141 photoelectric polarimeter using a mercury vapor source with filter for 365 mμ. The glycine itself acted as buffer and maintained pH within less than 0.1 unit during a single determination. Ionic strength of the buffers was brought to 0.5 by addition of potassium chloride. Initial penicillin concentration was 0.001 or 0.002 *M*. The temperature in these studies was maintained at 50° by water circulating through the polarimeter cell from a thermostated bath.

The pH of the buffers was measured at 50° with a Beckman expanded scale pH meter. The pKa′ of glycine at 50°, ionic strength 0.5, was determined from the pH of the buffers to be 9.10 ± 0.02.

The rates of alkaline hydrolysis of the penicillins in the absence of glycine were determined at 50° by measurement of the rate of acid production on a Radiometer TTT-1 titrator-pH stat equipped with SBR-2, SBU-1 recorder. Ionic strength in these runs was brought to 0.5 by addition of KCl. The rates for each penicillin were measured at several pH's.

RESULTS

Penicillenic Acid Formation.—The first-order rate constants for penicillenic acid formation were calculated by dividing the initial slope of a plot of absorbance at 322 mμ *versus* time by the initial concentration of penicillin.

$$k_{obs.} = \frac{\text{initial slope}/26{,}600}{\text{initial concn. of penicillin}}$$

The factor 26,600 is the molar absorptivity of penicillenic acid (5). Figure 1 shows these rate constants as a function of pH. These curves follow the same pattern previously observed for penicillin G (13) where the rate of penicillenic acid formation was found to be dependent upon the concentration of undissociated penicillin acid (HP):

$$\frac{d\,(\text{penicillenic acid})}{dt} = k_{obs.}\,(\text{HP}) = \frac{k_E(\text{H}^+)}{(\text{H}^+) + Ka}\,(\text{P})_{total}$$

where k_E represents the specific pH-independent rate constant. At high pH where $(H^+) \ll Ka$, the observed first-order rate constant will be directly proportional to (H^+) and, as shown in the dashed lines of Fig. 1, will decrease rapidly with increasing pH, and approach very low values at physiologic pH. There is a large difference in the magnitude of these rates for the various penicillins, the rate for methicillin being about 20 times that for oxacillin and about 50 times that of penicillin V. It is known that penicillin G fits in between oxacillin and methicillin, and much closer to the latter (13). Ampicillin produces penicillenic acid even more slowly than penicillin V (11).

Reaction of Penicillins with Glycine.—The optical rotation was followed until no further change

[1] The authors are grateful to Eli Lilly and Co., Indianapolis, Ind., for a supply of potassium penicillin V and to Bristol Laboratories for the other penicillins and 6-APA used in this work.

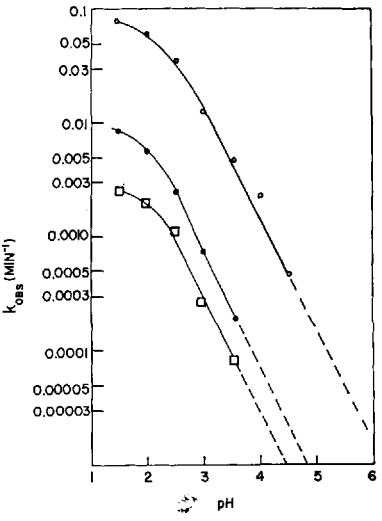


Fig. 1.—Rate of formation of penicillenic acid as a function of pH. Key: ○, methicillin; ●, oxacillin; □, penicillin V.

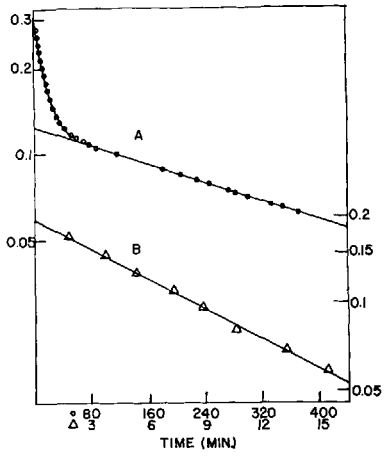


Fig. 2.—First-order plot for reaction of methicillin with 0.3 *M* glycine buffer, pH 9.47 at 50°. Key: ●, $(R - R_\infty)$ *vs.* *t*, left ordinate; △, residuals, right ordinate. Ordinates are log scale.

occurred and this value was designated R_∞. Plots of log $(R_t - R_\infty)$ against time were expected to show apparent first-order kinetics since the concentration of glycine was in large excess over that of penicillin. As seen in Fig. 2, curve A, large deviations from linearity were observed. This particular curve depicts the reaction of methicillin with 0.3 *M*, pH 9.48 glycine buffer, but is typical of all the studies.

It is known that reaction of penicillin with amines yields the α-penicilloamide which can undergo mutarotation to a mixture of diastereoisomers. The mixture is known to have a lower specific rotation than the α-diastereoisomer and the specific rotation of the penicillin is higher than that of the α-diastereoisomer (14). Essentially the following is the situation:

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \qquad \text{(Eq. 1)}$$

where *A* represents the original penicillin, *B* the α-diastereoisomer, and *C* the mixture of diastereoisomers. The apparent first-order rate constants are k_1 and k_2.

In this system:

$$A = A_0 e^{-k_1 t} \qquad \text{(Eq. 2)}$$

$$B = \frac{k_1 A_0}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \qquad \text{(Eq. 3)}$$

$$C = A_0\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \qquad \text{(Eq. 4)}$$

Let r_A, r_B, r_C represent the molar rotations of the respective species. The rotation at any time during the course of the reaction, R_t, will be the sum of the rotations of the individual species:

$$R_t = r_A(A) + r_B(B) + r_C(C) \qquad \text{(Eq. 5)}$$

$$R_t = r_A A_0 e^{-k_1 t} + \frac{r_B k_1 A_0}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) + r_C A_0\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \qquad \text{(Eq. 6)}$$

Combining terms with the same exponential:

$$R_t = \left[r_A A_0 + \frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_2}{k_1 - k_2}\right] e^{-k_1 t} - \left[\frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_1}{k_1 - k_2}\right] e^{-k_2 t} + r_C A_0 \qquad \text{(Eq. 7)}$$

At infinite time $R = r_C A_0 = R_\infty$

$$R_t - R_\infty = \left[r_A A_0 + \frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_2}{k_1 - k_2}\right] e^{-k_1 t} - \left[\frac{r_B k_1 A_0}{k_2 - k_1} + \frac{r_C A_0 k_1}{k_1 - k_2}\right] e^{-k_2 t} \qquad \text{(Eq. 8)}$$

Since k_1 is faster than k_2, at some time where $e^{-k_1 t}$ approaches zero there will remain a term in $e^{-k_2 t}$ and the plot of $\ln(R_t - R_\infty)$ *versus* time will be linear with slope $-k_2$. This linear portion of the curve may be extrapolated to zero time and the values of $(R_t - R_\infty)$ along this portion of the line represent the contribution of the term in $e^{-k_2 t}$ to the over-all value. Thus subtraction of this term from the observed $(R_t - R_\infty)$ will leave only the term in $e^{-k_1 t}$. Then a plot of logarithm of these residuals against time should be linear with slope $-k_1$, and this is shown as curve B in Fig. 2.

The rate constant k_1 was determined for each penicillin and 6-APA, and was corrected for alkaline hydrolysis in the absence of buffer:

$$k_C = k_1 - k_{OH}(OH^-) \qquad \text{(Eq. 9)}$$

It was found, for the penicillins, that k_C was not a linear function of glycine concentration at constant pH but was a linear function of the square of the glycine concentration as shown in Fig. 3. These particular lines represent reaction of ampicillin but are typical of those observed with all of the penicillins studied. In the case of 6-APA, on the other hand, there *was* a linear relationship between rate constant (k_C) and concentration of glycine as shown in Fig. 4.

The slopes of the lines in Fig. 3 represent the proportionality constant between k_C and the square of

the total glycine concentration and are designated k_G. It can be seen that k_G increases with increasing pH implicating the glycine anion, $NH_2CH_2COO^-$, as the reactive species. Figure 5 shows the direct dependence of k_G on the square of the fraction of the total glycine which exists as anion:

$$k_G = k_G^-(f_G^-)^2 \qquad \text{(Eq. 10)}$$

Thus the entire rate may be attributed to a third-order reaction with no second-order term in evidence.

In the case of 6-APA the slopes of the lines in Fig. 4 were found to be proportional to the fraction of the total glycine existing as anion:

$$k_G = k_G^-(f_G^-) \qquad \text{(Eq. 11)}$$

Table I gives both k_G and k_G^- for all the penicillins studied and 6-APA. Also included are the rate constants for alkaline hydrolysis. Each of the latter represents the average of 3 or 4 determinations.

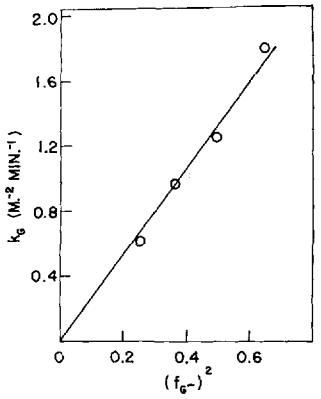


Fig. 5.—Plot showing glycine anion as reactive species.

DISCUSSION

The requirement of 2 molecules of glycine anion for reaction with the penicillins is indicative of a general base-catalyzed nucleophilic attack of glycine on the β-lactam carbonyl. General base-catalyzed aminolysis of esters (15, 16) and δ-thiolvalerolactone (17) have been reported. In the case of aminolysis of phenyl acetate by glycine (16) both general base-catalyzed and an uncatalyzed reaction were detected. In the present study an uncatalyzed aminolysis could not be detected, although one would suspect that such a reaction should occur in an aqueous medium. Probably the rate of such a reaction is so small that its contribution to the over-all rate could not be observed.

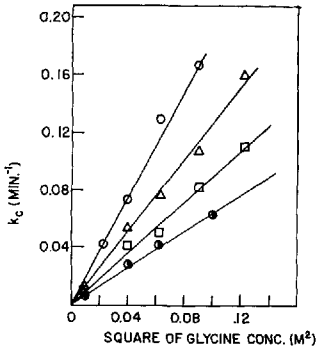


Fig. 3.—Dependence of rate of reaction of ampicillin with glycine upon the square of the glycine concentration. Key: O, pH 9.70; △, pH 9.48; □, pH 9.29; ◑, pH 9.10.

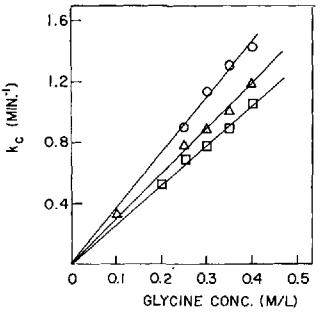


Fig. 4.—Dependence of rate of reaction of 6-APA upon glycine concentration. Key: O, pH 9.70; △, pH 9.48; □, pH 9.29.

Also absent from the rate law for reaction of penicillins with glycine was a term indicating general acid-catalyzed aminolysis as was noted with δ-thiolvalerolactone (17) in addition to the general base-catalyzed reaction. Again this might make too small a contribution to the over-all rate to be noticed or might be absent entirely.

A mechanism for general base-catalyzed aminolysis of penicillin may be described schematically as follows:

$$P + G^- \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} PG^-$$

$$PG^- + G^- \xrightarrow{k_4} G^{\pm} + \text{product} \qquad \text{(Eq. 12)}$$

where P represents penicillin, G^- the glycine anion, and $G^{\pm}$ the zwitter ion. The intermediate produced in the first reaction could be a tetrahedral addition product or a complex. The driving force in the second step is the removal of a proton from the intermediate by the second glycine anion. Assuming a steady state in the intermediate:

$$\frac{d(PG^-)}{dt} = k_3(P)(G^-) - [k_{-3} + k_4(G^-)]\,PG^- = 0 \qquad \text{(Eq. 13)}$$

$$(PG^-) = \frac{k_3(P)(G^-)}{k_{-3} + k_4G^-} \qquad \text{(Eq. 14)}$$

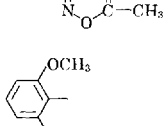
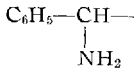
$$K_C = \frac{1}{P}\frac{dP}{dt} = \frac{k_4k_3(G^-)^2}{k_{-3} + k_4(G^-)} \qquad \text{(Eq. 15)}$$

when $k_{-3} \gg k_4(G^-)$

$$K_C = \frac{k_4k_3}{k_{-3}}(G^-)^2 \qquad \text{(Eq. 16)}$$

At sufficiently high (G^-) it might be thought from Eq. 15 that the rate would reduce to first order in

TABLE I.—k_G AND k_G^- FOR PENICILLINS AND 6-APA AND RATE CONSTANTS FOR ALKALINE HYDROLYSIS

Penicillin	Side Chain, R in I, Fig. 1	k_{OH} (L. mole^{-1} min.$^{-1}$)	k_G at Designated pH (L.^{2} mole^{-2} min.$^{-1}$) 9.70	9.47	9.29	9.10	k_G^- (L.^{2} mole^{-2} min.$^{-1}$)
G	$C_6H_5CH_2$—	43	1.48	1.10	0.82	0.56	2.3
V	$C_6H_5OCH_2$—	63	2.44	1.77	1.30	...	3.7
Oxacillin		52	1.34	1.00	0.77	0.56	2.1
Methicillin		32	1.18	0.76	0.58	0.37	1.6
Ampicillin		56	1.80	1.15	0.89	0.65	2.7
6-APA	...	12	0.036[a]	0.030[a]	0.026[a]	...	0.44[a]

[a] Units are L. mole^{-1} min.$^{-1}$.

glycine anion. This was not observed at concentrations up to 0.3 *M*, as would be expected for the rapid equilibration between reactants and intermediate which would take place in this case.

In the case of 6-APA only over-all second-order kinetics was observed. This might be due to intramolecular general base catalysis by the free amino group on the carbon adjacent to the β-lactam carbonyl, or a relatively large contribution by the uncatalyzed aminolysis, or both.

From these results one might speculate that compounds containing both general base and nucleophilic groups on the same molecule, diamines for example, would react much more rapidly with penicillin than glycine. This is currently under investigation, and preliminary results indicate that this is indeed the case. The results of these experiments will be the subject of a future communication (18). Intramolecular general base catalysis could provide a rationale for rapid reaction of penicillin with proteins at physiological pH, which is pertinent to the question of penicillin antigen formation. On protein molecules there could be many sites where intramolecular general base catalysis of aminolysis could occur at rates rapid enough to compete with the elimination of penicillins from the body where the half-life is about 30–60 min.

Also of pertinence to the mechanism of penicillin antigen formation is the fact that the rates of reaction of all these penicillins with glycine are of the same order of magnitude, a factor of about 2.3 separating the slowest (methicillin) from the most rapid (penicillin V). This shows a lack of influence of the side chain upon reactivity toward nucleophiles of the β-lactam carbonyl. This is also apparent in the values for k_{OH}. On the other hand, the side chain has a great effect on the rates of penicillenic acid formation from penicillins. Although clinical experience with some of these penicillins is rather limited, allergic reactions to all the penicillins have been reported (19). Even though many of these are probably due to cross-reaction with antibody to penicillin G, the new penicillin must still combine covalently with protein to provide an antigen. From the relative rates reported here it would seem more likely that the direct reaction between penicillin and protein is taking place rather than the penicillenic acid route.

While the side chain (R in I) appears to have little influence on reaction of penicillins with small molecules, the situation with proteins might be quite different where steric effects of the side chain might play a role. Such effects are observed in the relative affinities of the various penicillins for the enzyme penicillinase. Methicillin and oxacillin have very little affinity for the enzyme while penicillin G, penicillin V, and ampicillin are relatively tightly bound. Only a hint of steric effect was seen in the present work where the rate constant for reaction of oxacillin with glycine is slightly higher than that of penicillin G, while the reverse is true of the k_{OH} values. Oxacillin has the bulky phenyl group on the carbon adjacent to the point of attachment which might interfere somewhat with the approach of the glycine more than with hydroxyl ion. The magnitude of the differences is too small, however, to draw definite conclusions and further studies of steric effects are contemplated.

In contrast to the potentially relatively rapid rates of reaction of penicillins with proteins are the very low rates of penicillenic acid formation calculated for neutral pH. For example, the half-life for penicillenic acid formation from methicillin at pH 7.4 at 34° would be about 1000 days. It would seem then that if the penicillenic acid route to antigen is of any consequence the penicillenic acid would have to be present in a preparation *prior* to parenteral administration. This is possible as a result of decomposition under normal storage conditions particularly in liquid dosage forms. Since acid is produced upon hydrolysis of penicillins the pH will be lowered, thus increasing the rate at which penicillenic acid is formed. It would seem important, therefore, that parenteral penicillin preparations be buffered to minimize this route of degradation. With oral preparations penicillenic acid is probably formed quite rapidly in the acid stomach. The limiting factor would then become the absorption of penicillenic acid. Since oral administration of penicillins has produced the least number of clinical allergy problems of any route, this might be considered relatively unimportant.

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(2) Parker, C. W., *Federation Proc.*, **24**, 51(1965).
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(7) Thiel, J. A., Mitchell, S., and Parker, C. W., *J. Allergy*, **35**, 399(1964).
(8) Batchelor, F. R., Dewdney, J. M., and Gazzard, D., *Nature*, **206**, 362(1965).
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(18) Schwartz, M. A., unpublished data.
(19) Grieco, M. H., Dubin, M. R., Robinson, J. L., and Schwartz, M. J., *Ann. Internal Med.*, **60**, 304(1964); and references quoted therein.

Polarographic Study of Pteridines

By MILTON LAPIDUS and MARVIN E. ROSENTHALE

The electronegativity of the half-wave potentials of a series of pteridine congeners was found to be related to the substituent groups. The 2,4,7-triaminopteridines, 7-substituted 4-amino-2-aryl-6-pteridinecarboxamides, and 4,7-diamino-2-aryl-6-pteridinecarboxamides were characterized, in that order, by decreasingly lower electronegative half-wave potentials.

A NUMBER of attempts have been made to correlate pharmacological activity with the oxidation-reduction potentials of a homologous series of compounds (1). Relationships have seldom been demonstrated; however, there is a report that acridines with reduction potentials (E°_{h}) more negative than -0.400 v. have greater antiseptic activity (2) than those with less negative potentials. That study showed the active acridines to have reduction potentials so electronegative that no physiological system could reduce (inactivate) them. Evidently, with the acridines, the maintenance of the oxidized configuration is necessary for biological activity. Another study showed that the degree to which members of two homologous series of acridine antimalarials inhibit the diamine–oxidase enzyme system parallels the values of the reduction potential of the compounds (3). However, no evidence was found that inhibition of diamine–oxidase is necessary to antimalarial action (4).

The possible importance of the oxidation-reduction potential prompted the authors to determine it for a large number of pteridines which have been under pharmacological review. Some of these pteridines are useful diuretic agents (5–8).

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The authors acknowledge the technical assistance of Mr. D. D. Austin.

METHOD

Material.—The pteridines studied were synthesized by Osdene *et al.* (9).

Polarographic Analysis.—A Leeds and Northrup recording polarograph equipped with a dropping mercury electrode was used for determining reduction potentials. The H-type electrolysis cell consisted of a saturated calomel half-cell connected to the test solution through an agar bridge and a fritted-glass diaphragm. Under a potential of -0.50 v. the dropping mercury electrode delivered 2.43 mg. of Hg/sec. with a drop time of 4.31 sec. All measurements were made at 25°. Unless otherwise noted, the solutions for polarographic analysis contained 5×10^{-4} *M* pteridine, 0.1 *M* phosphate buffer (pH 2.0), and 0.005% gelatin. The calibrating solution, which contained zinc chloride (1×10^{-3} *M*) in place of the pteridine, gave an $E^{1}_{1/2}$

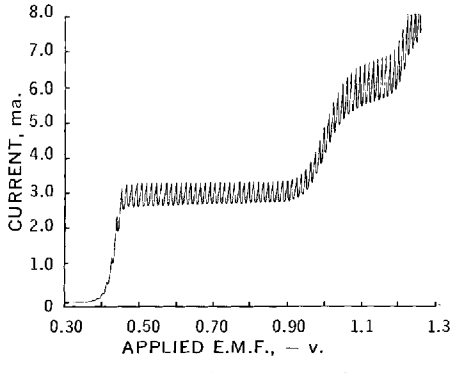

Fig. 1.—Polarographic reduction of Wy-4437 (pH 2.0).

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(1) Levine, B. B., and Ovary, Z., *J. Exptl. Med.*, **144**, 875 (1961).
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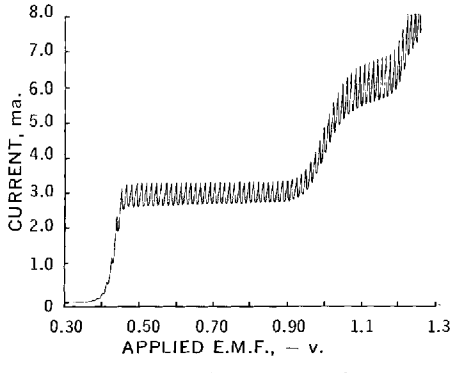
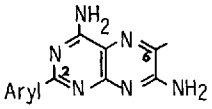
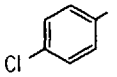
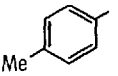
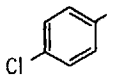
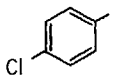
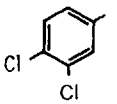


Fig. 1.—Polarographic reduction of Wy-4437 (pH 2.0).

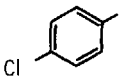
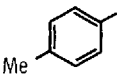
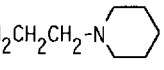
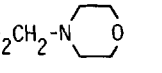
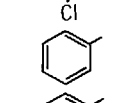
TABLE I.—HALF-WAVE REDUCTION POTENTIALS OF 4,7-DIAMINO-2-ARYL-6-PTERIDINECARBOXAMIDES (CLASS I)



Wy-No.	2	6	$E^1_{1/2}$	$E^2_{1/2}$
1512		-CONHMe	0.430	0.980
1841		-CONHCH$_2$CH$_2$CH$_2$NEt$_2$	0.430	1.010
1843		-CONHCH$_2$CH$_2$NEt$_2$	0.420	0.980
3580		-CONHCH$_2$CH$_2$NMe$_2$	0.430	1.000
3583		-CONH- 	0.405	0.965
3588		-CONEtCH$_2$CH$_2$NMe$_2$	0.445	1.000
3597		-CONMeCH$_2$CH$_2$NMe$_2$	0.440	1.020
3598		-CONHCH$_2$CH$_2$CH$_2$NBu$_2$	0.410	0.985
3599		-CON  NCH$_2$CH$_2$OH	0.440	1.015
3605		-CONHCH$_2$CH$_2$N(CHMe$_2$)$_2$	0.420	0.990
3654		-CONHCH$_2$CH$_2$N 	0.420	0.990
3665		-CONHCH$_2$CH$_2$CH$_2$NEt$_2$	0.410	0.985
3700		-CONHCH$_2$CH$_2$NEt$_2$	0.415	0.960
3873		-CONHCH$_2$CH$_2$CH$_2$NMe$_2$	0.420	0.990
4029		-CONHCH$_2$CH$_2$NEt$_2$	0.415	0.985
4119		-CONHCH$_2$CH$_2$N 	0.395	0.967
4153		-CONHCH$_2$CH$_2$OEt	0.415	0.990
4156		-CONHCH$_2$CH$_2$OMe	0.425	0.985
4196		-CONHCH$_2$CH$_2$NEt$_2$	0.390	0.987

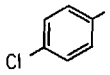
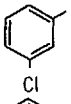
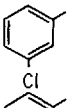
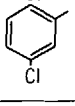
(*Continued on next page.*)

TABLE I.—(*Continued*)

Wy-No.	2	6	$E^1_{1/2}$	$E^2_{1/2}$
4276		-CONHCH$_2$CH$_2$NH$_2$	0.425	0.987
4277		-CONHCH$_2$CH(OEt)$_2$	0.430	1.020
4278		-CONHCH$_2$CH$_2$N(CH$_2$CH$_2$OH)$_2$	0.440	1.000
4376		-CONHCH$_2$CH(OEt)$_2$	0.455	0.950
4377		-CONHCH$_2$CH(OEt)$_2$	0.430	0.995
4436		-CONHCH$_2$CH$_2$CH$_2$-N 	0.440	1.025
4437		-CONHCH$_2$(CH$_2$)$_2$CH$_2$NMe$_2$	0.430	1.015
5121		-CONHCH$_2$(CH$_2$)$_2$CH$_2$NEt$_2$	0.440	1.010
5250		-CONHCH$_2$(CH$_2$)$_4$CH$_2$NMe$_2$	0.400	0.967
5330		-CONHCH$_2$(CH$_2$)$_5$CH$_2$NEt$_2$	0.405	0.995
5331		-CONHCH$_2$(CH$_2$)$_3$CH$_2$NMe$_2$	0.440	1.005
5365		-CONHCH$_2$(CH$_2$)$_3$CH$_2$NEt$_2$	0.410	0.990
5588		-CONHCH$_2$CH$_2$NEt$_2$	0.410	0.980
5589		-CONHCH$_2$CH$_2$-N 	0.425	1.000
6119		-CONHCH$_2$CH$_2$CH$_2$OEt	0.415	0.990
6520		-CONHCH$_2$CH$_2$NEt$_2$	0.410	0.995
7037		-CONHCH$_2$CH$_2$NEt$_2$	0.410	0.980
7038		-CONHCH$_2$(CH$_2$)$_2$CH$_2$NMe$_2$	0.427	1.035
7184		-CONHEt	0.425	1.000
7307		-CONHCH$_2$(CH$_2$)$_2$CH$_2$NMe$_2$	0.430	1.020

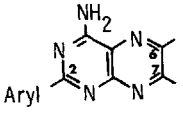
(*Continued on next page.*)

TABLE I.—(*Continued*)

Wy-No.	2	6	$E^1_{1/2}$	$E^2_{1/2}$
7322[a]		-CONHCH$_2$CH$_2$NMe$_2$	0.410	0.975
7323		-CONHCH$_2$CH$_2$NMe$_2$	0.405	0.980
7324[a]		-CONHCH$_2$CH$_2$CH$_2$NMe$_2$	0.400	0.975
7329		-CONHCH$_2$(CH$_2$)$_4$CH$_2$NMe$_2$	0.420	1.020
7330		-CONHCH$_2$(CH$_2$)$_3$CH$_2$NMe$_2$	0.420	1.015

[a] Less than 5×10^{-3} *M*.

TABLE II.—HALF-WAVE REDUCTION POTENTIALS OF 7-SUBSTITUTED 4-AMINO-2-ARYL-6-PTERIDINECARBOXAMIDES (CLASS II)



Wy-No.	2	6	7	$E^1_{1/2}$	$E^2_{1/2}$
4739		-CONHCH$_2$CH$_2$N 	-NHCH$_2$CH$_2$N 	0.480	1.070
4760		-CONHCH$_2$CH$_2$OEt	-NHCH$_2$CH$_2$OEt	0.480	1.050
5120		-CONHCH$_2$CH$_2$NEt$_2$	-NHCH$_2$CH$_2$NEt$_2$	0.490	1.100
5256		-CONHCH$_2$CH$_2$OMe	-NHCH$_2$CH$_2$OMe	0.500	1.070
5587		-CONHCH$_2$CH$_2$SEt	-NHCH$_2$CH$_2$SEt	0.480	1.050
5614		-CONHCH$_2$CH$_2$OMe	-NHCH$_2$CH$_2$OMe	0.515	1.085
5829		-CONHCH$_2$CH$_2$OEt	-NHCH$_2$CH$_2$OEt	0.485	1.055
6218		-CONHCH$_2$CH$_2$CH$_2$OEt	-NHCH$_2$CH$_2$CH$_2$OEt	0.510	1.100
7209		-CONHEt	-NHEt	0.470	1.050
7635		-CONHMe	-NHMe	0.565	1.080
7714		-CONHPr	-NHPr	0.500	1.020
7715		-CONHBu	-NHBu	0.500	...

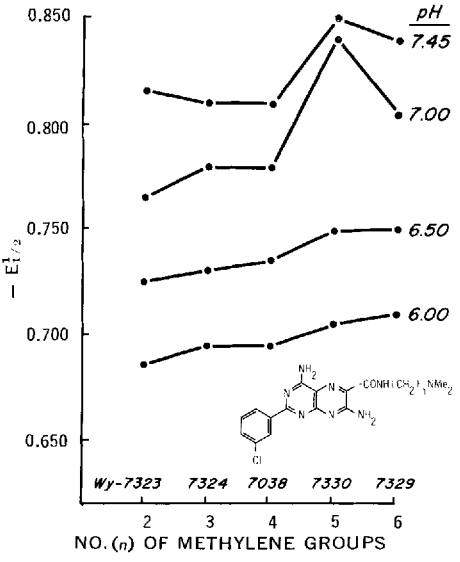


Fig. 2.—Half-wave potential profile for a series of 4,7-diamino-2-(*m*-chlorophenyl)-6-pteridine-carboxamides with varied length of methylene chain in 6-carboxamide substituent.

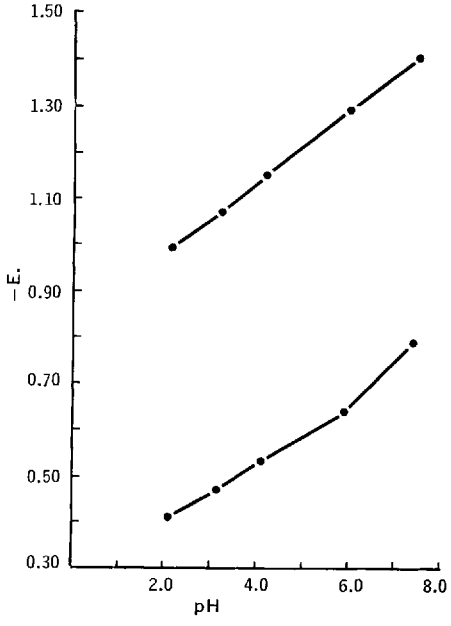


Fig. 3.—Variation in half-wave potential of Wy-4437 with change in pH. Key: top curve, $E^2_{1/2}$; bottom curve, $E^1_{1/2}$.

of −1.075, a value in agreement with the literature (10). Replicate samples varied ±0.015 v.

RESULTS

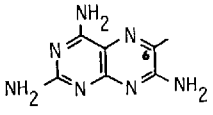
The pteridines gave well-formed 2-step polarographic waves (Fig. 1). The half-wave potentials, designated $E^1_{1/2}$ and $E^2_{1/2}$, were determined by expolation from the current (ma.) *versus* applied voltage (v.) record (11). Characteristic half-wave potentials, $E^1_{1/2}$, were found for each of the 3 general classes of pteridines at pH 2.0.

Class I.—*4,7-Diamino-2-aryl-6-pteridinecarboxamides.*—In this group the compounds had half-wave potentials of −0.390 to −0.460 (Table I).

Class II.—*7-Substituted 4-Amino-2-aryl-6-pteridine Carboxamides.*—Substitution in the 7 position resulted in an increase in the electronegativity of the half-wave potential ($E^1_{1/2}$ from −0.470 to −0.565, Table II).

Included in class II pteridines was a polarographic study investigating the influence of the N—N intra-

TABLE III.—HALF-WAVE REDUCTION POTENTIALS OF 2,4,7-TRIAMINO-6-ARYLPTERIDINES AND 2,4,7-TRIAMINO-6-PTERIDINECARBOXAMIDES (CLASS III)



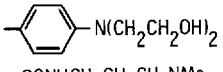
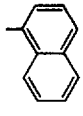
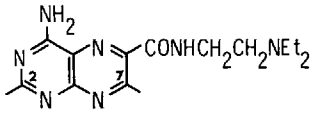
Wy-No.	6	$E^1_{1/2}$	$E^2_{1/2}$
1643		0.690	
1839	-CONHCH$_2$CH$_2$CH$_2$NMe$_2$	0.600	1.060
1840	-CONHCH$_2$CH$_2$NEt$_2$	0.570	1.020
3519		0.625	1.075
4151	-CONHCH$_2$CH$_2$CH$_2$OCHMe$_2$	0.595	1.105
4927		0.655	1.110

TABLE IV.—REDUCTION POTENTIAL OF REPRESENTATIVE COMPOUNDS OF THREE GENERAL CLASSES OF SUBSTITUTED PTERIDINES



Wy-No.	2	7	$E^{1}_{1/2}$
1843 (Class I)		$-NH_2$	0.420
5120 (Class II)		$-NHCH_2CH_2NEt_2$	0.490
1840 (Class III)	$-NH_2$	$-NH_2$	0.570

nuclear distance in the carboxamide structures in the 6 position of a homologous series of pteridines (Fig. 2). The half-wave reduction potential of this series was found to be a function of the N—N intranuclear distance as well as the pH. A methylene bridge of 5 carbons gave the maximal electronegative half-wave potential at pH 7.0 and 7.5.

Class III.—*2,4,7-Triamino-6-arylpteridines and 2,4,7-Triamino-6-pteridinecarboxamides.*—The electronegative potentials found in this general group were from −0.570 to −0.690 (Table III). The reduction potential of the 6-arylpteridines was more electronegative than the 6-pteridinecarboxamides.

Within each of the three general classes, the reduction potential of the pteridines increased in electronegativity with the increase in pH from 2.0 to 7.4. A typical half-wave potential *versus* pH curve is shown in Fig. 3.

DISCUSSION

Polarographic and spectrographic evidence has established that reduction of the pyrazine ring in folic acid and related pteridine structures occurs at the 5–6 and 7–8 double bonds. The reduction occurs in two steps and has been shown to be pH-dependent and to involve 2 electrons per step (12, 13). The authors compared the reduction record of 4,7-diamino-*N*-(4-dimethylaminobutyl)-2-phenyl-6-pteridinecarboxamide (class I, Wy-4437) with that of folic acid at the same molar concentration and pH and found the same 2-step reduction and the same limiting current. The authors consider this to be evidence for a 2-electron-per-step reduction mechanism for both structures (14).

Substitution on the pteridine nucleus had an over-all effect on the reductive process occurring at the 5–6 and 7–8 double bonds. The gain of electrons by the pteridine ring at the dropping mercury electrode and the simultaneous introduction of H+ occurred at voltages which were characteristic for each of the substituted pteridines. One can assume that the more difficult it is for a pteridine to gain electrons and be reduced, the more electronegative the applied voltage has to be. This effect, the ease or difficulty of reduction, involves not only the pteridine nucleus but the substituents as well.

The comparative electronegativity of the half-wave potential of the 3 general classes of pteridines studied was: class III > class II > class I. The substitution of a 2-phenyl group (Wy-1843) for the 2-amino group (Wy-1840) resulted in a decrease in electronegativity of the half-wave potential (Table IV). This decrease may be due to a withdrawal of electrons from the site of reduction by the phenyl group. The result of this resonance effect is to permit reduction to occur at a lower potential. The replacement of a primary amino group (Wy-1843) by a secondary amino group (Wy-5120) at position 7 resulted in an increase in electronegativity of the half-wave potential, indicating that the 2-diethylaminoethyl structure on the 7-amino group must act as an electron donor.

A polarographic study of the effect of pH on a homologous series of 4,7-diamino-2-*m*-chlorophenyl-6-pteridinecarboxamides (Fig. 2) revealed that an increase in the N—N intranuclear distance within the carboxamide tended to raise the electronegative half-wave potential. At acid pH's the protonation of amino and heterocycle nitrogen limited the resonance and inductive effects, but this leveling effect was minimized as the pH was increased. At neutrality the maximal reduction potential was recorded for a methylene chain length of 5 carbons.

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Moisture Stress Tests in Stability Programs

By J. THURØ CARSTENSEN, E. SERENSON ARON, D. C. SPERA, and J. J. VANCE

Although the effect of moisture in solid dosage forms is qualitatively recognized, the severity of this parameter is often much greater than anticipated. Quantization of interaction of moisture with active components is often possible and an apparent order of reaction can be established rather easily. The higher the order the more severe the effect of moisture content and the closer this variable must be controlled in stability programs. Vitamin A is used to exemplify these points.

TARDIF in a recent publication (1) touched on the effect of moisture on vitamin A stability in solid dosage forms. The moisture sensitivity of vitamin A is greatly affected by the presence of antioxidants, and as will be demonstrated below, can be exaggerated considerably depending on (*a*) the presence of antioxidants in the vitamin A beadlet and (*b*) the tablet base used. The sucrose base employed by Tardif is moderately severe. In our investigations we have found lactose to be a severe interacting substance that lends itself well to artificial testing of (*a*) the quantitative nature of the interaction of vitamin A with moisture and (*b*) as a screening agent for vitamin A beadlets of different matrix composition.

Higuchi and Reinstein (2) at an early date studied the order of reaction of vitamin A with water in hydroalcoholic systems and found the reaction order to be second order with respect to ethanol at high ethanol concentration, slowly approaching first order as the ethanol concentration was decreased. The order with respect to water was found to be complex, changing from much less than 1 in solvent containing 2% water, increasing to 1 at 5% water content, and then to 2 at 20% water content.

The authors have reported their findings in the lactose solid state system, which is a much cruder system than the systems employed by Higuchi and Reinstein.

Theory.—If vitamin A degrades by functional dependence of water content, then a $(1 + a)$ order reaction rate may be expressed as

$$dC_A/dt = -K \cdot C_A \cdot C^a_{H_2O} \qquad \text{(Eq. 1)}$$

where C denotes concentration and the subscripts denote vitamin A acetate and water. If the water is in sufficient excess, Eq. 1 will degenerate to the pseudo first order:

$$dC_A/dt = -K' \cdot C_A \qquad \text{(Eq. 2)}$$

or log (% retained) $= -K' \cdot$ (time) $+ 2$, where

$$K' = K \cdot C^a_{H_2O} \text{ or}$$
$$\log K' = \log K + a \cdot \log C_{H_2O} \qquad \text{(Eq. 3)}$$

"a" will appear as the slope of K' *versus* log C_{H_2O} plots regardless of whether Naperian or non-Naperian rate constants and logarithms are used. Non-Naperian data will be employed in the following.

EXPERIMENTAL

Samples of tablets were made of the following composition: 427.5 mg. of lactose, 5.0 mg. of calcium stearate, and 17.5 mg. of vitamin A acetate beadlets (containing no antioxidant).

The materials were predried at 37° for 24 hr. and various amounts of moisture were added to subparts of the granulation which was then compressed on a Stokes E machine using $^{13}/_{32}$ in. standard concave punches, the thickness being about 10.3 mm. The tableting of the wetter samples was not particularly satisfactory from a mechanical point of view.

The samples were stored in well-sealed bottles at 5, 25, 55, 70, and 85° and assayed at the periods shown in Table I.

Samples of vitamin A palmitate beadlets were incorporated in the same base. The vitamin A palmitate beadlets all contained antioxidants as opposed to the experimental lots of vitamin A acetate beadlets used. The results from storage testing of these are shown in Table III.

RESULTS

Table I yields storage results in terms of units of vitamin A/Gm. of tablet weight.

The table purposely omits longer term data to illustrate the rapidity and utility of the procedure. The use of longer periods is also questionable in view of recent findings (5) regarding equilibrium aspects of vitamin A beadlets, in particular in dryer formulations.

Extracting the K' values (in units of days^{-1}) from Table I yields the data in Table II. The $\log_{10}$ of K' is shown rather than K' to allow treatment by Eq. 3.

The data in Table II are shown graphically in Fig. 1, and analysis by the method of least squares show the lines (*a*) not to differ significantly with respect to slope and (*b*) to yield a best estimate of $a = 2.0 - 3.0$.

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The authors are indebted to Mr. E. DeRitter and Mr. L. Drekter for valuable assistance and suggestions.

TABLE I.—VITAMIN A ACETATE CONTENTS OF LACTOSE BASE TABLETS AT VARIOUS TEMPERATURES

Journal No. % Moisture	55-12A 0	55-12B 0.5	55-12C 1	55-12D 1.5	55-12E 2
Init.-Ref.	17,550	17,470	17,380	17,300	17,200
85°, 2 days	10,600	9,800	6,300	2,430	1,130
85°, 4 days	6,100	8,900	2,750	980	0
70°, 4 days	12,800	14,950	9,700	5,450	1,550
70°, 8 days	9,400	9,650	6,800	2,250	600
55°, 15 days	15,300	15,250	8,800	2,700	0
25°, 15 days	17,550	16,950	13,950	8,050	5,800
5°, 30 days			14,850	9,080	3,070

TABLE II.—LOG_{10} K'-VALUES AT VARIOUS TEMPERATURES

Journal No.	% H_2O Added	85° Log K'	70° Log K'	55° Log K'	25° Log K'	5° Log K'	Log (H_2O)[a]
55-12A	0	...	...	...	...	...	...
B	0.5	2.982–4	2.362–4	1.602–4	0.938–4		1.7–1
C	1	3.323–4	2.754–4	2.290–4	1.811–4	1.375–4	0.0–1
D	1.5	3.558–4	3.069–4	2.725–4	2.340–4	1.976–4	1.175–1
E	2	3.762–4	3.348–4		2.497–4	2.394–4	1.3–1

[a] It is assumed that the moisture present prior to water addition is bound; the water "concentration" is taken as moisture added.

The assays from 3 different lots of vitamin A palmitate with different beadlet composition are shown in Table III at the 2% moisture level, as an example of the screening aspects of the procedure. It should be noted that the beadlets referred to in Table III are all vitamin A *palmitate* beadlets containing antioxidants, whereas the beadlets referred to in Table I are vitamin A *acetate* beadlets containing no antioxidants.

DISCUSSION

It is apparent from the data that lactose plus moisture is a severe test for vitamin A. It hence may serve as a good agent for evaluating vitamin A beadlets made by various processes and compositions.

The data of Table II have been plotted graphically in Fig. 1 and yield the information that the over-all apparent interaction order is 4 ($a = 3$).

This is a relatively high order. For stability programs, therefore, there appears to be a need for preliminary determination of interaction order since the stability must be viewed in light of the moisture content. Such information should be secured early in the program.

Determination of the interaction order is rapidly obtained. Examplewise, data in Table I (which suffice for this purpose) were secured in about 1 month. The actual calculation of the a-value can be done graphically simply by determining the slope of the plot of log K' *versus* log (H_2O). For instance, the 25° data of Table II, taking points B and D would yield a slope (a-value) of $(2.340 - 0.938)/(1.175 - 0.700) = 2.96$.

Most of the data may be treated by Arrhenius plotting although in the case of the samples in Table I with high moisture contents, elevated temperatures give unreasonably high degradation rates and would have to be extrapolated differently (3) if such extrapolating were needed. The utility of the procedure outlined in stability programs and evaluations is quite apparent.

If the apparent order of the reaction is known it can be established whether losses are solely due to moisture or whether other factors are involved. If, for instance, one tablet formulation contained 2% moisture, another 1%, and the K-values depended on, *e.g.*, $K' = K \cdot C_{H_2O}^2$ then it may be con-

TABLE III.—VITAMIN A PALMITATE IN LACTOSE BASE TABLETS CONTAINING 2% MOISTURE

Tablet	A	B	C
Storage			
Initial	9890[a]	9300	7960
2 days at 85°	2480	2800	1420
k (days^{-1})	0.300	0.261	0.372
4 days at 70°	8300	7230	5840
k (days^{-1})	0.0190	0.0270	0.0342
15 days at 55°	7570	6420	3200
k (days^{-1})	0.00757	0.0107	0.0264
45 days at 45°	5660	6780	5000
k (days^{-1})	0.00543	0.00305	0.00446
180 days at 37°	7750	5560	1850
k (days^{-1})	0.000600	0.00124	0.00355
360 days at 25°	7755	7240	5715
k (days^{-1})	0.000300	0.000300	0.000396

[a] Assays are listed in units per tablet.

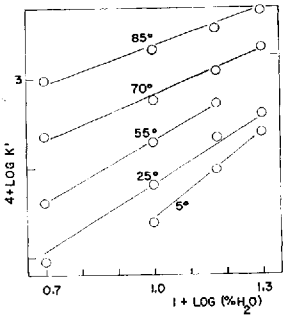

Fig. 1.—Vitamin A acetate-lactose tablets. Plot of the logarithm of the apparent first-order rate constant, K', as a function of amount of water added.

cluded that if K' (2%) = 4 K' (1%) the difference in K' values is solely due to moisture. Such a conclusion could not have been reached without knowledge of the apparent (third) order of reaction.

SUMMARY

1. Vitamin A acetate has been used as an example to demonstrate "order of interaction" in solid dosage forms.

2. Since moisture is a variable seldomly controlled within extremely narrow limits in stability programs, it may prove useful, in many cases, to establish the "order of interaction" between moisture and active component(s).

3. Screening bases for such studies can be selected by choosing the worst offenders in preliminary compatibility programs (4).

4. If a high-order interaction is established, stringent moisture control data should be obtained in the stability program.

5. The study of "order of interaction" may be carried out in a relatively short span of time (30 days).

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New Pharmacologic Aspects of β-Diethyl-aminoethyl 2,2-Diphenylpentanoate

By RICHARD A. CARRANO* and MARVIN H. MALONE

In a comparison with atropine and structurally related adiphenine using a gross *in vivo* screen in rats, β-diethyl-aminoethyl 2,2-diphenylpentanoate (SKF 525-A) appeared to act peripherally as a parasympatholytic and/or sympathomimetic. SKF 525-A apparently has some selective activity on the central nervous system (blepharoptosis, hypothermia) indicating a capacity to cross the blood-brain barrier. Drug–receptor interactions were studied on the isolated rat jejunum using furtrethonium as the agonist. SKF 525-A was primarily a noncompetitive antagonist with a competitive component and qualitatively different from the activities of atropine, adiphenine, and papaverine. The respective pA_2 and pD'_2 values are reported. The SKF 525-A receptor appears composed of the cholinergic receptor plus another spasmogen receptor. SKF 525-A did not inhibit the action of acetylcholinesterase, but was a potent inhibitor of monoamine oxidase at physiological concentrations.

MUCH OF the work previously reported on β - diethyl - aminoethyl 2,2 - diphenyl pentanoate (SKF 525-A) has been concerned with its ability to act as a multipotent inhibitor of various liver microsomal degradation reactions (1). The original observations made for SKF 525-A concerning potentiation of barbiturates and other central nervous system depressants (2–5) suggested possible potentiation by CNS mediation; however, Brodie (1) demonstrated that this agent was able also to prolong the activity of the central stimulant, amphetamine. This study was prompted by the chemical similarity between SKF 525-A and adiphenine, and also by the lack of comprehensive screening in the literature.

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The SKF 525-A and furtrethonium were provided courtesy of the Smith Kline & French Laboratories, Philadelphia, Pa., while adiphenine was generously supplied by the Ciba Pharmaceutical Co., Summit, N. J.
* Fellow of the American Foundation for Pharmaceutical Education.

EXPERIMENTAL

In Vivo Hippocratic Screening.—In accordance with the method of Malone and Robichaud (6), nonfasted albino rats (Wistar strain) in the weight range of 150–250 Gm. were injected intraperitoneally with 5 logarithmetically spaced doses of each drug tested (1 lethal, 1 essentially ineffective dose, and 3 effective log-dosages between those two). Observations were made using the standard worksheet (6) at 5, 10, 15, 30, and 60 min. postinjection, 2, 4, and 24 hr. postinjection, and 2, 4, and 7 days postinjection.

Mechanism of Drug-Receptor Interaction.—Using the methods of Ariëns (7), van Rossum (8), and van Rossum and van den Brink (9), cumulative dose-response curves were made utilizing rat jejunum and furtrethonium iodide as the reference agonist. The bath solution was standard Tyrode's oxygenated with 95% oxygen and 5% CO_2 and containing the calcium disodium salt of ethylenediaminetetraacetic acid in a concentration of 1×10^{-5} Gm./L. The jejunum was mounted in the bath (37.5°) using a modified Magnus technique. All drug concentrations were calculated in terms of drug base. When an antagonist was tested it was

cluded that if K' (2%) = 4 K' (1%) the difference in K' values is solely due to moisture. Such a conclusion could not have been reached without knowledge of the apparent (third) order of reaction.

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1. Vitamin A acetate has been used as an example to demonstrate "order of interaction" in solid dosage forms.

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New Pharmacologic Aspects of β-Diethyl-aminoethyl 2,2-Diphenylpentanoate

By RICHARD A. CARRANO* and MARVIN H. MALONE

In a comparison with atropine and structurally related adiphenine using a gross *in vivo* screen in rats, β-diethyl-aminoethyl 2,2-diphenylpentanoate (SKF 525-A) appeared to act peripherally as a parasympatholytic and/or sympathomimetic. SKF 525-A apparently has some selective activity on the central nervous system (blepharoptosis, hypothermia) indicating a capacity to cross the blood-brain barrier. Drug–receptor interactions were studied on the isolated rat jejunum using furtrethonium as the agonist. SKF 525-A was primarily a noncompetitive antagonist with a competitive component and qualitatively different from the activities of atropine, adiphenine, and papaverine. The respective pA_2 and pD'_2 values are reported. The SKF 525-A receptor appears composed of the cholinergic receptor plus another spasmogen receptor. SKF 525-A did not inhibit the action of acetylcholinesterase, but was a potent inhibitor of monoamine oxidase at physiological concentrations.

MUCH OF the work previously reported on β - diethyl - aminoethyl 2,2 - diphenyl pentanoate (SKF 525-A) has been concerned with its ability to act as a multipotent inhibitor of various liver microsomal degradation reactions (1). The original observations made for SKF 525-A concerning potentiation of barbiturates and other central nervous system depressants (2–5) suggested possible potentiation by CNS mediation; however, Brodie (1) demonstrated that this agent was able also to prolong the activity of the central stimulant, amphetamine. This study was prompted by the chemical similarity between SKF 525-A and adiphenine, and also by the lack of comprehensive screening in the literature.

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EXPERIMENTAL

In Vivo Hippocratic Screening.—In accordance with the method of Malone and Robichaud (6), nonfasted albino rats (Wistar strain) in the weight range of 150–250 Gm. were injected intraperitoneally with 5 logarithmetically spaced doses of each drug tested (1 lethal, 1 essentially ineffective dose, and 3 effective log-dosages between those two). Observations were made using the standard worksheet (6) at 5, 10, 15, 30, and 60 min. postinjection, 2, 4, and 24 hr. postinjection, and 2, 4, and 7 days postinjection.

Mechanism of Drug-Receptor Interaction.—Using the methods of Ariëns (7), van Rossum (8), and van Rossum and van den Brink (9), cumulative dose-response curves were made utilizing rat jejunum and furtrethonium iodide as the reference agonist. The bath solution was standard Tyrode's oxygenated with 95% oxygen and 5% CO_2 and containing the calcium disodium salt of ethylenediaminetetraacetic acid in a concentration of 1×10^{-5} Gm./L. The jejunum was mounted in the bath (37.5°) using a modified Magnus technique. All drug concentrations were calculated in terms of drug base. When an antagonist was tested it was

allowed to incubate with the tissue for 10 min. prior to the production of the cumulative dose-response curve.

In Vitro Acetylcholinesterase Inhibition Studies.—The manometric techniques of Umbreit *et al.* (10) were used for this study with acetyl-β-methylcholine (0.03 *M*) as the substrate for acetylcholinesterase. Physostigmine was used in concentrations ranging from 10^{-6} to 10^{-9} *M* as a standard acetylcholinesterase inhibitor. SKF 525-A was used in concentrations ranging from 10^{-3} to 10^{-6} *M*. The inhibitor and acetylcholinesterase were incubated (37°) together for 20 min. prior to mixing with the substrate. The total flask volume was 3 ml. and pH was kept at 7.4 with Krebs-Ringer bicarbonate buffer. Molar I_{50} and I_{80} values were calculated. All drug concentrations were expressed in terms of drug base.

In Situ Monoamine Oxidase Inhibition Studies.—Rat liver MAO activity was determined by the spectrophotometric disappearance of the substrate kynuramine (11). Iproniazid was used as the reference MAO inhibitor. All concentrations were calculated in terms of the drug base.

Radiometric determinations were carried out using the method of Wurtman and Axelrod (12) with incubations at 37° in 15-ml. glass-stoppered centrifuge tubes for 20 min. A young male rat was sacrificed by decapitation, and 200 mg. of liver rapidly excised and homogenized thoroughly with 10 ml. of cold isotonic KCl. One milliliter of this homogenate was diluted to 10 ml. with isotonic KCl and rehomogenized so that a final concentration of 2 mg./ml. was obtained. The total volume of each incubation tube was 3 ml. made up of the appropriate ingredients. At the termination of the incubation, 0.2 ml. of 2 *N* HCl was added to each tube followed by 6 ml. of toluene. The ^{14}C derivative was extracted into the toluene layer by shaking and the two layers separated by centrifugation. Four milliliters of the toluene layer was taken from each tube and placed into a liquid scintillator vial containing 10 ml. of liquid scintillator solution (0.5 Gm. POPOP, 4 Gm. PPO, add 1 L. of toluene), and counted for 10 min. in a Packard tricarb counter. Counting efficiency of each vial was determined by the internal standard method. A wide range of log-concentrations was used for each agent with concentrations expressed in terms of drug base.

RESULTS AND DISCUSSION

In Vivo Hippocratic Screening.—Dosages of 32, 56, 100, 178, 316 mg./Kg. were injected for each of the following: SKF 525-A, structurally related adiphenine, and atropine. The qualitative nature of the observable symptoms which exhibit dose-response relationships permit an estimate of possible mechanism of action (6).

Atropine produced excellent dose-response activity for the following acute effects: mydriasis, decreased skeletal muscle coordination and tone, lowering of rectal temperature, tremors and convulsions coupled with xerostomia, and decreased lacrimation. An increase in blood pressure was suggested by the presence of exophthalmos. The well-known parasympatholytic activity appeared to be detected by the screening technique and all activities were in agreement with reports in the literature. At 100–316 mg./Kg. dosages, there was an apparent blockade of transmission at the level of the motor end plate of skeletal muscle. The dosage of 316 mg./Kg. was lethal within 30 min. Recovery from 178 mg./Kg. of atropine was essentially complete by +24 hr.

Adiphenine produced effects very similar to those observed with atropine. However, mydriasis was not as pronounced and stimulation of both salivation and lacrimation was noted at doses of 100 and 178 mg./Kg. Lack of xerostomia suggests a different mechanism of action at these higher dosage levels, possibly indicating a decreased affinity of the adiphenine molecule to the parasympathetic receptor, and possibly the extension of its action to another closely related receptor or receptor system.

SKF 525-A was found to be a strong mydriatic but not as powerful as atropine. It had the ability to decrease motor activity, although paralysis of skeletal muscle and loss of screen grip (6) were not seen. Fine tremors of skeletal muscle were pronounced at the two lower dosages. Lacrimation was greatly increased. The most striking effect of SKF 525-A and with an excellent dose-response relationship was hypothermia (−6.9° at +1 hr. with a dosage of 178 mg./Kg.). At the lowest dosage level of 32 mg./Kg., SKF 525-A produced a hypothermia (−3.3° at +1 hr.) equivalent to that noted with 178 mg./Kg. of atropine and adiphenine. Associated with hypothermia was an effect of palpebral ptosis and enophthalmos. The dosage of 316 mg./Kg. was lethal within 30 min. and was accompanied by convulsions. The animals receiving 178 and 100 mg./Kg. died within 60 and 95 hr. of dosing and death was associated with increased skin plasticity, pilomotor erection, and profound loss of body weight. Since only 1–2 rats per dosage are required for the Hippocratic screening (6), it is a qualitative technique whereby the evaluation of the activity of the test material is based on the complete log-response profile of the entire animal population receiving the 5 log-dosages rather than on one animal or on one selected log-dosage. The Hippocratic technique is used as an initial directive screen to suggest avenues for further more specific pharmacologic evaluation. In considering the total profile of acute symptoms produced by SKF 525-A, one could postulate either parasympatholytic or sympathomimetic activity. Increased lacrimation and mydriasis accompanied by skeletal muscle tremors suggest sympathomimetic activity. Salivation was not stimulated concurrent with lacrimation. The hypothermia appeared due to a selective CNS activity—this was suggested considering two factors. First, the "true" palpebral ptosis indicative of possible hypothalamic depression was correlated directly with the period of strong hypothermic activity; and second during the ptotic effects the animals were obviously conscious. Since death with SKF 525-A came on slowly accompanied by weight loss, this agent could produce general metabolic inhibition.

It was evident from this gross *in vivo* screen that SKF 525-A may have important pharmacologic actions independent of metabolic inhibition and actions quite different from those seen with atropine and adiphenine. In general, the data indicated a decrease in parasympatholytic activity stepwise

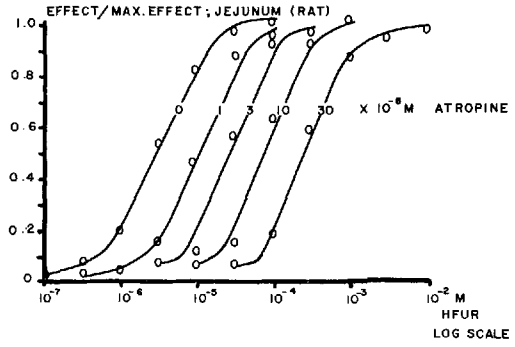

Fig. 1.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of atropine.

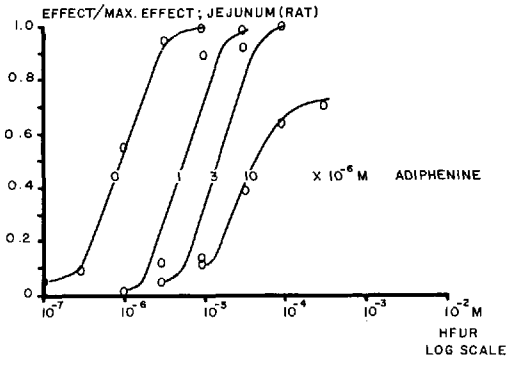

Fig. 2.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of adiphenine.

from atropine to adiphenine to SKF 525-A. A shifting of mechanism of activity or site of action may be pertinent to this structural progression.

Drug Receptor Interactions.—The results for SKF 525-A were compared with those for atropine, adiphenine, and papaverine. Figure 1 illustrates the cumulative dose-response curves for furtrethonium in the presence of increasing log-increments of atropine. A constant level of atropine was maintained for each dose-response curve of furtrethonium. The conclusion that must be drawn is that atropine is a pure competitive antagonist of furtrethonium for the cholinergic receptor of rat jejunum. There is a concentration-related decrease in the affinity of the furtrethonium molecule for the cholinergic receptor. The influence of atropine can be overcome by an increase in the concentration of furtrethonium and eventually furtrethonium occupies all receptors, thus producing a maximal effect. The complementarity between the cholinergic receptor site and the atropine molecule is similar to that between furtrethonium and the cholinergic receptor site, but not equivalent since atropine has no intrinsic activity.

Figure 2 illustrates similar cumulative dose-response curves with adiphenine as the antagonist. Here the competitive nature of the drug–receptor interactions can also be seen. However, with a concentration of 10×10^{-6} *M* for adiphenine, the maximum response obtainable for the furtrethonium cumulative dose-response curve is reduced. This implies not only a decrease in the affinity, but also a decrease in the intrinsic activity of the furtrethonium molecule for the cholinergic receptor. A noncompetitive component has been introduced into adiphenine's action. This noncompetitive component may be the determinate factor responsible for the decrease in gross parasympatholytic activity (as compared to atropine) noted in the *in vivo* screening data.

Figure 3 demonstrates the furtrethonium dose-response curves in the presence of increasing log-increments of the musculotropic antagonist papaverine. A "pure" noncompetitive antagonism is illustrated. The papaverine molecule and the cholinergic receptor do not have any degree of complimentarity; however, the interaction of papaverine with components (7) of the contractile mechanism of the muscle has a blocking influence on the action produced by furtrethonium on the cholinergic receptor of the jejunum.

Figure 4 illustrates that SKF 525-A, in part, appears to be a noncompetitive antagonist of furtrethonium on the cholinergic receptor. It is not correct to assume that SKF 525-A acts in the same way as papaverine on this biological preparation. Examination of the curves in Fig. 4 reveals that there is a shift in the dose-response curves for furtrethonium occurring along the log-dose axis

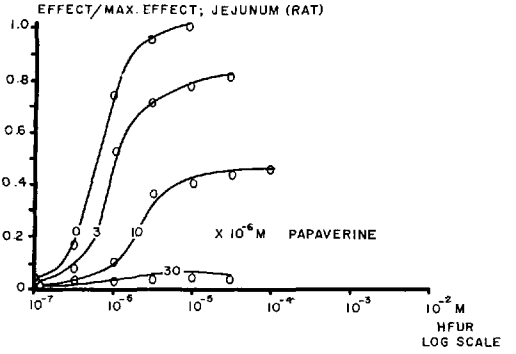

Fig. 3.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of papaverine.

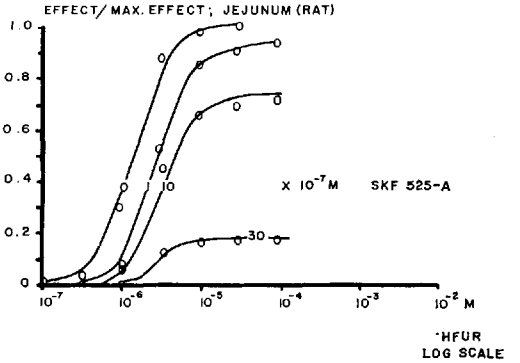

Fig. 4.—Cumulative log-concentration curves of furtrethonium (HFUR) in the presence of SKF 525-A.

TABLE I.—PARAMETERS OF PARASYMPATHETIC AGENTS TESTED ON ISOLATED RAT JEJUNUM (10 min. INCUBATION) WITH FURTRETHONIUM AS THE AGONIST

	Intrinsic Activity	Log Affinities		
		pD_2	pA_2	pD'_2
Furtrethonium[a]	1	5.90 (5.90)[b]	...	...
Atropine	0	...	8.42 (8.80)[b] (8.37–8.77)[c]	...
Adiphenine	0 (−1)	...	6.69 (6.80)[b]	4.58[d] (4.70)[e]
SKF 525-A	?[f]	...	...	6.06
Papaverine	−1	...	...	5.00 (4.80)[b]

[a] While all calculations are in terms of the base molecules, furtrethonium iodide and atropine sulfate were the respective salts used with all other agents as hydrochloride salts. [b] Parenthetical value was reported by van Rossum and van den Brink (9); the incubation time was not listed. [c] Parenthetical values were reported by Schild (17, 18) for a 2 and 14-min. incubation time, respectively. [d] Incubated at 1×10^{-5} *M* concentration. [e] Parenthetical value was reported by Ariëns and van Rossum (13); the incubation time was not listed. [f] See text for discussion.

prior to the depression of maximal effect. This is not demonstrated in Fig. 3 with papaverine. According to Ariëns and van Rossum (13), van Rossum (8), and Ariëns (7), when a noncompetitive antagonist interferes with the induction of the stimulus its presence may result in a shift of the dose-response curves of the agonist followed by a decline of the maximum attainable effect. In such a case the antagonist may be expected to exhibit a certain specificity with respect to the agonist. A reserve in receptors for the agonist may be the cause of this shift. Since SKF 525-A is a reversible antagonist, the small shift noted is significant. Therefore, SKF 525-A although noncompetitive in action like papaverine acts by way of a different mechanism. Papaverine's action is nonspecific whereas SKF 525-A has a certain specificity with respect to the furtrethonium molecule and the cholinergic receptor and, therefore, may be considered to interfere with the actual induction of the stimulus itself. Table I gives the values for the log-affinities calculated according to the method of van Rossum and van den Brink (9). An examination of Table I shows that the values obtained in this laboratory are in agreement with those reported by others. Atropine has the greatest affinity (pA_2) for the cholinergic receptor, even greater than furtrethonium. However, since its intrinsic activity is equal to zero it cannot cause an effect. The pronounced affinity of atropine to the cholinergic receptor is understood in light of the fact that it is effective in concentrations around 1×10^{-8} *M*. Furtrethonium produces its effects at about 3×10^{-6} *M*; but since it has an intrinsic activity, it is capable of eliciting a stimulus and a subsequent response of the biologic object. Adiphenine has an affinity (pA_2) to the cholinergic receptor less than atropine but greater than furtrethonium. The inability of adiphenine to produce a stimulus is due to a molecular configuration that destroys its intrinsic activity. The affinity of SKF 525-A to the cholinergic "neighboring" receptor site (pD'_2) is higher than both papaverine and adiphenine. The value for the affinity is indirectly related to the effective concentrations. Table I lists a question mark for the intrinsic activity of SKF 525-A. As a strong noncompetitive antagonist with some competitive characteristics, it may have two intrinsic activities of 0 and −1, as does adiphenine. On several occasions when SKF 525-A was introduced into the bath, it initiated a stimulus and produced an effect which rapidly subsided. Whether this stimulatory effect was induced by interaction with receptors is questionable. However, such a response was never noted for any of

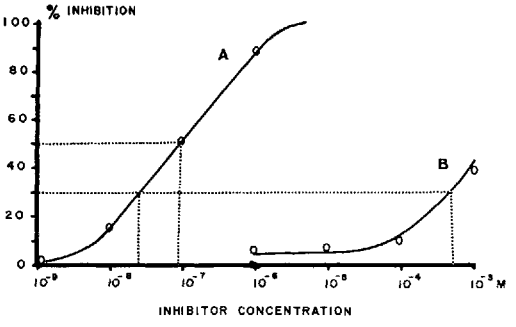

Fig. 5.—Acetylcholinesterase inhibition by physostigmine and SKF 525-A. Key: A, physostigmine ($M\ I_{50} = 9.50 \times 10^{-8}\ M$; $M\ I_{30} = 2.65 \times 10^{-8}\ M$); B, SKF 525-A ($M\ I_{30} = 8.25 \times 10^{-4}\ M$).

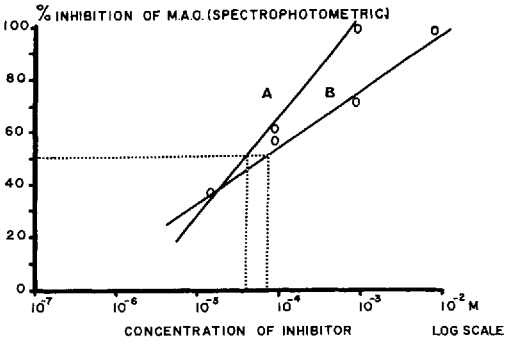

Fig. 6.—Spectrophotometric determination of $M\ I_{50}$ for iproniazid ($8.5 \times 10^{-5}\ M$) and SKF 525-A ($5.3 \times 10^{-5}\ M$) on monoamine oxidase. Key: A, SKF 525-A; B, iproniazid.

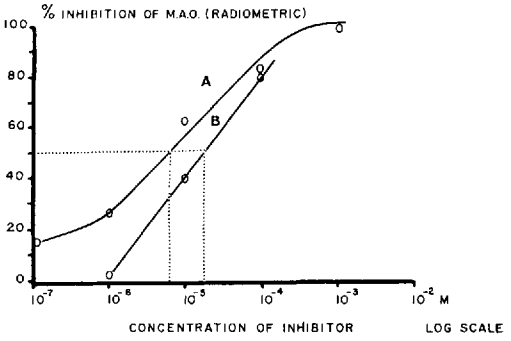

Fig. 7.—Radiometric determination of M I_{50} for iproniazid (7.5×10^{-6} M) and SKF 525-A (1.9×10^{15} M) on monoamine oxidase. Key: A, iproniazid; B, SKF 525-A.

the other antagonists utilized. Important implications may be derived from these observations in connection with the rate theory proposed by Paton (14). In reference to this theory, Paton has suggested that if the rate of occupation of the receptor is an important factor for the intrinsic activity, it may be expected that not only the mimetics but also the lytics will induce an initial response (contraction) before acting as a blocking agent. The unique action of SKF 525-A in blocking the interactions of furtrethonium with the cholinergic receptor is strong indication that the receptor site for SKF 525-A is composed of certain components of the cholinergic receptor and another spasmogen receptor.

In Vitro Acetylcholinesterase Inhibition.—Since SKF 525-A is considered to be a multipotent enzyme inhibitor and since most of the antiacetylcholinesterases, especially the reversible ones, are structurally related to acetylcholine, the action of SKF 525-A on acetylcholinesterase was compared with physostigmine. Figure 5 shows that SKF 525-A was not an effective acetylcholinesterase inhibitor at physiological concentrations. The data for the standard inhibitor, physostigmine, were in excellent agreement with that reported in the literature (15).

In Situ Monoamine Oxidase Inhibition.—The possibility of sympathomimetic effects due to MAO inhibition was considered to be possible in light of the Hippocratic screening. Figure 6 illustrates the data obtained from the spectrophotometric method and shows that SKF 525-A has a lower molar I_{50} than the standard, iproniazid. Figure 7 illustrates the results from the radiometric technique for SKF 525-A and iproniazid. The molar I_{50} values for both procedures are in good general agreement. The concentrations used are in the physiological range. The radiometric method is regarded generally to be the more sensitive and more reliable of the two assays. Allmark (16) demonstrated that large doses of isoniazid and related compounds (iproniazid) prolonged the sleeping time of barbiturates. This provides a correlation between SKF 525-A activity and MAO inhibition since the first major action of SKF 525-A that was studied was its capacity for barbiturate potentiation. In any case it is possible that inhibition of MAO may account for the sympathomimetic effects seen when SKF 525-A alone is administered to intact unanesthetized rats.

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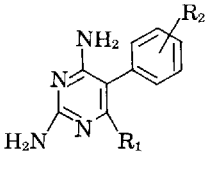
Pyrimidine Derivatives VIII

5-Arylazopyrimidines and Their Inhibitory Effects Against Transplantable Mouse Tumors

By MERVYN ISRAEL, HERBERT N. SCHLEIN*, CHARLOTTE L. MADDOCK, SIDNEY FARBER, and EDWARD J. MODEST

Twenty-five substituted 5-arylazopyrimidine derivatives have been prepared as potential "small molecule" antagonists of folic acid *via* reaction of 5-unsubstituted pyrimidines with appropriate diazotized aniline derivatives. The antineoplastic activity of 19 of these compounds has been examined *in vivo* by means of a primary screening program utilizing five transplantable mouse tumors. At nontoxic dosages, several of these agents showed effective tumor growth inhibition, as evidenced by reduction in mean tumor size. Histopathological examination of mice treated with 2,4-diamino-6-hydroxy-5-phenylazopyrimidine revealed intestinal irritation and evidence of nephrotoxicity, as well as incomplete absorption of the drug.

THE EFFECTIVENESS of aminopterin (4-aminopteroylglutamic acid) against acute leukemia in children (1) has led to the synthesis of a variety of folic acid antagonists as potential tumor inhibitory substances. Included among these are the so-called "small molecule" antifolics (*i.e.*, compounds less closely related to the structure of folic acid but retaining antifolic activity),[1] which are represented by the following examples: the 2,4-diamino-5-arylpyrimidines (I) (2), the 2,4-diamino-5-aryloxypyrimidines (II) (3), the 2,4-diamino-5-benzylpyrimidines (III) (4), and the 2,4-diamino-5-aryl-*as*-triazines (IV) (5), all synthesized by Hitchings and his collaborators, and the 4,6-diamino-1-aryl-1,2-dihydro-*s*-triazines (V) (6), prepared in these laboratories. These antimetabolites all approximate the 2,4-diamino-5-arylpyrimidine structure. A more complete comparison of these "small molecule" antifolic structures has already appeared elsewhere (7). Various substituted 5-arylazopyrimidines (VI), which structurally resemble the aforementioned compounds, have been prepared by us for evaluation as potential antitumor agents. Preliminary communications on the synthesis (8) and microbiological activity (9) of these compounds have appeared; the latter report was published simultaneously, by mutual arrangement, with that of Timmis and co-workers (10), whose independent studies paralleled our own. At about the same time, Tanaka and his colleagues also described the biological activity of some 5-arylazopyrimidines (11), and various reports have since appeared concerning the antifolic and tumor inhibitory activity of this type of compound (12–15). The authors now describe the synthesis of some hitherto unreported derivatives of this series and summarize their experimental antitumor properties.



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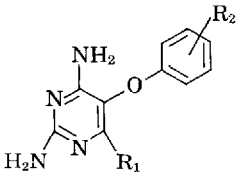

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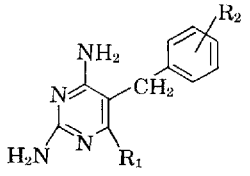

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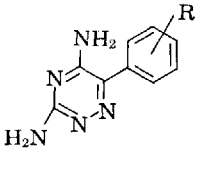

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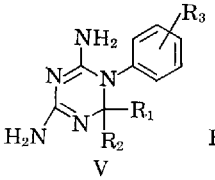

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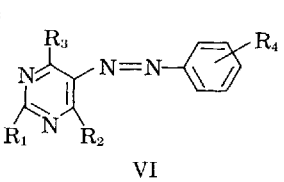

VI

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* Present address: New England Laboratories, Ipswich, Mass.

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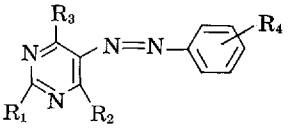
DISCUSSION

The 5-arylazopyrimidines prepared during this investigation (Tables I and II) were obtained by reaction of a 5-unsubstituted pyrimidine with an appropriate diazotized aniline derivative. Compounds *1–14* represent a series of derivatives of 2,4-diamino-6-hydroxypyrimidine with modification in the arylazo substituent (R_4). In this series, the coupling reaction was accomplished at pH 5.0–5.5 in 10% sodium acetate, following essentially the conditions of Benson, Hartzel, and Savell (16); the yields varied from 50–100%. With other pyrimidines, no general pH optimum was found for diazo coupling. Some pyrimidines (*e.g.*, 2,4-diamino-6-chloropyrimidine) coupled best at a slightly acid pH; others (*e.g.*, 2,4-diamino-6-mercaptopyrimidine) required an alkaline pH for coupling. In some instances (*e.g.*, 2,4-diaminopyrimidine), coupling did not occur at any pH tried. In two instances (compounds *19* and *21*), the coupling reaction was most efficient when the pH was kept constant during the entire course of the reaction by means of a buffered system. It appears, therefore, that optimal conditions for diazo coupling represent a compromise between the solubility of the pyrimidine and its relative reactivity toward electrophilic substitution, on the one hand, and the concentration of the diazonium salt and its rate of decomposition on the other.

The crude products were purified initially by thorough washing with alcohol, water, and ether, and then, if they contained hydroxy or mercapto substituents, by precipitation from alkaline solution by the addition of dilute acid; material purified to this stage was sufficiently pure for biological evaluation. Analytically pure samples were obtained by one of three general methods: sublimation, recrystallization, or reprecipitation. The method of choice, wherever applicable, was high vacuum sublimation; this technique afforded analytically pure, anhydrous material directly. Second choice was recrystallization. Arylazopyrimidines are, in general, insoluble in most common organic solvents. However, a limited few were crystallizable from hydrophilic solvents such as ethanol or 2-ethoxyethanol. The third choice was repeated precipitation either from basic solution by the addition of acid or from acetone solution by the addition of water. This procedure usually returned hydrates of variable composition which retained

TABLE I.—5-ARYLAZOPYRIMIDINES (VI)



Compd.	R_1	R_2	R_3	R_4	Method of Prepn.[a]	Yield, %[b]	Purification[c]	M.p., °C.	Color
1[d,e]	NH_2	OH	NH_2	...	*A*	90	S (250–275)	>300	Yellow
2	NH_2	OH	NH_2	3′—Cl	*A*	95	A	>300	Yellow-orange
3[e]	NH_2	OH	NH_2	4′—Cl	*A*	65	C	>300	Yellow
4	NH_2	OH	NH_2	3′,4′—Cl_2	*A*	80	S (280–300)	273–274	Yellow
5	NH_2	OH	NH_2	2′—Br	*A*	85	P	>300	Orange
6[e]	NH_2	OH	NH_2	4′—NO_2	*A*	88	P	>300	Red
7[f,g]	NH_2	OH	NH_2	4′—SO_3Na	*E*	95	W[h]	>300	Yellow
8	NH_2	OH	NH_2	2′—CH_3	*A*	60	S (285)	>300	Yellow
9	NH_2	OH	NH_2	4′—CH_3	*A*	80	S (300)	>300	Yellow
10	NH_2	OH	NH_2	2′,6′—$(CH_3)_2$	*A*	86	A	257–258	Yellow-orange
11	NH_2	OH	NH_2	2′—OCH_3	*A*, *C*	65	S (290)	>300	Yellow
12[e]	NH_2	OH	NH_2	4′—OCH_3	*A*, *C*	85	P	>300	Yellow
13	NH_2	OH	NH_2	2′,3′—Benzo	*C*	80	P	>300	Red-orange
14	NH_2	OH	NH_2	3′,4′—Benzo	*C*	70	P	>300	Red-orange
15[i]	NH_2	Cl	NH_2	...	*D*	72	A	242–243	Yellow
16[j]	NH_2	Cl	NH_2	4′—Cl	*D*	68	C-W	271–272	Yellow
17[j]	NH_2	NH_2	NH_2	...	*A*, *D*	90	C-W	262–263	Yellow
18[k]	NH_2	NH_2	NH_2	4′—Cl	*A*, *D*	87	C-W	253.5–255	Yellow
19[i]	NH_2	SH	NH_2	...	*F*	67	E	257–259 dec.	Pale orange
20	NH_2	SH	NH_2	4′—Cl	*B*	65	P	>300	Red
21[l]	NH_2	SCH_3	NH_2	...	*G*	39	A	180.5–182	Yellow
22[e]	OH	NH_2	NH_2	...	*B*	25	P	>300	Pale yellow
23	SCH_3	NH_2	NH_2	...	*A*	73	S (245)	235 dec.	Yellow
24	OH	NH_2	OH	...	*A*	52	P	>300	Yellow
25	OH	OH	OH	...	*A*	63	A	288–289	Yellow

[a] See under *Experimental*. [b] Percentage yield figures for compounds *1–14*, *19*, *20*, *22*, *24*, and *25* are based upon material which had been once purified by precipitation at pH 4–5 from an alkaline solution; yields for the remaining compounds are based on product purified according to the method indicated under *Purification*. [c] Purification procedure used to obtain the analytical sample. Crystallization solvents: A, 95% ethanol; C, cellosolve (2-ethoxyethanol); C-W, cellosolve–water; E, ether; W, water; S, sublimation in high vacuum (sublimation temperature in °C. in parentheses); P, precipitation at pH 4–5 from alkaline solution. [d] Previously reported as a hydrate (16). [e] Cited in Parker, R. P., and Webb, J. S., U. S. pat. 2,543,333 (Feb. 27, 1951); however, no analytical values are given. [f] The sesquihydrate is obtained on drying at 85° for 48 hr. *in vacuo*; drying at 140° for 72 hr. afforded the hemihydrate. [g] Free acid previously reported by Hartzel, L. W., and Benson, F. R., *J. Am. Chem. Soc.*, **76**, 2263(1954). [h] Containing 5% sodium chloride. [i] *Reference 19*. [j] Also prepared by Timmis *et al.* (10). [k] Reported (10) m.p. 262°. [l] *Reference 21*.

TABLE II.—ANALYTICAL DATA FOR NEW COMPOUNDS LISTED IN TABLE I

Compd.	Formula	Anal., % Calcd. C	Calcd. H	Calcd. N	Found C	Found H	Found N
1	$C_{10}H_{10}N_6O$	52.17	4.38	...	52.25	4.6	...
2	$C_{10}H_9ClN_6O$	45.38	3.43	31.76	45.70	3.38	31.56
3	$C_{10}H_9ClN_6O$	45.38	3.43	31.76	45.71	3.59	31.80
4	$C_{10}H_8Cl_2N_6O$	40.15	2.70	28.10	39.97	2.85	27.80
5	$C_{10}H_9BrN_6O$	38.85	2.93	...	39.38	2.62	...
6	$C_{10}H_9N_7O_3$	43.64	3.30	35.63	43.50	3.50	35.20
7[a]	$C_{10}H_9N_6NaO_4S.1.5\ H_2O$	33.43	3.37	9.12[b]	33.39	3.43	9.27[b]
8	$C_{11}H_{12}N_6O$	54.09	4.95	34.41	53.89	5.20	34.20
9	$C_{11}H_{12}N_6O$	54.09	4.95	...	53.86	5.10	...
10	$C_{12}H_{14}N_6O$	55.80	5.46	32.54	55.83	5.62	32.75
11	$C_{11}H_{12}N_6O_2$	50.76	4.65	32.30	50.31	4.78	32.40
12	$C_{11}H_{12}N_6O_2$	50.76	4.65	32.30	50.46	4.90	31.90
13	$C_{14}H_{12}N_6O$	59.99	4.32	...	59.73	4.20	...
14	$C_{14}H_{12}N_6O$	59.99	4.32	...	59.55	4.50	...
18	$C_{10}H_{10}ClN_7$	45.54	3.82	37.19	45.56	3.99	36.88
20	$C_{10}H_9ClN_6S$	42.78	3.23	29.94	42.62	3.17	29.80
22	$C_{10}H_{10}N_6O$	52.17	4.38	...	51.70	4.09	...
23	$C_{11}H_{12}N_6S$	50.75	4.65	32.29	50.86	4.82	31.90
24	$C_{10}H_9N_5O_2$	51.94	3.92	...	51.72	4.21	...
25	$C_{10}H_8N_4O_3$	51.73	3.47	24.13	51.88	3.62	24.42

[a] Analysis for hemihydrate.—Calcd. for C, 35.19; H, 2.95. Found: C, 35.12; H, 3.15. [b] Per cent sulfur.

TABLE III.—INHIBITORY ACTIVITY OF NONTOXIC DOSAGES OF 5-ARYLAZOPYRIMIDINES AGAINST TRANSPLANTABLE MOUSE TUMORS[a]

Compd.	L1210 Dose	L1210 S, %	P1534 Dose	P1534 S, %	P1534 I, %	C1498 Dose	C1498 S, %	C1498 I, %	DBRB Dose	DBRB S, %	DBRB I, %	S180 Dose	S180 S, %	S180 I, %
1	80	+5	80	−8	+60 (*11*)[c]	20	+33[b]	+72 (*12*)[c]	80	+5	+60 (*8*)[c]	80	+11	+21 (*10*)
2	500	+5	500[d]	−29	+61 (*11*)[c]	125	+4	+45 (*12*)	125	−4	+38 (*13*)	500	−15	+53 (*12*)[c]
3	500	0	...	...	...	500	−11	+38 (*12*)	...	...	...	...	...	...
4	125	+15	...	...	...	125[d]	−27	+61 (*12*)[c]	...	...	...	...	...	...
7	125	−5	6.25	0	+52 (*11*)[c]	100	+3	+100 (*11*)[c]	25	−7	+9 (*11*)	6.25	+4	+14 (*7*)
8	125	+3	5	−14	+26 (*11*)	20	−1	+4 (*14*)	5	−3	−9 (*11*)	20	−4	+12 (*13*)
9	125	−2	31.25	−19	+59 (*11*)[c]	320	−9	+21 (*14*)	1000	+13	+72 (*8*)[c]	500[d]	−26	+17 (*13*)
10	500	−3	...	...	...	...	...	...	...	...	...	...	...	...
11	125	−5	31.25	−12	+1 (*11*)	7.8	−8	−5 (*10*)	7.8	+22[b]	−5 (*11*)	7.8	+12	+12 (*10*)
12	500	−11	...	...	...	125	−16	+32 (*12*)	...	...	...	125	−24	+47 (*17*)
13	31.25	+6	...	...	...	...	...	...	...	...	...	...	...	...
14	500	+3	...	...	...	31.25	+3	+50 (*12*)[c]	...	...	...	...	...	...
15	80	0	...	...	...	100	−22	+91 (*12*)[c]	...	...	...	...	...	...
16	125	+29[b]	...	...	...	125	−16	+60 (*14*)[c]	...	...	...	125	−24	+27 (*13*)
17	6.25	−3	8	−8	+32 (*9*)	8	+15	+11 (*12*)	0.5	−22	+8 (*8*)	8	+34[b]	+30 (*10*)
18	31.25	−3	31.25	−2	+34 (*9*)	31.25	−10	+23 (*14*)	31.25	−17	+76 (*8*)[c]	31.25	−3	+8 (*13*)
20	31.25	+3	7.8	−4	+34 (*11*)	31.25	−7	+41 (*12*)	31.25	+6	+15 (*11*)	7.8	−2	−5 (*10*)
23	500	+14	31.25	−2	+26 (*11*)	500	−10	+68 (*12*)[c]	500	+23[b]	+25 (*11*)	125	+17	+33 (*13*)
24	31.25	−2	125	−24	+11 (*13*)	125	−8	+85 (*14*)[c]	31.25	+23[b]	+19 (*15*)	...	...	...

[a] Transplantable mouse tumors employed: L1210 ascitic lymphatic leukemia in the BDF/1 hybrid; P1534 lymphatic leukemia in the DBA/2 inbred strain; C1498 myelogenous leukemia in the C57BL/6 inbred strain; DBRB mammary adenocarcinoma in the DBA/1 inbred strain; S180 spindle cell sarcoma in the CAF/F1 hybrid. Dosages are in mg./Kg./day. A suspension of the agent in 10% polysorbate 80 was administered i.p. once daily starting with the first post-tumor inoculation day and continuing until the death of the last animal. *S*, per cent change in mean survival of treated mice compared with controls; *I*, per cent change in mean tumor size of treated mice compared with controls. Numbers in parentheses indicate day of tumor measurement. [b] Significant survival increase ($S > +20\%$). [c] Significant tumor inhibition ($I > +50\%$). [d] Toxic dose ($S < -25\%$).

water tenaciously; extreme drying conditions were then necessary to obtain anhydrous material.

Nineteen of the 25 compounds reported here were examined for antineoplastic activity against transplantable mouse tumors following the standard assay procedures employed at this foundation (17). (Table III.) All 19 were evaluated against the L1210 ascitic lymphatic leukemia in the BDF/1 mouse and against as many other primary screen tumors as availability of sample would permit. These include the following mouse tumors: P1534 lymphatic leukemia in the DBA/2 inbred strain, C1498 myelogenous leukemia in the C57BL/6 inbred strain, DBRB mammary carcinoma in the DBA/1 inbred strain, and S180 spindle cell sarcoma in the CAF/1 hybrid. The agent was administered intraperitoneally as a suspension in 10% polysorbate 80[2] once daily starting with the first post-tumor inoculation day; injections were continued until the death of the last experimental animal.

At nontoxic dosages,[3] only one agent (compound *16*) showed significant activity against the L1210 leukemia as evidenced by an increase in mean survival time. A number of agents effected moderate to marked tumor inhibition in the other assay systems, but few showed a concomitant increase in mean survival time. These data are summarized in Table III.

[2] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

[3] A dose level was regarded as toxic if the mean survival time of mice administered this dose was 25% or below the mean survival time of control mice.

Tanaka and his associates have claimed that positions 2, 4, and 6 of the pyrimidine ring of VI should all be substituted with amino groups for maximum activity against mouse tumors (14). However, the authors' results (Table III) indicate that, although the 2,4,6-triamino derivatives are active (compounds *17* and *18*), the 2,4-diamino-6-hydroxy derivatives (compounds *1*, *7*, and *9*) and the 2,4-diamino-6-chloro derivatives (compounds *15* and *16*) are more active. A more accurate but nevertheless tentative generalization might be that the 2,4-diamino-5-arylazopyrimidine configuration is required for experimental antitumor activity.

When compound *1* was administered at 20, 40, and 80 mg./Kg./day × 20 i.p. to mice bearing the P1534 leukemia, and compound *24* at 31.25, 62.5, and 125 mg./Kg./day × 12 i.p. to mice bearing the C1498 leukemia, gross inspection at autopsy performed 1 day later in these groups of mice revealed incomplete absorption of compound from abdominal cavities. In addition, compound *1* at the 2 higher doses, and compound *24* at all 3 doses produced intestinal irritation, as manifested by adhesions between surfaces of peritoneal viscera (liver, spleen, and kidney), and capsular thickening. Compound *24*, at the 2 higher dose levels, showed histological evidence of nephrotoxicity, with hyaline casts in dilated proximal and distal convoluted tubules, and degenerating renal epithelium.

In an acute oral toxicity experiment, compound *1* was administered to normal BAF/1 mice by gavage at single doses of 500, 750, and 1000 mg./Kg. The mice were sacrificed 4 days later and, on autopsy, the agent was still evident in the stomachs of the treated animals. The kidneys were pale and enlarged; histology indicated that pigment casts were present in both collecting and convoluted tubules. Additional casts of an amorphous, pink-staining material with dilatation of convoluted tubules pointed to nephrotoxicity.

As mentioned earlier, the compounds have also been examined for antimetabolic activity in microbiological systems and have been found to exhibit antifolic and antipurine properties (9). These results and a detailed analysis of the structure-activity correlations will be the subject of the next paper in this series.

EXPERIMENTAL[4]

Starting Materials.—The aromatic amines were Eastman Kodak white label grade and were used without purification, except for aniline, which was redistilled from zinc prior to use.

The pyrimidines were obtained as follows: (*a*) 2,4-diamino-6-hydroxypyrimidine, 2,4-diamino-6-chloropyrimidine,[5] 2,4-diamino-6-mercaptopyrimidine,[5] and 4-amino-2,6-dihydroxypyrimidine by procedures recently described (19); (*b*) 2,4,6-triaminopyrimidine by the method of Traube (20) from malononitrile and guanidine free base; (*c*) 2,4-diamino-6-methylthiopyrimidine by methods developed in these laboratories (21); (*d*) 4,6-diamino-2-hydroxypyrimidine by hydrolysis of 4,6-diamino-2-mercaptopyrimidine with chloroacetic acid following the procedure of Bendich, Tinker, and Brown (22); (*e*) 4,6-diamino-2-methylthiopyrimidine from Dougherty Chemical Co., Richmond Hill, N. Y., and barbituric acid from Matheson Coleman and Bell, East Rutherford, N. J.

Preparation of 5-Arylazopyrimidines.—The compounds listed in Table I were prepared by the following methods. Although many of the arylazopyrimidines can be made by more than one of the procedures indicated in Table I, the method specified for each compound is the optimal method found during this study. In particular, compounds *7* (*Method E*), *15* and *16* (*Method D*), *19* (*Method F*), and *21* (*Method G*) are best prepared by the specific procedures indicated. Analytical data for the new compounds prepared are given in Table II.

Method A.—This is essentially the method of Benson, Hartzel, and Savell (16). The diazotized aromatic amine in hydrochloric acid solution was added to a cold, stirred solution of an equimolar amount of the pyrimidine in 10% sodium acetate; the pH at the end of the reaction was 5–6. For alkali-soluble products, the work-up was modified to include an initial purification step: the crude product was dissolved in warm 1 *N* sodium hydroxide, the solution filtered free of tarry or insoluble impurities, and the product precipitated at pH 5 by the addition of acetic acid.

Method B.—This procedure differs from *Method A* in that the diazonium intermediate was added to a suspension of the pyrimidine (0.05 mole) in 450 ml. of 10% sodium acetate. The mixture was stirred for 1 hr. at room temperature and the product collected by suction filtration, washed, and dried.

Method C.—In this modification, 0.1 mole of the aromatic amine in 100 ml. of 6 *N* hydrochloric acid was diazotized and coupled with 2,4-diamino-6-hydroxypyrimidine (0.1 mole) dissolved in 500 ml. of 10% sodium carbonate.

Method D.—A diazotized amine solution, prepared in the usual manner and freed of excess nitrous acid by the addition of urea, was added to a cold, stirred solution of the pyrimidine in 3 *N* acetic acid. Immediately after admixture, solid sodium hydroxide was added to adjust the pH to 4; the pH was then brought to 5.5 by the addition of a large quantity of solid sodium acetate. The reaction mixture was stirred for at least 3 hr., the temperature gradually rising to room temperature. The product was collected, washed with water, and dried.

Method E.—A suspension of 0.2 mole of diazotized sulfanilic acid (23) was added at room temperature to a solution of 0.2 mole of 2,4-diamino-6-hydroxypyrimidine in 160 ml. of 1 *N* sodium hydroxide. A bright yellow precipitate formed instantaneously and the resulting suspension was stirred for 15 min. The final pH was 5.0. The product was collected, washed sparingly with cold water, and dried for 17 hr. at 60° *in vacuo*. Isolation of the sodium salt of compound *7* was accomplished by addition of an equal volume of absolute ethanol to a solution of 20 Gm. of the crude material in 600 ml. of 1% aqueous sodium bicarbonate.

[4] Melting points were taken by the capillary method at a rate of heating of 2°/min. in a modified Wagner-Meyer melting point apparatus (18) and are uncorrected. Drying of analytical samples was carried out at 70–100° for 17 hr. *in vacuo* over phosphorus pentoxide. Analyses were performed by Dr. Stephen Nagy and his associates, Microchemical Laboratories, Massachusetts Institute of Technology, Cambridge, Mass., and by the Scandinavian Microanalytical Laboratory, Herlev, Denmark.

[5] Larger quantities of this material were obtained through the courtesy of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Bethesda, Md., and were prepared by Aldrich Chemical Co., Milwaukee, Wis., according to the procedures outlined in *Reference 19*.

Method F.—The coupling reaction was achieved at a constant pH of 9.6 in an ammonium hydroxide–ammonium chloride buffer following the procedure recently described (19).

Method G.—Coupling was accomplished at constant pH of 5.0 in a citric acid–disodium phosphate buffer; this procedure is described in *Reference 21.*

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Effects of Pentobarbital, Acetylsalicylic Acid, and Reserpine on Blood Pressure and Survival of Rats Subjected to Experimental Stress

By JOSEPH P. BUCKLEY, EUGENE E. VOGIN*, and WILLIAM J. KINNARD

Sodium pentobarbital, 20 mg./Kg. *per os* daily, and acetylsalicylic acid (ASA), 100 mg./Kg. *per os* daily, failed to prevent the development of hypertension in rats subjected to experimental stress. ASA enhanced the lethal effects of the stressors and potentiated the effects of the stress conditions on gastric mucosa. Reserpine phosphate, 0.1 mg./Kg. (base) i.p. daily, administered after the animals had been subjected to the stress conditions for 6 weeks, did lower the blood pressure to control levels.

THE DEVELOPMENT of hypertension in animals exposed to experimental stress has been reported by numerous investigators (1–5). Buckley *et al.* (5) found that reserpine phosphate, 0.1 mg./Kg. i.p., and chlorpromazine hydrochloride, 4 mg./Kg. i.p., administered 1 hr. prior to subjecting rats to a 4-hr. variable stress program not only failed to decrease the pressor effects induced by the stressors over a 27-week period but also appeared to potentiate the lethal effects of the stressors. This present study was undertaken to investigate the effects of pentobarbital and acetylsalicylic acid (ASA) on animals subjected to chronic variable stress programs, and the effects of reserpine phosphate[1] administered to the experimental animals after physiological effects of stress exposure were evident.

EXPERIMENTAL

The stress chambers utilized were semisoundproof rooms designed by the Industrial Acoustics Co., New York, N. Y. (5). The stress program consisted of (*a*) flashing 150-w. spotlights (installed in each corner of the cage) which were on for $^1/_4$ sec. and off for $^3/_4$ sec. (in alternate pairs); (*b*) audiogenic stimulation at 5-min. intervals for 0.5-min. periods produced by amplifying a tape recording of noxious sound so that the intensity was approximately 100 decibels at the center of the cage; and (*c*) oscillation at the rate of 140/min. A simple conditioned avoidance response program utilizing automatic pole climbing units (4) was also utilized in the stress program. The cycle was initiated every 2.75 min. and consisted of a low tone for 15 sec., followed by the delivery of an electric shock (3 ma.) to the grid

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[1] Kindly supplied by Dr. William E. Wagner, Ciba Pharmaceutical Co., Summit, N. J.

Method F.—The coupling reaction was achieved at a constant pH of 9.6 in an ammonium hydroxide–ammonium chloride buffer following the procedure recently described (19).

Method G.—Coupling was accomplished at constant pH of 5.0 in a citric acid–disodium phosphate buffer; this procedure is described in *Reference 21.*

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Effects of Pentobarbital, Acetylsalicylic Acid, and Reserpine on Blood Pressure and Survival of Rats Subjected to Experimental Stress

By JOSEPH P. BUCKLEY, EUGENE E. VOGIN*, and WILLIAM J. KINNARD

Sodium pentobarbital, 20 mg./Kg. *per os* daily, and acetylsalicylic acid (ASA), 100 mg./Kg. *per os* daily, failed to prevent the development of hypertension in rats subjected to experimental stress. ASA enhanced the lethal effects of the stressors and potentiated the effects of the stress conditions on gastric mucosa. Reserpine phosphate, 0.1 mg./Kg. (base) i.p. daily, administered after the animals had been subjected to the stress conditions for 6 weeks, did lower the blood pressure to control levels.

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EXPERIMENTAL

The stress chambers utilized were semisoundproof rooms designed by the Industrial Acoustics Co., New York, N. Y. (5). The stress program consisted of (*a*) flashing 150-w. spotlights (installed in each corner of the cage) which were on for $^1/_4$ sec. and off for $^3/_4$ sec. (in alternate pairs); (*b*) audiogenic stimulation at 5-min. intervals for 0.5-min. periods produced by amplifying a tape recording of noxious sound so that the intensity was approximately 100 decibels at the center of the cage; and (*c*) oscillation at the rate of 140/min. A simple conditioned avoidance response program utilizing automatic pole climbing units (4) was also utilized in the stress program. The cycle was initiated every 2.75 min. and consisted of a low tone for 15 sec., followed by the delivery of an electric shock (3 ma.) to the grid

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floor for 30 sec. or until the animal leaped onto the Plexiglas pole. Control blood pressures were obtained twice weekly in both studies, utilizing a photoelectric tensometer (Metro Industries, Long Island, N. Y.) and the animals acclimated to this apparatus over a 4-week pre-experimental training period.

Study 1.—Fifty-nine male Wistar rats, weighing approximately 175 Gm., were randomly divided into 6 groups. The groups to be stressed contained 15 rats each and received the following treatment: group *A*, distilled water, 1 ml./Kg.; group *B*, sodium pentobarbital, 20 mg./Kg.; and group *C*, acetylsalicylic acid (ASA), 100 mg./Kg. Three unstressed control groups were divided as follows: group *D*, 5 animals treated with distilled water, 1 ml./Kg.; group *E*, 5 rats receiving sodium pentobarbital, 20 mg./Kg.; and group *F*, 4 rats receiving ASA, 100 mg./Kg. The animals receiving pentobarbital and distilled water were treated by oral intubation 0.5 hr. prior to subjecting the animals to the 4-hr. stress program. Acetylsalicylic acid was suspended in 1% carboxymethylcellulose and was administered by gastric intubation 1 hr. prior to subjecting the animals to the stress.

The stressors were applied singly or in combination, and the stressor schedule altered daily to prevent acclimation to the stimuli. The animals were stressed 4 hr./day, 6 days/week, from weeks 1–17 and 6 hr./day, 6 days/week, from weeks 18–30. The blood pressure of each animal was obtained once a week prior to administering drugs and subjecting the animals to the stress chamber. The animals in the 3 stressed groups were subjected to ten 2.75-min. cycles in the avoidance response pole climbing apparatus once weekly starting with the twelfth week. These animals were not exposed to the stress chamber on that particular day and received water or drug treatment prior to being subjected to the avoidance-escape situation.

Study 2.—A second study was conducted utilizing 68 male albino Wistar rats, which were divided as follows: group *A*, 24 rats treated with 0.1 ml./Kg. of saline i.p.; group *B*, 25 rats treated with 0.1 ml./Kg. of saline i.p., weeks 1–6; and reserpine phosphate, 0.1 mg./Kg. (base) i.p., 0.5 hr. prior to the experiment, from week 7 on; group *C*, 9 rats treated with saline, 0.1 ml./Kg. i.p.; and group *D*, 10 rats treated with saline, week 1–6 and reserpine phosphate, 0.1 mg./Kg. (base) i.p., from week 7 on. The animals in groups *A* and *B* were subjected to 2 hr. of variable stress daily, 6 days/week; and starting with the tenth week were also subjected to the pole-climbing stress once weekly. Groups *C* and *D* were utilized as nonstressed controls. Blood pressures and body weights were obtained once weekly on all animals prior to treatment and subjection to stress.

RESULTS

Study 1.—The effects of the stressors on blood pressures of the experimental animals are summarized in Fig. 1. The blood pressures of the stressed animals rose steadily reaching maximum values during the sixteenth and seventeenth week (group *A*, 158 mm. Hg ± 3.08; group *B*, 154 mm. Hg ± 2.25; and group *C*, 154 mm. Hg ± 2.0), whereas the mean blood pressures of the nonstressed animals ranged from 118–124 mm. Hg. When these data were subjected to the Student *t* test, the differences between the mean blood pressures of the stressed group and the nonstressed control group were highly significant ($P < 0.01$).

Increasing the duration of stress from 4 to 6 hr. during the eighteenth week did not appear to induce a further increase in blood pressure. At the end of the thirtieth week, none of the stressed rats of group *A* were dead, whereas the mortality rate was 13% for the pentobarbital treated stressed rats and 67%

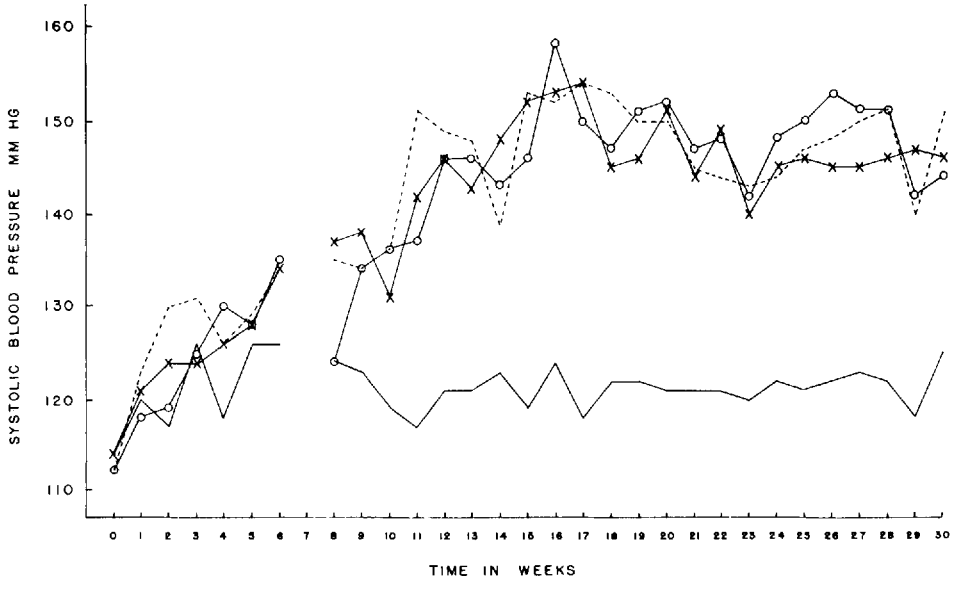

Fig. 1.—Effects of experimental stress on the systolic blood pressure of male Wistar rats in *Study 1*. Group *A*, distilled water, 1 ml./Kg., stressed; group *B*, pentobarbital, 20 mg./Kg., stressed; group *C*, ASA, 100 mg./Kg., stressed; group *D*, distilled water, 1 ml./Kg., nonstressed controls. Key: ○, group *A*; ×, group *B*; — — —, group *C*; ————, group *D*.

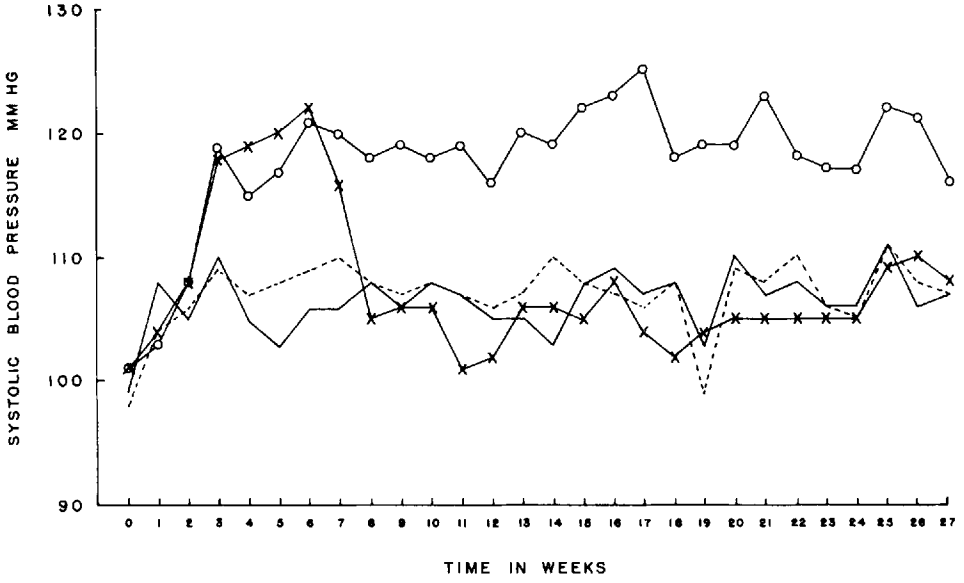

Fig. 2.—Effects of experimental stress on the systolic blood pressure of male Wistar rats in *Study 2*. Group *A*, saline, 0.1 ml./Kg., stressed; group *B*, saline, 0.1 ml./Kg., weeks 1–6, and reserpine PO_4, 0.1 mg./Kg., weeks 7–27, stressed; group *C*, saline, 0.1 ml./Kg., nonstressed controls; group *D*, saline, 0.1 ml./Kg., weeks 1–6, and reserpine PO_4, 0.1 mg./Kg., weeks 7–27, nonstressed controls. Key: ○, group *A*; ×, group *B*; — — —, group *C*; ———, group *D*.

for the ASA-treated stressed rats. The majority of deaths occurred at night between stress sessions. All of the animals in group *C* (ASA treated) which died during the stress session had marked ulceration of the stomach and small intestines with evident bleeding of the gastric mucosa. There was also a marked difference in the behavior of the various groups. The animals in each group were initially docile and very easy to handle; however, by the fifteenth week, the rats in group *A* (treated with distilled water) were extremely difficult to handle and demonstrated a high degree of activity. The animals in group *B* (pentobarbital treated) appeared to be more docile than those in group *A* but did show signs of irritability and became more aggressive during the latter portion of the study. The animals in group *C* (ASA treated) demonstrated the greatest change in that they were extremely difficult to handle and fought the feeding tube continuously. All of the animals in this group had pronounced diarrhea, excessive salivation, and appeared to be extremely sensitive to touch. The nonstressed controls remained fairly docile throughout the experimental period; however, group *F* which received daily administration of ASA appeared to be more excitable than the other two control groups, and most of the animals in this group had periods of diarrhea throughout the study.

Study 2.—The blood pressures of the animals in the two stressed groups gradually increased and by the sixth week of stress averaged 20 mm. Hg higher than initial blood pressures, whereas the blood pressures of the two control groups increased by less than 8 mm. Hg (Fig. 2). Reserpine phosphate, administered once daily starting with the seventh week produced mild diarrhea and ptosis in the animals of groups *B* and *D*. The mean blood pressure of the animals in group *B* dropped from 122 mm. Hg in week 6 to 105 mm. Hg in week 8, and ranged from 101–110 mm. Hg throughout the remainder of the experiment. The blood pressure of the saline-treated stressed group reached a maximum of 125 ± 2.9 mm. Hg during the seventeenth week of the experiment, whereas the reserpine-treated stressed animals in group *B* had a mean blood pressure of 104 ± 1.4 mm. Hg ($P < 0.05$). The number of deaths occurring in this study was relatively low in comparison to previous studies (4% in group *A*, 12% in group *B*, 11% in group *C*, and 20% in group *D*), and the hypertensive effect occurring during the chronic exposure to the stressors was also much less than has

Table I.—Conditioned-Avoidance Response in Male Albino Rats Subjected to Experimental Stress

Wk.	Response Latencies, sec. ± S.E. Group *A* Saline, 0.1 ml./Kg.	Group *B* Reserpine, 0.1 mg./Kg.
10	25.1 ± 1.57	36.0 ± 2.35
11	14.4 ± 1.72	25.9 ± 3.41
12	11.2 ± 1.11	27.8 ± 3.40
13	10.6 ± 1.43	29.1 ± 3.56
14	11.3 ± 1.27	28.4 ± 3.05
15	8.9 ± 1.34	29.1 ± 2.92
16	9.1 ± 0.92	24.9 ± 3.11
17	8.6 ± 1.09	20.9 ± 2.57
18	8.3 ± 1.21	19.6 ± 2.85
19	8.7 ± 0.75	19.7 ± 2.73
20	8.2 ± 0.96	20.4 ± 2.65
21	7.1 ± 0.97	19.7 ± 1.82
22	7.6 ± 1.56	15.6 ± 1.63
23	9.0 ± 1.36	15.6 ± 0.65
24	8.6 ± 1.15	14.4 ± 1.07
25	8.3 ± 1.06	14.7 ± 1.23
26	8.1 ± 0.99	15.1 ± 1.06

previously been obtained. The response latencies of the saline and reserpine-treated stressed animals in the avoidance-escape test are summarized in Table I. The response time of the reserpine-treated animals was always much greater than the saline-treated animals; however, there was a gradual decrease in response time over a 12-week period from 36 sec. to slightly over 15 sec.

DISCUSSION

The hypertensive effects induced by exposure to the stressors in the first study were similar to those previously obtained in this laboratory (3-5). The responses obtained in the second study, however, were relatively mild; and after reaching a peak in the seventeenth week, the animals appeared to be acclimated to the experimental conditions as demonstrated by a plateauing of the blood pressure over the next 10 weeks. Several additional studies have since been conducted, and this variation in the blood pressure response has also occurred. The physiological response of the animal to the experimental stressors appears to vary among samples obtained from the same strain and from an identical source at different times, but there is only a slight variation in the response of animals within a particular sample.

In the first study, pentobarbital and ASA failed to protect the experimental animals from the effects of the stressors. The stressors apparently potentiated the toxicity of ASA, an effect which was most apparent on the gastrointestinal tract. The oral administration of single doses of ASA (100–500 mg./Kg.) has been reported to cause small gastric mucosal erosions to fasted guinea pigs within 30 min. of dosing; however, they appeared to heal within 24 hr. (6). Many investigators have also associated ingestion of ASA with the development of gastric mucosal erosions in mice (7, 8). Selye (9) has reported that exposure of rats to one or more stressors will induce gastric ulceration. The intensity of the stressors utilized in this current study have been controlled so that gastric ulceration would not occur in untreated stressed rats. The data indicate that the effects of ASA on gastric mucosa are greatly enhanced by environmental stress. Selye (10) has suggested that stressors induce the discharge of ACTH which stimulates the release of both mineral corticoids and glucocorticoids from the adrenal cortex, and Timmer (11) has reported a marked increase in free plasma corticosterone of rats subjected to severe acute stress. Salicylates have also been reported to increase the plasma level of 17-hydroxycorticosteroids in rats (12), and it is, therefore, possible that this assumed increase in plasma corticosteroids may be responsible for the gastric ulceration. However, it appears more likely that the observed ulcerations were due to a combination of the local effect of the ASA on the gastric mucosa and an increase in free plasma corticosteroids.

Reserpine phosphate did reverse the effects of the experimental stressors on the blood pressure of rats (*Study 2*). These data are opposite to those obtained in a previous study (5) in which reserpine, 0.1 mg./Kg. i.p., not only failed to protect the rats from the pressor response induced by chronic stress but potentiated the lethal effects of the stressors. Epstein *et al.* (13) investigated the effects of reserpine on the stress of reduced barometric pressure in rats and also found that rather than protect the rats against the stress of altitude reserpine acted as an added stress factor. The experimental design in the previous study was different than the one presently being reported. In the present study, reserpine was not administered until the seventh week of stress to allow for the development of the stress reaction; whereas in the previous study, reserpine treatment was initiated immediately. These data suggest that the effects of reserpine and, for that matter, almost any compound may vary greatly, depending upon the time interval following the initiation of the stress program that treatment is started. However, it is also possible that the relatively mild physiological response to the stressors was responsible for the effect observed with reserpine.

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Micromeritics of Granular Pharmaceutical Solids II

Factors Involved in the Sieving of Pharmaceutical Granules

By DALE E. FONNER, JR., GILBERT S. BANKER, and JAMES SWARBRICK

This report deals with basic studies concerned with the use of sieving as an accurate means of classifying granular pharmaceutical solids. Attention was focused on the effects of sieve shaker speed, initial load and particle size distribution of the granulation on the rate of particle size reduction and the cumulative per cent of material passing a sieve. The size and concentration of the smaller-than-mesh particles were shown to exert a profound influence on the equilibrium time of a sieve. Using a nest of sieves, an equilibrium time technique was developed to obtain the particle size distribution and permit calculation of various statistical parameters dependent on the observance of the log-normal law. An equilibrium time procedure such as developed here permits the quantitative classification of granular pharmaceutical solids by sieving, with a better assurance that separation has been achieved with the minimum of attrition.

THERE ARE few reports in the literature concerning the sieving of granular pharmaceutical solids for the purposes of classification or size analysis. It would appear that, in the past, such important considerations as size of the initial load, the sieve shaker speed, time of sieving, and the hardness, particle size distribution, and shape of the material being sieved have either been left to chance or substantially neglected. Such fundamental parameters as the type of particle size distribution and the magnitude of various statistical diameters on a weight or count basis have also not been applied to pharmaceutical granulations.

Since sieving is the easiest technique available for classifying particles in the size range of granular pharmaceutical solids, it finds wide industrial application. However, sieving errors can arise from a number of different variables. According to Herdan (1), the most predominant are variations in sieve loading, duration of sieving, random orientation of particles, fluctuation through sampling, errors of observation and experiment, and the effect of different equipment and operations. Consequently, if sieving is to be applied as an accurate, quantitative technique for classification, it is necessary to obtain more information as to the effect of the parameters previously enumerated on both the sieving process and the particle size distributions obtained.

Whitby (2) has carried out an extensive study of the physical laws which govern the sieving of fine particles. It was shown that the sieving curve under nonsteady-state conditions can be divided into two distinctly different regions, with a transition region between. The sieving curve was obtained by plotting the cumulative per cent by weight passing the sieve against time on either log-log or log-probability paper. A typical example of the former case is shown in Fig. 1. Whitby found that the rate at which the material passes a sieve in region 1 is a constant closely following the relationship:

$$\% \text{ material passing} = at^b \qquad \text{(Eq. 1)}$$

where t is the sieving time, b is a constant very nearly equal to 1, and a is a sieving rate constant. Region 2 was found to follow the log-normal law. In this region all the particles much smaller-than-mesh size have already passed the sieve; consequently, the particles now passing the sieve are of a constant mesh size. For all practical purposes the sieve is considered to be at equilibrium at that time when the second linear portion (region 2) begins, since the slight positive slope of the line is due mostly to the attrition of larger-than-mesh size particles (Fig. 1).

The purpose of this report is to (*a*) present useful techniques and theories developed in other fields of study for the computation of various statistical diameters, (*b*) investigate the magnitude of particle size reduction which occurs on a vibrating sieve, (*c*) investigate the effects of load, shaker speed, and particle size distribution on the cumulative per cent of material passing a sieve, and (*d*) suggest a method for determining the sieving time for a nest of sieves.

EXPERIMENTAL

Preliminary Controls.—Brass screens[1] constructed to A.S.T.M. specifications were used in all cases. A Cenco-Meinzer[2] sieve shaker was employed with the speed setting at position 5, except for those studies concerned with the effect of varying the sieve

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Previous paper. Fonner, D. E., Jr., Banker, G. S., and Swarbrick, J., *J. Pharm. Sci.*, **55**, 181(1966).

[1] Fisher Scientific Co., New York, N. Y.
[2] Central Scientific Co., Chicago, Ill.

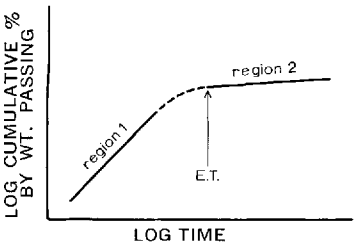

Fig. 1.—Typical plot of the cumulative per cent by weight of material passing a sieve as a function of time.

shaker speed. A nylon brush was used to clean the sieves. Each granulation batch was gently mixed prior to sampling by placing the granulation on a sheet of paper and lifting the corners in sequence toward the center of the sheet. The preparation and properties of all granules used have been described elsewhere (3).

Particle Size Reduction on a Single Sieve.—Granules prepared using a hand screen and an oscillating granulator were examined (3). No sieve cuts were used and the initial load for sieve analysis was 100 Gm. The cumulative per cent by weight passing a No. 16 sieve was plotted against time on log-probability paper over an extended shaking period.

Effect of Load and Shaker Speed on Granule Breakdown.—Granules produced from an oscillating granulator (3) were employed. A 16-mesh sieve was used and no sieve cuts were taken. The effect of varying the initial load was studied between the limits of 50 and 150 Gm. The effect of altering the speed setting was studied using a standard load of 100 Gm. and varying the speed between the limits of positions 2 and 8 on the shaker speed control.

Particle Size Reduction on a Nest of Sieves.—The effect of shaking time on the estimation of the geometric mean diameter on a weight basis, $\bar{M}$, was determined using 16, 20, 40, and 60 screens in a nest. An initial 100-Gm. load of granules prepared using the hand screen was used and no sieve cuts were taken. The particle size distributions obtained at various shaking time intervals were plotted on log-probability paper. The values for $\bar{M}$ were then plotted against time on log-log paper.

Influence of Particle Size on the Equilibrium Time of a Sieve.—This investigation was carried out using a No. 20 mesh sieve and a commercially prepared calcium sulfate base granulation.[3] Three systems of granulations were studied. System *1* consisted of 12/16 mesh particles together with various concentrations of 40/60 mesh particles over the range from 12.5–100% by weight. System *2* contained 12/16 mesh particles and 30/40 mesh particles in concentrations ranging from 25–100% by weight. System *3* was composed of 12/20 mesh particles plus various concentrations of 20/40 mesh particles ranging from 12.5–100% by weight. The equilibrium times were obtained, from log-log plots of the cumulative per cent passing the sieve as a function of time, as that time at which the second linear portion of the plot began. This is shown as E.T. in Fig. 1. (See also Fig. 6.)

Characterization of Particle Size Distribution.—Various statistics were computed from the distribution of particle sizes obtained using two techniques.

Standard Procedure.—The standard procedure consisted of sieving a 200-Gm. sample of the lactose granulation (3), prepared using the hand screen, for 10 min. on a nest composed of a No. 16, 20, 30, 40, 60, and 80 mesh sieve. The cumulative per cent less-than-stated size was plotted against stated size on log-probability paper.

Equilibrium Time Procedure.—The equilibrium time procedure was based upon a sequential analysis developed from the equilibrium time values of the individual sieves in the nest. This approach was necessary because the upper sieves in a nest will unload their smaller-than-mesh particles before those sieves near the bottom of the nest. Consequently, the equilibrium time for a sieve in a nest can only be computed when the smaller mesh screens are at nonsteady-state conditions. Therefore, the sieve immediately below the sieve whose equilibrium time was being determined was covered with a sheet of paper. The material collected after the upper sieve had come to equilibrium was then used as the sample for the previously covered sieve; the next lower sieve in the nest was covered in this case. The equilibrium times for the individual sieves, obtained from log-log plots of the cumulative per cent passing the sieve as a function of time, are presented in Table I.

On the basis of these results, the following procedure was used to obtain the particle size distribution of 200 Gm. of the lactose granulation (3). The same nest of sieves was employed but no sieves were covered. The No. 16 sieve was removed after 90 sec. of shaking and its contents weighed. It was then emptied and returned to the nest. After 150 sec. of shaking, the 20- and 30-mesh sieves were removed and their contents weighed. The screens were emptied and then returned to the nest. Finally, the last three screens were removed and weighed after a total of 200 sec. of shaking (this time was obtained by adding the individual equilibrium times together). Selection of the times for removal of the sieves is not highly critical, although the selected time must *not* be less than the computed equilibrium time for the sieve being removed.

RESULTS AND DISCUSSION

Particle Size Reduction on a Sieve.—The data from extended shaking time studies performed on particles from the oscillating granulator were found to follow the log-normal law as predicted by Whitby (2), for sieving times beyond the equilibrium time value previously defined. Figure 2 represents a plot of cumulative per cent passing *versus* time on log-probability paper. It is evident that a considerable number of particles are still passing the sieve after 140 min. of shaking. Since it is not likely that undersize particles would take this long to reach the classification area, it must be assumed that particle size reduction is occurring.

Table I.—Equilibrium Times for the Individual Sieves in a Nest[a]

Mesh size	16	20	30	40	60	80
Equilibrium time, sec.	45	30	25	20	36	44

[a] 200 Gm. of the lactose granulation prepared by the hand screen method (3) was used.

[3] Miles Laboratories Elkhart, Ind.

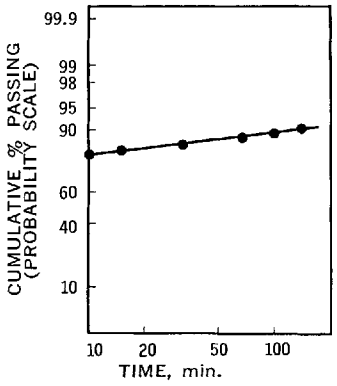

Fig. 2.—Log-probability plot of the rate of attrition of particles from the oscillating granulator. Load: 100 Gm.; speed setting: 5; sieve size: 16 mesh.

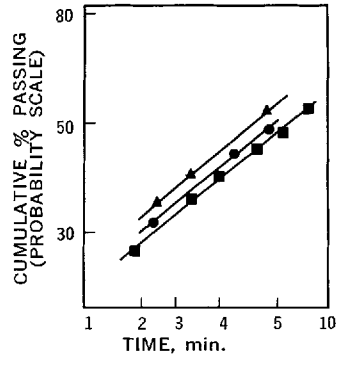

Fig. 3.—Effect of initial load upon the rate of attrition of particles from the oscillating granulator. Speed setting: 5; sieve size: 16 mesh. Key: ▲, 50 Gm.; ●, 100 Gm.; ■, 150 Gm.

Effect of Load on Granule Breakdown.—Figure 3 illustrates the results obtained for the three different initial loads studied. Since the curves for each initial load are virtually parallel, the attrition rate constant is apparently unaffected by the initial load over the range of values studied. However, it is evident from Fig. 3 that the cumulative per cent passed at any one time increases as the initial load decreases over the range of values studied. This means that the smaller loads have passed a greater percentage of their particles at equilibrium time than have the higher loads, *i.e.*, the smaller the load, the more efficient is the sieving process. This phenomenon is probably due to less "blinding" on the sieve and greater maneuverability of the particles at these smaller loads.

Effect of Shaker Speed on Granule Breakdown.—The results obtained from this portion of the study are plotted in Fig. 4. Since the actual vibration rates of the shaker at the various speed settings could not be determined, it was not possible to plot attrition rate as a function of vibration rate. Nevertheless, it is apparent from Fig. 4 that higher speeds of shaking may markedly influence the reduction in size of particles on a sieve.

Effect of Sieving Time on the Geometrical Mean Diameter by Weight.—Plots of the geometrical mean diameter by weight, $\bar{M}$, for particles from the oscillating granulator, at various sieving times are shown in Fig. 5. These data were obtained from studies on the particle size reduction on a nest of sieves.

It is apparent from Fig. 5 that much care must be exercised when selecting a sieving time, especially if the ultimate objective is to characterize the distribution of particle sizes. The mean particle size by weight obtained from sieve analysis will continually decrease as the sieving time increases. Similar data were obtained for particles prepared from the hand screen; however, the slopes of the two lines were different. Thus, the slope of the sieving curve for particles from the oscillating granulator was 0.096 μ/sec. and the slope for particles from the hand screen was 0.174 μ/sec. This implies that the particles prepared from the oscillating granulator are harder than those from the hand screen, a finding which is in agreement with the hardness index values determined for these granulations in earlier work (3).

Influence of Particle Size on the Equilibrium Time of a Sieve.—The effects of different proportions by weight of three sieve fractions of smaller-than-mesh size calcium sulfate granules on the equilibrium time of a 20-mesh sieve are shown in Fig. 6. Each point on the curve represents the mean of two equilibrium

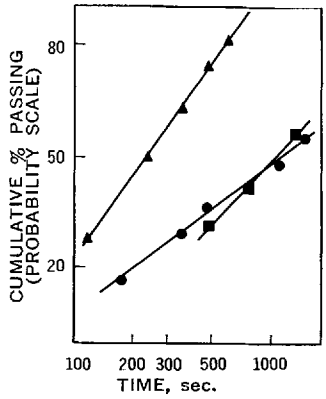

Fig. 4.—The effect of the speed setting of the sieve shaker upon the rate of attrition of particles from the oscillating granulator. Load: 100 Gm.; sieve size: 16 mesh. Key: ■, speed setting 2; ●, speed setting 5; ▲, speed setting 8.

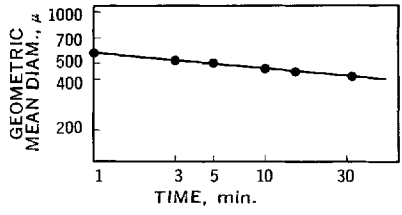

Fig. 5.—Plot of the geometric mean diameter by weight against sieving time for particles from the oscillating granulator. Load: 100 Gm.; speed setting: 5; sieve sizes: 16, 20, 40, and 60 mesh.

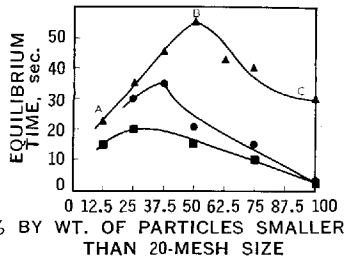

Fig. 6.—Effect of various particle size fractions upon the equilibrium time of a 20-mesh sieve using a commercial calcium sulfate granulation. Load: 200 Gm.; speed setting: 5. Key: ■, system *1* (12/16 and 40/60-mesh fractions); ●, system *2* (12/16 and 30/40-mesh fractions); ▲, system *3* (12/20 and 20/40-mesh fractions).

times. These were obtained graphically and, in most cases, were identical. System *3* was chosen because it contained many particles at, or near, mesh size.

From Fig. 6, it can be seen that the equilibrium times go through a maximum which is displaced to a lower percentage of smaller-than-mesh size particles as these smaller particles decrease in size. At the same time, the height of the peak decreases. In this particular study, all the variables were held constant with the exception of the per cent by weight of particles smaller-than-mesh size. The time taken for all the smaller-than-mesh size particles to pass the sieve is obviously a function of the number of these particles as well as the number of particles too large to pass which will hinder the passage of the smaller particles. Thus,

$$\text{E.T.} = f(n_u, n_0) \qquad \text{(Eq. 2)}$$

where E.T. is the equilibrium time in seconds, n_u is the number or weight of particles which can pass the screen, and n_0 is the number or weight of the greater-than-mesh size particles.

Increasing n_u while keeping n_0 constant would be expected to increase E.T. because of the increased number of particles which must now pass through the larger-than-mesh size particles and the sieve. On the other hand, holding n_u constant and decreasing n_0 would permit the smaller-than-mesh size particles to pass more rapidly through the sieve because the hindrance effect of the larger-than-mesh size particles is being progressively reduced.

In the system under discussion, we are increasing the weight concentration of the smaller-than-mesh size particles at the expense of the larger-than-mesh size particles, *i.e.*, n_u is increasing and n_0 is decreasing simultaneously. This reasoning may now be applied to the results shown in Fig. 6. Thus, it is postulated that over the region AB, the E.T. is increasing because the increase in n_u is of more significance than the decrease in n_0. However, at B, the effect of increasing n_u (tending to increase E.T.), and the effect of decreasing n_0 (tending to decrease E.T.), are equal and a maximum point is obtained. Over the region BC, the value for E.T. falls because the decreasing n_0 is now the controlling factor. We would expect, furthermore, the point at which the equilibrium time begins to decrease, after reaching the maximum B, to occur at lower concentrations of the smaller sized component, as the size of the latter decreases. This is because the hindrance of the smaller-than-mesh size particles by the larger particles decreases as the size of the former decrease. This is shown to occur in Fig. 6.

It is also evident from Fig. 6 that the maximum equilibrium time increases as the smaller-than-20-mesh particles increase in size. This is presumably due to the fact that the movement of the smaller sized particles through the void spaces in the granular bed becomes more difficult as the size of the smaller particle fraction increases. The large increase in equilibrium time for system *3* (12/20 mesh and 20/40 mesh particles), is most likely due to "blinding," since this system contained many particles at, or near, mesh size. Particles in this size range will reduce the effective open area of the sieve. Therefore, when selecting a sieving time, much thought should be given not only to the size of the smaller-than-mesh particles, but also to the *concentration* of such particles.

It is interesting to note the 100% point on the curves for the three systems shown in Fig. 6. At this point, there are no particles larger than mesh size present on the sieve. Therefore, an equilibrium time near zero is to be expected. This was not the case for system *3*, containing 20/40 mesh particles, where 6.7% of the granules by weight were still on the sieve after 15 min. of shaking. This phenomenon was checked by resieving the bulk sample of 20/40 mesh particles and running the determination again. The moisture content of the granules was checked before resieving and immediately following the 15 min. shaking period; it was constant at 1.6%. The same result was obtained, *i.e.*, approximately 7% by weight of the initial load of 200 Gm. did not pass. It is postulated that reduction in the

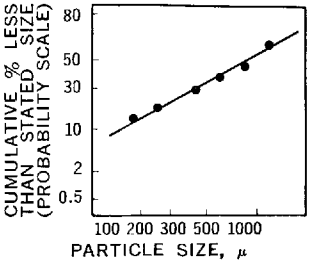

Fig. 7.—Plot of cumulative per cent less-than-stated size against stated size using the equilibrium time procedure for hand-granulated material. Load: 200 Gm.; speed setting: 5.

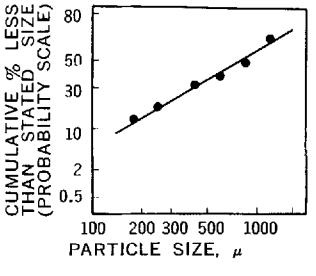

Fig. 8.—Plot of cumulative per cent less-than-stated size against stated size using a 10-min. sieving time for hand granulated material. Load: 200 Gm.; speed setting: 5.

TABLE II.—STATISTICAL PARAMETERS CALCULATED FROM SEIVING STUDIES[a]

Parameter	Eq. Used (4)	Sieving Procedure: Std., 10 min.	Sieving Procedure: Equilibrium Time
Geometric mean diam. by wt., $\bar{M}$	50% of stated size from plot on log-probability paper	840 μ	900 μ
Geometric S.D., σ_g	$\frac{84.13\%\text{ stated size}}{50\%\text{ stated size}}$ from plot on log-probability paper	3.67	4.12
Geometric mean diam. by count, d_g	$\log d_g = \log \bar{M} - 6.908 \log^2 \sigma_g$	5.13 μ	2.19 μ
Mean surface diam. by count, d_s	$\log d_s = \log \bar{M} - 4.605 \log^2 \sigma_g$	28.73 μ	16.29 μ
Mean vol. diam. by count, d_v	$\log d_v = \log \bar{M} - 3.454 \log^2 \sigma_g$	66.81 μ	44.40 μ
Arithmetic mean diam. by count, d_{av}	$\log d_{av} = \log \bar{M} - 5.757 \log^2 \sigma_g$	12.36 μ	5.97 μ
No. of particles/Gm., N	$\log N = \log \frac{1}{\rho \alpha_v} - \log d_g^3 - 10.362 \log^2 \sigma_g$	1.369×10^7	4.661×10^8

[a] 200 Gm. of the lactose granulation prepared by the hand screen method was used.

effective open sieve area, due to the large number of near-mesh-size particles present, was probably the major cause of this unexpected result.

Comparison of Various Statistical Diameters.—Using the standard and equilibrium time procedures for sieve analysis, it was possible to make a comparison of various statistical diameters. These were computed from the respective plots of size distribution by weight on log-probability paper shown in Figs. 7 and 8. In both cases, the distributions closely follow the log-normal law. Table II summarizes the results obtained from Figs. 7 and 8.

Owing to the increased duration of sieving, it was expected that those statistical diameters obtained by sieving for 10 min. would be smaller than those using the equilibrium time procedure. However, this was only true for the diameter calculated on a weight basis, $\bar{M}$. The expected order of statistical diameters by count was reversed because of the high value for σ_g in the equilibrium time analysis. (See Table II.) From this portion of the study, it can be concluded that although diameters on a weight basis will always decrease with increasing sieving times, those calculated diameters by count cannot always be assumed to decrease with increasing sieving times. In actuality, these parameters do decrease, but the computed values are arrived at from a consideration of the entire range of particle sizes. Since sampling of particle sizes was possible only over a finite range, the error in σ_g arising from lack of data at the very small sizes was probably responsible for the results shown in Table II.

Particle size reduction has been shown to occur when sieving is continued well beyond the equilibrium time, and this probably accounts for the widely varying values of $\bar{M}$ and σ_g shown in Table II. Therefore, to lessen the error introduced by particle breakdown, the equilibrium time procedure is suggested for the characterization of size distribution of pharmaceutical granular solids. Such an approach automatically takes account of the variable equilibrium times observed for the separate sieves. (See Table I.)

CONCLUSIONS

Basic studies on the sieving mechanism were carried out. It was found that particle size reduction occurred during sieving and was markedly influenced by the sieve shaker speed setting. The rate of particle size reduction was apparently independent of the initial load; however, the efficiency of the sieving operation is influenced by the initial load, as evidenced by Fig. 3. The equilibrium time of a sieve was found to be markedly dependent on the particle size of the sample used. This was particularly so when the size of the particles approach the mesh size of the sieve. Also shorter equilibrium times were observed when the concentration of smaller-than-mesh size particles was either fairly high or low in comparison to the greater-than-mesh size particles. At intermediate concentrations of the smaller-than-mesh particles, the equilibrium times can be expected to increase.

Comparative studies, using two different techniques to obtain particle size distribution curves, showed that the computed statistical diameters were influenced by the time of sieving. It is recommended that the equilibrium time procedure be used for the characterization of granular pharmaceutical solids by sieving. This in turn must entail prior knowledge of the equilibrium times of each individual sieve since, as is seen in Table I, these vary with screen mesh size. Only by the use of the equilibrium time method can the operator be confident that particle breakdown has been kept to a minimum and that equilibrium conditions on each sieve in the nest have been attained.

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Increasing Dissolution Rates and Gastrointestinal Absorption of Drugs *Via* Solid Solutions and Eutectic Mixtures IV

Chloramphenicol–Urea System

By ARTHUR H. GOLDBERG*, MILO GIBALDI†, JOSEPH L. KANIG, and MICHAEL MAYERSOHN

The dissolution rates of a number of chloramphenicol–urea samples were studied. Solubility studies indicated that urea increased significantly the solubility of chloramphenicol; this resulted in a large increase in the initial dissolution rate of chloramphenicol from physically mixed samples of the drug with urea. The α solid solution of chloramphenicol in urea was found to dissolve twice as rapidly as a physical mixture of the same composition, and almost 4 times as rapidly as the pure drug.

THE IMPORTANCE of particle size reduction as a means of increasing dissolution rates is well established. The methods by which a drug may be presented to the gastrointestinal fluids in finely divided form has been reviewed by Levy (1). Among the various ways to obtain microcrystalline dispersions *in vivo* is to administer a eutectic mixture composed of the drug and a substance which readily dissolves in water (2). This approach has been employed to enhance the dissolution rate of chloramphenicol (3). The results of this study were explained on the basis of particle size reduction of chloramphenicol in the drug–urea fused mixture (3).

Goldberg *et al.* (4) have raised a number of theoretical questions concerning the proposed mechanism of this phenomenon. Alternatively, these authors suggested that the enhanced dissolution rate was attributable to the presence of solid solutions in the system rather than simple eutectic formation. Indeed, the sample prepared by Sekiguchi *et al.* (3) at the eutectic composition manifested no enhancement in the dissolution rate of chloramphenicol as compared to the pure drug. An increase in dissolution rate became apparent only when a sample containing urea in excess of the eutectic composition was investigated.

The failure of the chloramphenicol–urea eutectic mixture to display increased dissolution of the antibiotic drug raises doubts concerning the general utility of the simple eutectic mixture in modifying dissolution. These doubts are heightened by a recent study by Goldberg and co-workers (5) on acetyl *p*-aminophenol–urea mixtures. This binary system showed practically no solid solubility. Examination of the results of this investigation indicated that particle size reduction in the eutectic mixture played a negligible role in enhancing dissolution. Conversely, a subsequent study (6) convincingly demonstrated the importance of solid solutions in modifying dissolution characteristics. The griseofulvin–succinic acid solid solution was found to dissolve 6–7 times faster than the pure drug.

The purpose of this present investigation was to examine the dissolution properties of various mixtures of chloramphenicol and urea in order to elucidate the mechanism involved in the reported enhancement of the rate of solution of chloramphenicol from these mixtures.

EXPERIMENTAL

Sample Preparation.—The fused mixtures of chloramphenicol[1] and urea were prepared by adding the powdered blend to a stainless steel crucible immersed in a temperature-controlled silicone fluid bath preheated to the melting point of the mixture. The mixture was constantly stirred until a homogeneous liquid resulted. The molten material was then cast immediately on chrome-plated stainless steel plates and allowed to congeal. The solidified mass was crushed with a mortar and pestle and then sieved through standard screens using a Syntron shaker.[2] Those particles passing through a No. 50 standard screen but retained on a No. 60 screen were used in the dissolution studies. The particle size of pure chloramphenicol was increased in the same manner. The samples investigated are listed in Table I. The chloramphenicol content of each sample was verified by spectrophotometric analysis.

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Previous paper: Goldberg, A. H., Gibaldi, M., and Kanig, L., *J. Pharm. Sci.*, **55**, 487(1966).

[1] Chloramphenicol used was generously supplied by Parke, Davis & Co., Detroit, Mich.
[2] Syntron TSS-25 Test Shaker, Syntron Co., Homer City, Pa.

Table I.—Chloramphenicol and Chloramphenicol–Urea Samples Prepared for Dissolution Studies

Sample	% Compn.	Description	% α Solid Soln.
A, Chloramphenicol	100	...	...
B, Chloramphenicol	92	Fused, β solid soln.	0
Urea	8		
C, Chloramphenicol	76	Fused, eutectic mixture	23
Urea	24		
D, Chloramphenicol	55	Fused	58
Urea	45		
E, Chloramphenicol	43	Fused	78
Urea	57		
F, Chloramphenicol	26	Fused, α solid soln.	100
Urea	74		
G, Chloramphenicol	26	Physical mixture	...
Urea	74		

Solubility Studies.—The solubility of chloramphenicol as a function of urea concentration was studied in aqueous solution. An excess of chloramphenicol was added to 30 ml. of distilled water containing various concentrations of urea, in 60-ml. screw-top vials. The vials were then placed in an incubator shaker[3] and maintained at 37° until equilibrium was established.

Solubility studies were also conducted with sample *F* (α solid solution) and sample *G* (a physical mixture corresponding in composition to the α-solid solution). An excess of each sample was placed in 30 ml. of water and incubated until the system reached equilibrium. This experiment was conducted to insure that fusion did not result in decomposition of the active ingredient.

Dissolution Rate Studies.—The dissolution rate of chloramphenicol from each of the samples listed in Table I was determined by means of the tape method (7). The quantity of material dusted on the adhesive surface varied with the individual sample but in each case corresponded to 10 mg. of chloramphenicol. The choice of a constant amount of drug is based on the assumption that if no interaction occurs, then the drug crystallizes from the melt to form particulates, within the mass, of approximately the same size regardless of urea concentration. If this hypothetical situation did exist, then all fused samples should show the same dissolution rate since the urea rapidly dissolves and leaves behind about 10 mg. of drug in the form of equal-sized particulates, having the same surface area. Under such conditions it would not be reasonable to maintain sample size constant since the apparent dissolution rate would decrease as the concentration of diluent increases and the corresponding effective surface area decreases.

The dissolution fluid consisted of 400 ml. of distilled water maintained at 37° in a 600-ml. beaker which was immersed in a constant-temperature water bath. The stir paddle was rotated in the fluid at a constant rate of 53.5 r.p.m. After immersion of the tape frame, 1-ml. samples were withdrawn at 3 and 5 min.

Assay Procedure.—Chloramphenicol concentration was determined spectrophotometrically. Each sample was diluted suitably with distilled water and the absorbance determined at 274 mμ using a Beckman DB recording spectrophotometer. Concentrations were calculated from a previously prepared Beer's law plot.

[3] Gyrotory Incubator Shaker, model G 25, New Brunswick Scientific Co., New Brunswick, N. J.

RESULTS AND DISCUSSION

Phase Diagram.—As noted by Goldberg *et al.* (4) the chloramphenicol–urea system exhibits a great deal of solid solubility. This is manifested by the existence of regions α and β in the phase diagram depicted in Fig. 1. At the eutectic point the mixture contains 76% chloramphenicol which is present as part of 2 distinct saturated solid solutions. The saturated α solid solution contains 30% chloramphenicol, while the saturated β solution contains

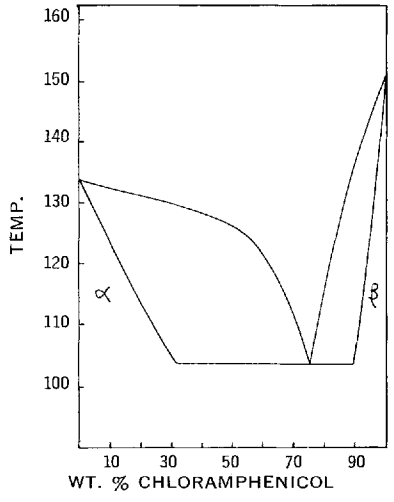

Fig. 1.—Phase diagram for chloramphenicol–urea system (3).

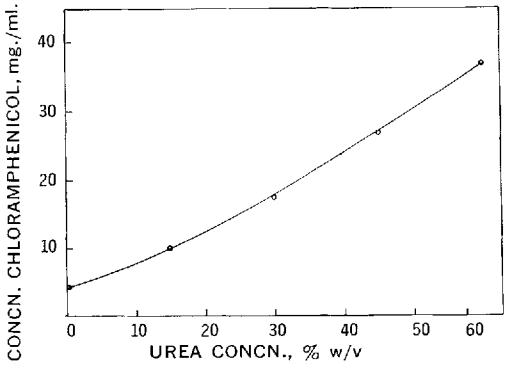

Fig. 2.—Solubility of chloramphenicol in aqueous solutions of urea at 37°.

TABLE II.—DISSOLUTION STUDIES OF CHLORAMPHENICOL FROM FUSED AND PHYSICAL MIXTURES WITH UREA

Sample[a]	Amt. Dissolved, mg./400 ml. 3 min.	5 min.	Relative Dissolution Rate at 3 min.
A, Pure drug	1.3	1.8	1.0
B, β solid soln.	1.3	1.7	1.0
C, Eutectic	1.7	2.3	1.3
D, 58% α solid soln.	2.4	3.0	1.8
E, 78% α solid soln.	2.8	3.8	2.2
F, α solid soln.	4.9	6.2	3.9
G, Physical mixture	2.4	2.9	1.8

[a] Refer to Table I for description of samples.

90% of the drug. The eutectic mixture actually consists of 23% α and 77% β solid solution. The α and β solid solutions account for 7 and 69 parts, respectively, of the total 76 parts of chloramphenicol present in the eutectic mixture.

Solubility Studies.—The data presented in Fig. 2 clearly demonstrate the significant effect of urea on the solubility of chloramphenicol. A greater than sevenfold increase in the solubility of the drug was observed over the urea concentration range studied.

The solubility of the α solid solution was found to be identical with that of a physical mixture of the same composition. This finding is indicative of the absence of chemical reaction between the drug and carrier which could occur during the fusion process. Therefore, the samples differ only with respect to their physical state.

Dissolution Rate Studies.—The results of the dissolution studies are shown in Table II. Inspection of each of these rates reveals a number of interesting relationships as well as an insight to the complexities involved in the dissolution of chloramphenicol from the fused binary mixtures.

The chloramphenicol–urea eutectic was found to dissolve somewhat faster than the pure drug with a comparable particle size. Sekiguchi *et al.* (3) were unable to detect differences in the dissolution rate of the eutectic mixture and the pure drug. The experimental discrepancies between the former study and the present work may be ascribed to differences in the method of determining dissolution rate. The method employed by Sekiguchi and coworkers involved a higher degree of agitation than employed in this investigation. The use of high shear in *in vitro* dissolution investigations tends to obviate differences arising from microenvironmental factors which would be significant *in vivo*. Three individual (or possibly concerted) factors are involved in the dissolution of chloramphenicol from the eutectic. These include local solubilization, particle size reduction, and the presence of a significant amount of the rapidly soluble α solid solution.

The importance of the microenvironmental effect of urea on the dissolution of chloramphenicol may be appreciated by considering the dissolution rate of the physically mixed chloramphenicol–urea sample. As noted in Table II, the relative dissolution rate of the drug from sample *G* is almost twice as rapid as from the pure chloramphenicol. The solubility of chloramphenicol is significantly higher in the microenvironment (which approximates a saturated solution of urea) than in the bulk and the drug dissolves rapidly.

The significance of solid solution formation in the enhancement of dissolution can be realized by comparing the results obtained from samples *F* and *G*. Both samples are identical with respect to composition but differ in that sample *F* is a fused mixture and is actually composed of a homogeneous solid solution of chloramphenicol in urea. One would anticipate that the local effect of urea would be approximately the same in both samples or perhaps somewhat lower in the fused sample where urea exists in a more hydrophobic solid environment and conceivably dissolves at a slower rate. Despite this seeming equality, the initial dissolution rate of chloramphenicol from the α solid solution is more than twice that of the physical mixture and almost 4 times greater than the rate of solution of the pure drug. These differences may only be ascribed to the physical state of the chloramphenicol in the fused sample.

In a previous paper (4), Goldberg *et al.* theorized that the β solid solution of urea in chloramphenicol may demonstrate a strong crystal lattice. The results of dissolution studies conducted with the β solid solution indicate that despite the presence of a significant quantity of urea in the sample the solution rate is approximately equal to that of the pure drug. This is rather surprising in that the mere presence of a material as soluble as urea in the crystal would tend to increase the wetting of the particle and in this manner alone increase effective surface area and thereby increase dissolution rate. The inability to demonstrate this effect may perhaps be attributable to the formation of a crystal lattice in which the chloramphenicol is bound at least as tightly as in the pure crystal.

Comparison of the results obtained with samples *B*, *C*, *D*, *E*, and *F* reveals an interesting relationship. These samples range in α solid solution content from 0% (pure β solid solution) to 100% (pure α solid solution). The rate of dissolution of chloramphenicol from these mixtures was found to be a direct function of the α solid solution content of the sample. With an increase in the per cent α solid solution in the sample there was a corresponding increase in the rate of solution of the drug.

The findings of these investigations once again point out the potential importance and biopharmaceutical significance of solid state molecular dispersions in the enhancement of dissolution rate.

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Dermatitic Effect of Nonionic Surfactants I

Gross, Microscopic, and Metabolic Changes in Rabbit Skin Treated with Nonionic Surface-Active Agents

By M. MEZEI, R. W. SAGER, W. D. STEWART*, and A. L. DERUYTER†

Nonionic surface-active agents were applied to rabbit skin daily in an attempt to determine the physiological properties and irritative potential of some selected surfactants. Three methods of evaluation were used: gross examination, microscopical examination, and measurement of the respiratory metabolic activity of the treated skin. The gross and microscopical evaluations of these tests both indicated that the greatest irritation was produced by the polyoxyethylene ethers. These substances produced gross inflammation in even short periods of time (3 days) in the weakest dilutions (1 per cent) in which they were tested. Polysorbates caused more irritation than sorbitans. The metabolic measurements indicated a two, three, and fourfold increase in the oxygen consumption of the inflamed, treated skin sample, depending on the length of the treatment and the type of agent used.

IN THE PAST 20 years there has been a considerable increase in the utilization of synthetic surfactants as household and industrial cleansing products, and as solubilizers and emulsifiers in both pharmaceutical and cosmetic preparations. Consequently, because of this frequency of contact among the general population, the effects on the human skin of these surfactants become of extreme importance.

At present a literature survey presents a wide variety of results. In 1963, Treon (1) conducted standard patch tests with a large number of nonionic surfactants, including those that were used in the present experiment. He reported no irritation with human skin and only mild irritation on rabbit skin in the cases of sorbitan trioleate,[1] polysorbate 80,[2] and polyoxyethylene esters 52, 56, and 72.[3] The other nonionic surfactants caused no irritation in rabbit skin. The same year Choman (2) published observations on the effects of aqueous nonionic surfactants on excised calf and human skin, in which he indicated that irritation or cellular structural alteration did not occur.

Contrary to these reports there are several studies claiming carcinogenic effects of some of the same nonionic surfactants in *in vivo* mouse skin experiments (3–9).

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[1] Marketed as Span 85 by Atlas Chemical Industries, Inc., Wilmington, Del.
[2] Marketed as Tween 80 by Atlas Chemical Industries, Inc., Wilmington, Del.
[3] Marketed as Brij 52, 56, and 72 by Atlas Chemical Industries, Inc., Wilmington, Del.

This presentation will consider the gross anatomic, histological, and metabolic changes of rabbit skin caused by the daily application of selected nonionic surface-active agents.

EXPERIMENTAL

Three main groups of nonionic surface-active agents were selected for this study: (*a*) partial esters of sorbitan fatty acids, sorbitan series; (*b*) partial esters of polyoxyethylene sorbitan fatty acids, polysorbate series; and (*c*) polyoxyethylene ethers.[3]

A detailed list of these surfactants is in Table I. These surfactants were used both in undiluted and in diluted forms, using distilled water, hydrophilic

TABLE I.—NAME AND CHEMICAL COMPOSITION OF SURFACTANTS USED

Partial Esters of Sorbitan Fatty Acids (Sorbitan Series)
Sorbitan monolaurate
Sorbitan monostearate
Sorbitan monooleate
Sorbitan trioleate
Partial Esters of Polyoxyethylene Sorbitan Fatty Acids (Polysorbate)
Polyoxyethylene (20) sorbitan monolaurate (Polysorbate 20)
Polyoxyethylene (20) sorbitan monostearate (Polysorbate 60)
Polyoxyethylene (20) sorbitan monooleate (Polysorbate 80)
Polyoxyethylene (20) sorbitan trioleate (Polysorbate 85)
Polyoxyethylene Ethers
Polyoxyethylene (4) lauryl ether
Polyoxyethylene (2) cetyl ether
Polyoxyethylene (10) cetyl ether
Polyoxyethylene (2) stearyl ether
Polyoxyethylene (2) oleyl ether
Polyoxyethylene (10) oleyl ether[a]

[a] Marketed as Brij 96 by Atlas Chemical Industries, Inc., Wilmington, Del.

TABLE II.—GROSS OBSERVATIONS AFTER 3 DAYS OF APPLICATION[a]

Substance	60%				10%				5%				1%
	100%	in Water	in H.O.[b]	in H.P.[c]	in Water	in H.O.	in H.P.	in Pet.[d]	in Water	in H.O.	in H.P.	in Pet.	in Pet.
Sorbitan monolaurate	±			+			0	+					0
Sorbitan monostearate				0			0	0					0
Sorbitan monooleate	+			0			0	0					
Sorbitan trioleate	+							+					+
Polysorbate 20	+				0	+		+					+
Polysorbate 60	+				+	+		+		+			+
Polysorbate 80	+				+	+			0	+			
Polysorbate 85	+				+	+		±		+			+
Polyoxyethylene ether 30	+++	++	+++		+	++		++					+
Polyoxyethylene ether 52	++	++		++	+			++			+	+	+
Polyoxyethylene ether 56	++	++	++		+	+		++	+	+		+	+
Polyoxyethylene ether 72		±			+		++	++	+		++		+
Polyoxyethylene ether 92	+++	++	+++					++					±
Polyoxyethylene ether 96								++		±		±	±
H.O.	0												
H.P.	0												
Pet.	0												

[a] 0, no visible change; +, erythema, edema; ++, thickening; +++, hyperkeratinization; ++++, formation of fissures and open lesions; ± to ++++, underline signifies more intense irritation. [b] H.O., hydrophilic ointment U.S.P. [c] H.P., hydrophilic petrolatum U.S.P. [d] Pet., petrolatum U.S.P.

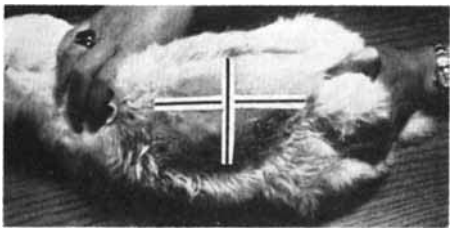

Fig. 1.—There is erythema and thickening of the lower left quadrant of the shaved area (polyoxyethylene ether 56, 5% in hydrophilic ointment) and crusting with induration of the lower right quadrant (polyoxyethylene ether 30, 10% in hydrophilic ointment). The upper right quadrant is the control area. One week of application.

ointment U.S.P., hydrophilic petrolatum U.S.P., and white petrolatum U.S.P. as diluents. The diluents were also applied alone as controls.

Increasing concentrations from 1–100% of the above surfactants were prepared and applied to the trunks of New Zealand white rabbits. Each trunk, clipped free of hair by an electric clipper (Oster model A2), provided 8 test areas. On each rabbit, one area was untreated and one area was treated with only an ointment base, to act as control sites. Fifty rabbits were used in this investigation, providing 400 separate areas. About 0.3 Gm. of each different preparation was evenly applied and then gently rubbed in for 3 sec. with a hard rubber spatula to the appropriate area, once a day. For the evaluation of histological changes biopsy specimens were taken from representative sites after application for 10 and 30 days and at the completion of the experiment. After each biopsy, that area was discontinued for use as an experimental site. With forceps, the skin was elevated, and a full thickness biopsy was taken by scissors. The specimens were kept in a 10% formol–saline solution until routine histological slides were prepared, stained with hematoxylin and eosin, and examined microscopically.

The oxygen consumptions of the treated and control skin samples were determined by the direct Warburg method (10). The animals were killed by fracturing the neck. The test areas were washed with water quickly to remove the remainder of the substances previously applied, and the samples were taken with the aid of the Castroviejo keratotome (11) set to cut 0.2 mm. thickness of skin. The skin slices were cut to small pieces with cold scissors and were immediately weighed on an analytical balance (referred to as wet weight), then transferred to Warburg flasks containing 3.0 ml. of Kreb's Ringer phosphate–glucose solution (10) and 0.2 ml. of 20% KOH solution in the center well. The measurement was carried out at 37°, after a 30-min. equilibration period. Room air was used as the gas phase. At the end of the measurement the samples were removed from the flasks, rinsed in distilled water, and placed in preweighed crucibles. Dry weights of the samples were obtained by drying overnight in an oven at 105°. The oxygen consumption was calculated in microliters per milligram of skin (dry) per hour: this value is the QO_2.

RESULTS AND DISCUSSION

The samples were evaluated by three methods: (*a*) direct inspection, (*b*) histological examination, and (*c*) measurement of the respiratory metabolic activity of the treated skin.

Gross observations were made and recorded daily, but representative tables are made up for intervals of 3 days and 10 days after starting applications. After 3 days of application, the first gross changes, namely erythema and edema, resulting in a hard induration began to appear where certain of the polyoxyethylene ethers were being applied, even in a dilution of 10%. Irritation was also observed, but to a lesser degree (Table II), where 100% concentrations of polysorbates were applied. At the sites of application of 10% concentrations of polysorbates, only a slight erythema was noticeable. With the exception of sorbitan monolaurate[4] and sorbitan trioleate, no changes were seen at the sorbitan areas. As the treatment continued, a de-

[4] Marketed as Span 20 by Atlas Chemical Industries, Inc., Wilmington, Del.

TABLE III.—GROSS OBSERVATIONS AFTER 10 DAYS OF APPLICATION[a]

Substance	100%	60%			10%			5%			1%
	in Water	in H.O.	in H.P.	in Water	in H.O.	in H.P.	in Pet.	in Water	in H.O.	in H.P.	in Pet.	in Pet
Sorbitan monolaurate	+		++			+++	+|+|+|+|+|+|+|+					++++++++
Sorbitan monostearate			+++									
Sorbitan monooleate	++											
Sorbitan trioleate	+++											
Polysorbate 20	++++			+++	++							
Polysorbate 60	++++			+++	+++							
Polysorbate 80	++++			+++	+++			0	+++			
Polysorbate 85	+++	++		+++	+++		++					
Polyoxyethylene ether 30	+|+|+	++		++	+		++					
Polyoxyethylene ether 52	+|+|+	++	+	+++	++		+++	+++		++	+|+	+++
Polyoxyethylene ether 56	+|+|+	++	+	+++	++		+++	+++		++	+|+	+++
Polyoxyethylene ether 72	+|+		+	++		+	++			+	+	+
Polyoxyethylene ether 92	+|+		+	++		+	++			+	+	++
Polyoxyethylene ether 96	+|+		+	++		+	++			+	+	++
H.O.	0								+++			
H.P.	+											
Pet.	+											

[a] See footnotes under Table II.

Fig. 2.—Control skin, plain hydrophilic ointment application only (−).

gree of irritation became apparent in most treated areas. The skin treated with polyoxyethylene ethers of all concentrations showed scaling and thickening, with marked induration and fissuring. A type of crust formation, shown microscopically to consist of necrotic and sloughing epidermis, often developed after 1 week of treatment with various concentrations of polyoxyethylene ether, (as illustrated by Fig. 1). Area 1 (polyoxyethylene ether 56, 5%) and area 3 (polyoxyethylene ether 30,[5] 10%) showed considerable irritation. Area 4, the untreated skin, showed no sign of irritation. The inflamed necrotic and irreversibly damaged epidermis sloughed off after 2 or 3 weeks of polyoxyethylene ethers application, but reepithelialization of the surface followed rapidly.

Table II illustrates the gross evaluations after 3 days of treatment. It is evident from this table that the surfactants belonging to the polyoxyethylene ether series produced irritation even after 3 days of application, and in a dilution of 1%. Table III records observations made after 10 days of application, where even the areas treated with surfactants belonging to the sorbitan series begin to show some irritation. An interesting observation was the increased rapidity of hair growth, chiefly in areas of application of the polysorbates. New skin was formed satisfactorily from remaining epidermal appendages in almost all the areas which had sloughed off after treatment with the polyoxyethylene ether-type of surfactants.

The microscopic appearances in the treated areas paralleled the degree of gross irritation observed. Figures 2, 3, 4, and 5 illustrate the degrees of inflammation and irritation after 10 days of application. The microscopic changes which were observed are illustrated by Tables IV and V.

The respiratory metabolic activity of selected samples of surfactant-treated skin was studied using oxygen consumption measured by the Warburg method. It has been reported (12–14) that in *in vitro* tests the surfactants influence enzymatic reactions, damaging the function of the respiratory chain. The authors found that the surfactant-treated skin consumed 2, 3, or 4 times as much oxy-

[5] Marketed as Brij 30 by Atlas Chemical Industries, Inc., Wilmington, Del.

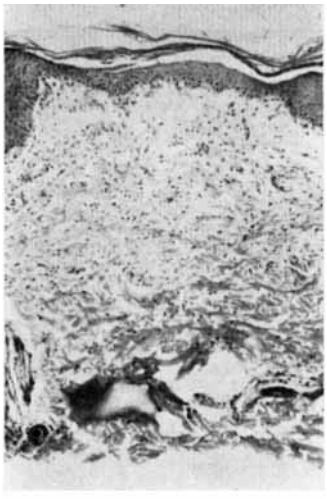

Fig. 3.—Polysorbate 80 in 100% concentration; there is irregular acanthosis of the epidermis and edema and inflammation in the dermis (2+).

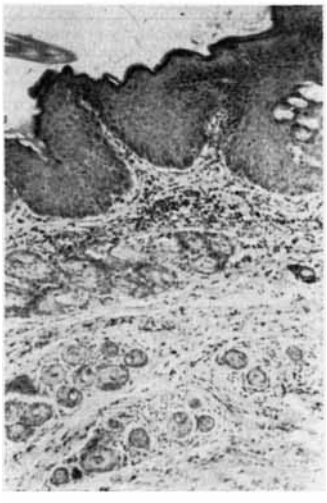

Fig. 4.—Polyoxyethylene ether 52 in 5% concentration. There is acanthosis and hyperplasia of the epidermis, together with some edema and inflammation of the dermis (3+).

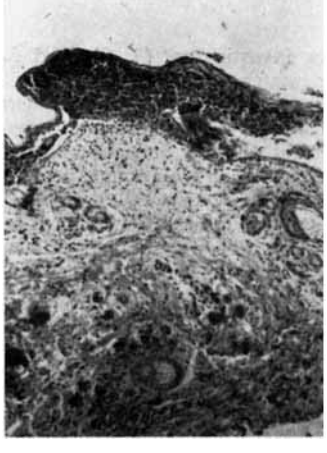

Fig. 5.—Polyoxyethylene ether 30 in 60% concentration; the epidermis is destroyed. It is replaced by eosinophilic staining material filled with many polymorphonuclear leucocytes. These cells extend to the dermis and in some instances dip down into the sebaceous glands and hair follicles. There are scattered polymorphonuclear leucocytes and indication of edema in the dermis (4+).

gen as the control skin, depending on the length of time of the treatment and on the type of agent used. The results are illustrated by Figs. 6 and 7.

Figure 6 represents the QO_2 values of the control and treated skin samples obtained from 13 rabbits at a period of 3- to 15 days (1 rabbit a day) after starting application. Figure 7 illustrates the QO_2 values of skin samples taken from the second group of rabbits, 15 in number, within the period of 30–81 days after starting application. During this period the samples were taken 30, 31, 40, 42, 43, 44, 45, 50, 51, 52, 55, 58, 77, 78, and 81 days after starting the application.

A specific explanation for the increased respiration rate due to the topical application of surfactants has not yet been found. The surfactants can directly influence enzymatic reactions as reported (12–14). On the other hand, the permeability changes of cell membranes induced by the surfactants could also be the source of the altered respiratory activity. The effect of these surfactants on the cell membranes, and in a smaller scale, on the membrane of the mitochondrion is very probable, due to their hydrophilic and lipophilic properties. By these properties they might be able to disturb the balance of the cell system, *e.g.*, they can weaken the hydrophobic bond, which is a key to the structure and function of the membrane of the mitochondrion, consequently causing alteration in the oxidative metabolism.

One of the difficulties in measuring skin respiration is to provide samples containing mainly the epidermis and relatively small amounts of dermis. The oxygen consumption of the epidermis, which is ordinarily actively proliferating, is significantly higher than that of the dermis. Usually, skin respiratory results refer to milligrams of dry skin or milligrams of tissue nitrogen. While the dry weight is a rather poor standard, due to the possible variations in amounts of epidermis, dermis, or physiologically inert constituents present, no other standard has been shown to be superior. Tissue nitrogen varies directly with the dry weight and is, in addition, more time consuming. With the aid of the Castroviejo keratotome samples were obtained containing relatively uniform structural components of the skin. The definite pattern in the authors' results, and the reproducibility of repeated measurements during these investigations, indicate that with this technique, the dry weight (imperfect as it may be) is the most practicable standard of reference.

A number of rabbits, of which the gross and histological skin-change data were presented above, were being treated with surfactants for several months in a long-term study to ascertain whether any carcinogenic effect can be observed arising from application of the above surfactants, as has been reported in mouse skin experiments (3–9). The authors found no gross or microscopic signs of carcinogenic effect of these agents in 3 rabbits which were treated with sorbitans and polysorbates for a period of 5 months and of 22 rabbits which were treated for more than 2 months.

The authors' results are in conflict with previous reports regarding the irritative potential of these substances.

In these reports (1, 2), no irritation was reported caused by sorbitan monolaurate, sorbitan mono-

TABLE IV.—MICROSCOPIC OBSERVATIONS AFTER 10 DAYS OF APPLICATION

		60%		10%		5%	
	100%	in Water	in Ointment Base	in Water	in Ointment Base	in Water	in Ointment Base
Sorbitan monolaurate	+[a]				0		0
Sorbitan monostearate							
Sorbitan monooleate	+				0		0
Sorbitan trioleate	+						
Polysorbate 20	+			+	+	0	
Polysorbate 60	+			+	+	+	
Polysorbate 80	++			+	+	+	
Polysorbate 85	++			+	+	+	
Polyoxyethylene ether 30	++++ N[b]	++++ N	++++ N	++	++ H[c]		
Polyoxyethylene ether 52		+++ N		++			+++ H
Polyoxyethylene ether 56		+++ N		+++	++ H	++	++
Polyoxyethylene ether 72		+++		++	++	++	++ H
Polyoxyethylene ether 92	++++ N	++++ N	++++ N				
H.O.	0						
H.P.	0						
Untreated skin	0						

[a] + to ++++, inflammation, from minimally increased numbers of inflammatory cells in the dermis, chiefly perivascular to marked degrees of polymorphonuclear and round cell infiltrate throughout the depth of the dermis. [b] Necrosis, a complete destruction of the normal cellular structure of the epidermis, replaced by an eosinophilic amorphous mass thoroughly infiltrated by polymorphonuclear leucocytes. This process involved hair follicular epidermis and sebaceous glands in severe cases. It was followed in all instances, when the rabbit lived, by re-epithelialization from the remaining appendages. [c] Acanthosis, an irregular acanthosis of the normally flat, thin, and regular epidermis.

TABLE V.—MICROSCOPIC OBSERVATION AFTER 1 MONTH OF APPLICATION[a]

		60%		10%		5%	
	100%	in Water	in Ointment Base	in Water	in Ointment Base	in Water	in Ointment Base
Sorbitan monolaurate	+				+		+
Sorbitan monostearate							
Sorbitan monooleate	+++				+		
Sorbitan trioleate	++ H						
Polysorbate 20	++			+	+		
Polysorbate 60	++ H			++++ N		++	
Polysorbate 80	+++ N H			+++	++	++	
Polysorbate 85	+++ H					++	
Polyoxyethylene ether 30	++++ N H	++++ N	+++ N		++		
Polyoxyethylene ether 52		+++					++
Polyoxyethylene ether 56	++ N	++ N		+++ H	+++ H	+++	+++ H
Polyoxyethylene ether 72	+++	+++ H			++ H	++	++
Polyoxyethylene ether 92	++++ N H	+++ H	++++ N H				
H.O.	+						
H.P.	+						
Untreated skin	0						

[a] See footnotes under Table IV.

stearate,[6] sorbitan monooleate,[7] polysorbate 20,[8] polysorbate 60,[9] polysorbate 80; and polyoxyethylene ethers 30 and 92;[10] and only mild irritation caused by sorbitan trioleate, polysorbate 85,[11] and polyoxyethylene ethers 52 and 72. The difference in the degree of irritation, which in our case was extreme, and in some instances, resulted in complete destruction of the epidermis, may be due to the different methods by which the surfactants were applied. The above investigators (1, 2) used *in vitro* tests and/or patch tests. The authors' method was an attempt to reproduce the frequent application of dermatologic and of cosmetic preparations by the general public. It is not always advisable to apply the results of animal experiments to humans and the fact that these evidences of irritation have been produced in rabbits must be kept in mind in assessing their significance.

The closed patch test applied for 48 hr. is less desirable as a measure of capacity of a primary irritant than a daily application of the test material for a period of at least 10 days.

Studies have not proved that synthetic detergents are entirely responsible for the reported increase in primary irritant dermatitis and "housewife's dermatitis" cases (15–18). Evidence has been presented that surface-active agents cause denaturation of keratin (19) and produce harmful

[6] Marketed as Span 60 by Atlas Chemical Industries, Inc., Wilmington, Del.
[7] Marketed as Span 80 by Atlas Chemical Industries, Inc., Wilmington, Del.
[8] Marketed as Tween 20 by Atlas Chemical Industries, Inc., Wilmington, Del.
[9] Marketed as Tween 60 by Atlas Chemical Industries, Inc., Wilmington, Del.
[10] Marketed as Brij 92 by Atlas Chemical Industries, Inc., Wilmington, Del.
[11] Marketed as Tween 85 by Atlas Chemical Industries, Inc., Wilmington, Del.

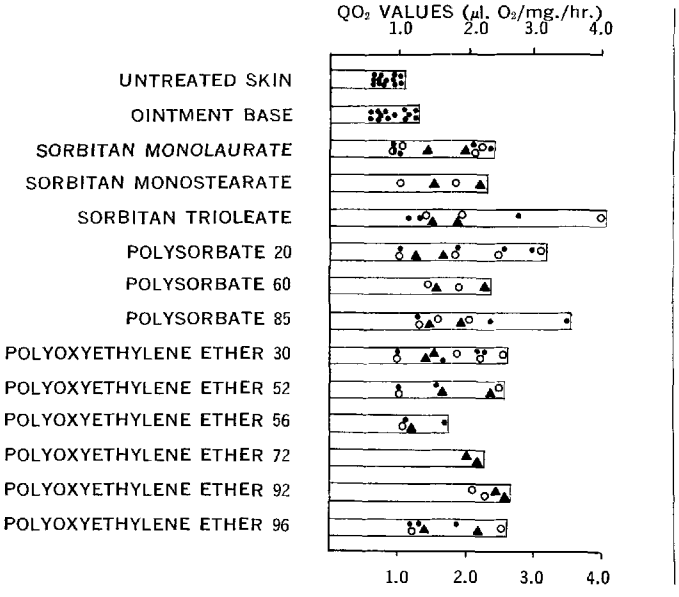

Fig. 6.—The oxygen consumption of control and treated skin samples taken from 3–13 days after starting application. Key: ●, control, ointment base alone, or 100% surfactant; ○, 10% surfactant in ointment base; ▲, 1% surfactant in ointment base.

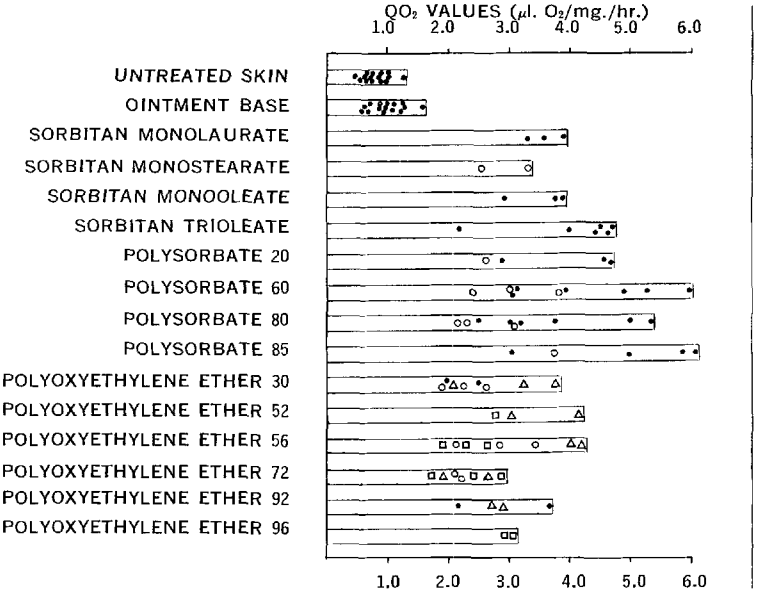

Fig. 7.—The oxygen consumption of control and treated skin samples taken from 30–81 days after starting application. Key: ●, control, ointment base alone, or 100% surfactant; Δ, 60% surfactant in ointment base; ○, 10% surfactant in ointment base; □, 5% surfactant in ointment base.

effects on the horny layer as defatting agents by removal of the lipids and other substances (20–23). The surface-active agents are usually present in cosmetic and dermatological preparations in concentrations varying from 1–10%. Considering the above studies (15–23) and these results, the possibility arises that, if the increased number of cases of hand dermatitis in females cannot be explained by the increased use of synthetic household detergents then the surfactants present in hand lotions and other cosmetic or dermatological preparations could possibly be one factor in some of the cases of hand dermatitis in the female population. There are, as yet, only animal tests to prove that a number of polyoxyethylene ether surfactants, a common component of hand lotions, have a distinct potential to irritate the skin. Certain other surfactants, namely the polysorbates and sorbitans, also have undesirable influences on the skin, if used in daily applications for a longer period.

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Until 1918, all species of deadly amanitas occurring in the U. S. were generally referred to *A. phalloides*, which was considered to represent a single polymorphic species. For example, Murrill (1), referring to *A. phalloides* in 1916, wrote of "The variety of colors assumed by this species—white, yellow, green, gray, brown, blackish . . ." In 1918, Atkinson (2) recognized that the most common *Amanita* species in the eastern U. S., usually interpreted as a dark brown form of *A. phalloides*, was actually a different species. He subsequently described it and assigned the name *Amanita brunnescens* Atk. The various color forms were gradually sorted out with the passing years, the white forms being identified as *Amanita verna* (Fr.) Vitt. s. Boud., *Amanita virosa* Secr., or *Amanita bisporigera* Atk., the yellow or green as *Amanita citrina* S. F. Gray, and the blackish or gray as *Amanita porphyria* (Fr.) Secr. Finally, none remained which could actually be designated *A. phalloides*.

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Fig. 1.—*Amanita phalloides* carpophore collected in Seattle, Wash.

produced by amanita toxins was reported from California in 1965 (8). The species responsible was identified as *A. phalloides*, but botanical details were not presented. In October 1965, Mrs. Betty Dorland collected specimens on Mercer Island, Seattle, Wash., which were identical to the 1959 collection from Oregon. The carpophores (Fig. 1), found growing in sandy grass-covered soil under old birch trees, were identified as *A. phalloides*[1] (Table I).

This discovery of specimens of deadly amanitas in the Pacific Northwest prompted the present study with its dual objectives: (*a*) to determine if the specimens of *A. phalloides* collected in the U. S. were similar in their toxin content to specimens of European origin and (*b*) to evaluate the quality and quantity of toxins in other American collections of deadly amanitas.

EXPERIMENTAL

Analysis of Washington A. phalloides.—Dried carpophore tissue (550 mg.) was exhaustively extracted with methanol in a Soxhlet apparatus and the resulting extract (*A*) concentrated to a volume of 2.0 ml. To insure complete extraction, the dried marc was subsequently re-extracted with 70% ethanol, and this extract (*B*) was evaporated to dryness, and the residue redissolved in 1.0 ml. of 70% ethanol.

Quantities of these extracts ranging from 1–10 μl. were spotted on thin-layer plates prepared with Silica Gel G and were chromatographed in a solvent mixture composed of methanol–methyl ethyl ketone (1:1), as described by Sullivan *et al.* (9). For reference, α- and β-amanitin[2] were chromatographed concurrently and in admixture with the extracts. Spraying the chromatograms with 1% cinnamaldehyde in methanol followed by exposure to hydrochloric acid vapor revealed the amanita toxins as violet-colored spots. As little as 0.3 mcg. of pure α- or β-amanitin could be detected with certainty by this procedure. The results, including average R_f values and approximate quantities determined by visual comparison of the size and intensity of the spots with those of the reference standards, are summarized in Table II.

Quantities (< 20 μl.) of both extracts *A* and *B* and of the reference compounds were also chromatographed on Silica Gel G plates with *n*-butanol–acetic acid–water (4:1:1) as described by Benedict *et al.* (10) and on Whatman No. 1 and No. 3 filter papers with methyl ethyl ketone–acetone–water–*n*-butanol (20:6:5:1) as recommended by Block *et al.* (3). In all of these systems extract *A* yielded relatively large amounts of β-amanitin; extract *B* contained a very small amount of β-amanitin and a trace only of α-amanitin.

Analysis of Other Deadly Amanitas.—Additional *Amanita* species which have been suspected of containing amanita toxins include *A. bisporigera*, *A. tenuifolia* Murr., *A. verna*, and *A. virosa* (11). Of these, samples of *A. tenuifolia* and *A. verna* were examined chromatographically by Block *et al.* (3); the former was found to contain only β-amanitin but the latter both α- and β-amanitin. However, several specimens identified as *A. verna* contained no amanita toxins.

For their studies the authors were able to obtain several samples of *A. bisporigera*,[3] *A. phalloides* (European origin), *A. phalloides* (Oregon origin), *A. verna*,[3] and *A. virosa*. Details of these collections are presented in Table I. One-hundred-milligram samples of *A. bisporigera* and 500-mg. samples of *A. phalloides*, *A. verna*, and *A. virosa* were extracted with methanol in a Soxhlet apparatus and quantities of the concentrated extracts were spotted and chromatographed in the methanol–methyl ethyl ketone system as previously described. The amanitins were detected with cinnamaldehyde spray and verified, in doubtful cases, with Pauly's reagent (12). R_f values and estimated quantities of toxins detected are described in Table II.

RESULTS AND DISCUSSION

Chromatographic examination of extracts of the specimen of *Amanita* collected in the fall of 1965 in Seattle, Wash., revealed the presence of amanita toxins. The results were similar but not identical to those obtained by analysis of a similar specimen collected in Oregon in 1959 (6). β-Amanitin predominated in both samples, but only traces of α-amanitin could be detected with difficulty in the Seattle specimen following a second extraction of the carpophore with aqueous ethanol. A somewhat larger amount of α-amanitin was present in the Oregon mushroom. Although the total concentrations of these two amanitins (1.9–2.43 mg./Gm.)

[1] The authors are indebted to Prof. D. E. Stuntz, Department of Botany, University of Washington, Seattle, for authoritative identification of all Pacific Northwest and Texas specimens utilized in this investigation.

[2] Supplied through the courtesy of Prof. Dr. Theodor Wieland, Institut für Organische Chemie, der Universität Frankfurt am Main, Frankfurt am Main, West Germany.

[3] Two collections of *A. bisporigera* (No. 27518 and 27653) and four of the collections of *A. verna* (No. 5362, 22065, 27055, 25698) were supplied through the courtesy of Dr. L. R. Hesler, University of Tennessee, Knoxville.

TABLE I.—TIME AND PLACE OF DEADLY *Amanita* COLLECTIONS

Collection No.	Species	Collection Site	Date
27518	*A. bisporigera* Atk.	Toxaway Gorge, N. C.	7/29/1961
B2	*A. bisporigera*	Cades Cove, Tenn.	7/27/1963
27653	*A. bisporigera*	Gatlinburg, Tenn.	8/6/1963
E1	*A. phalloides* (Fr.) Secr.	Harz Mountains, Germany	9/11/1963
O1	*A. phalloides*	Ashland, Ore.	Fall 1959
W1	*A. phalloides*	Seattle, Wash.	10/24/1965
5362	*A. verna* (Fr.) Vitt. s. Boud.	Knoxville, Tenn.	8/19/1934
22065	*A. verna*	Highlands, N. C.	8/24/1955
27055	*A. verna*	Bergen, N. Y.	10/11/1962
25698	*A. verna*	Cades Cove, Tenn.	7/27/1963
B1	*A. verna*	Fall City Falls, Tenn.	8/3/1963
Ta	*A. verna*	Huntsville State Park, Tex.	11/8/1964
Tb	*A. verna*	Huntsville State Park, Tex.	11/8/1964
T2a	*A. verna*	Huntsville State Park, Tex.	11/14/1965
T2b	*A. verna*	Huntsville State Park, Tex.	11/14/1965
T2c	*A. verna*	Huntsville State Park, Tex.	11/14/1965
T1a	*A. virosa* Secr.	Huntsville State Park, Tex.	11/8/1964
T1b	*A. virosa*	Huntsville State Park, Tex.	11/8/1964
T4	*A. virosa*	Huntsville State Park, Tex.	11/14/1965

TABLE II.—R_f VALUES AND ESTIMATED CONCENTRATIONS OF AMANITA TOXINS DETECTED IN DEADLY *Amanita* SPECIES

Species Investigated	Av. R_f Values (MEK:MeOH)		Estimated Quantity, mg./Gm.	
	α-Amanitin	β-Amanitin	α-Amanitin	β-Amanitin
A. bisporigera No. 27518	0.45	0.21	1.75	0.5
A. bisporigera No. B2	0.47	0.24	3.5	1.5
A. bisporigera No. 27653	0.46	0.23	2.63	0.75
A. phalloides No. E1	0.47	0.24	1.2	0.8
A. phalloides No. O1	0.44	0.21	0.93	1.5
A. phalloides No. W1				
Ext. A	...	0.24	} tr.	1.9
Ext. B	0.47	0.23		
A. verna No. 5362	...	...	0	0
A. verna No. 22065	0.45	0.22	0.28	tr.
A. verna No. 27055	0.46	...	<0.1	0
A. verna No. 25698	0.45	...	<0.1	0
A. verna No. B1	...	...	0	0
A. verna No. Ta	0.46	0.22	1.4	0.3
A. verna No. Tb	0.46	0.23	0.7	0.3
A. verna No. T2a	0.47	0.24	0.58	0.25
A. verna No. T2b	0.46	0.22	0.72	tr.
A. verna No. T2c	0.47	0.23	1.4	0.3
A. virosa No. T1a	...	...	0	0
A. virosa No. T1b	0.44	...	<0.1	0
A. virosa No. T4	0.45	...	<0.1	0
Reference amanitins	0.46	0.23		

approximated that detected in a typical European collection of *A. phalloides* (2.0 mg./Gm.), the unusually high proportions of β- to α-amanitin (1.9/trace, 1.5/0.93) are quite different from that detected in European specimens (about 2/3).

However, since morphological differences between the Pacific Northwest collections and European specimens of *A. phalloides* are essentially nonexistent, it appears most useful to describe the former as members of a chemical race of *A. phalloides* in which β-amanitin is the predominant amanitin. Until large numbers of single carpophores of the European species can be investigated for their content of both α- and β-amanitin, it is not possible to determine if this chemical race was transferred relatively recently to the Pacific coast together with the various introduced trees (*Betula, Populus*), under which it has been found and with which it probably forms mycorrhizal associations, or if it is a race originating in the Pacific Northwest and restricted there in its occurrence. In any event, the evidence indicates conclusively the existence of a chemical race of *A. phalloides* distinguished by a high proportion of β-amanitin, specimens of which are rare in Washington, Oregon, and presumably California.

It is apparent from the data in Table II that *A. bisporigera* is the most toxic American mushroom yet examined. Its total content of α- and β-amanitins was found to range from 2.25–5.0 mg./Gm. in contrast to 1.9–2.43 mg./Gm. for the native *A. phalloides*, 0–1.7 mg./Gm. for *A. verna*, and 0–<0.1 mg./Gm. for *A. virosa*. Singer (13) does not classify *A. bisporigera* in section 6, *Euamanita*, of subgenus II, *Euamanita*, together with other amanita toxin-containing species, but instead lists it under section 5, *Amidellae*. On the basis of its high concentration of α- and β-amanitin, as well as its botanical affinities to *A. virosa* (14), it would seem that the species should properly be classified in section 6, *Euamanita*.

The finding of variable concentrations of toxins in *A. verna* was not unexpected. Although the complete absence of toxins from collection No. 5362 may

be attributed to its excessive age and prolonged storage (31 years) preceding analysis, failure to detect toxins in the more recent collection No. B1 must be due to natural variablility. Block *et al.* (3) report similar results from their study of toxin-containing *Amanita* species in which several specimens identified as *A. verna* produced no toxic symptoms in mice nor could toxins be detected in them chromatographically. It is not clear whether the differences observed by Block *et al.* and by the authors are due to environmental conditions, ontogenetic considerations, or genetic factors, but it must be concluded that the amanita-toxin content of *A. verna* carpophores is extremely variable and, in general, appreciably less than that of *A. bisporigera* or *A. phalloides*.

No amanitins were detected in one sample of *A. virosa* (Tla), but another carpophore (Tlb) collected at the same time from the same site contained a very small amount of α-amanitin (<0.1 mg./Gm.), as did another, more recent collection (T4). Although standard reference works (15, 16) all refer to *A. virosa* as a deadly poisonous species, apparently the only previous experimental work on the subject was that of Ford (17). He found an extract to be toxic to guinea pigs and concluded that the toxins of the species were identical to those of *A. phalloides*. Identification of small amounts of α-amanitin in two carpophores from different collections confirms this earlier finding. However, as in the case of *A. verna*, the effects of environment, ontogenesis, and genetics must all be investigated before a definite explanation can be given for the irregular low-level occurrence of toxins in *A. virosa*.

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Kinetics and Mechanisms of Action of Antibiotics on Microorganisms V

Chloramphenicol and Tetracycline Affected *Escherichia coli* Generation Rates

By EDWARD R. GARRETT, GEORGE H. MILLER, and M. R. W. BROWN*

Total and viable count methods were used to study *Escherichia coli* generation rates at various temperatures and tetracycline and chloramphenicol concentrations. The negative dependence of apparent first-order generation rate constants on concentrations of these antibiotics was determined for all temperatures. The coincidence of the heat of activation for *E. coli* growth in antibiotic-free media and for the inhibitory rate constants was observed. The facile reversibility of antibiotic effects to predictable rates of *E. coli* generation in the subinhibitory concentration ranges of these antibiotics was demonstrated. A quantitative model consistent with the observed concentration dependencies and observed reversibilities is proposed which relates antibiotic partitioning from the media, a critical value for protein synthesis that results in microbial generation, and the present concept that these antibiotics compete for ribosomal binding sites and so inhibit the function of messenger RNA in protein synthesis.

THE CLINICAL importance of chloramphenicol and tetracycline has resulted in many investigations of their biochemical mode of action (1–4) and is generally ascribed to the inhibition of protein synthesis which has been principally observed under conditions of complete inhibition of growth. A more complete understanding of the action of these antibiotics could be realized if the kinetics of bacterial generation was more completely elucidated in antibiotic concentrations less than those that result in complete growth inhibition, *i.e.*, subinhibitory concentrations.

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Kinetics and Mechanisms of Action of Antibiotics on Microorganisms V

Chloramphenicol and Tetracycline Affected *Escherichia coli* Generation Rates

By EDWARD R. GARRETT, GEORGE H. MILLER, and M. R. W. BROWN*

Total and viable count methods were used to study *Escherichia coli* generation rates at various temperatures and tetracycline and chloramphenicol concentrations. The negative dependence of apparent first-order generation rate constants on concentrations of these antibiotics was determined for all temperatures. The coincidence of the heat of activation for *E. coli* growth in antibiotic-free media and for the inhibitory rate constants was observed. The facile reversibility of antibiotic effects to predictable rates of *E. coli* generation in the subinhibitory concentration ranges of these antibiotics was demonstrated. A quantitative model consistent with the observed concentration dependencies and observed reversibilities is proposed which relates antibiotic partitioning from the media, a critical value for protein synthesis that results in microbial generation, and the present concept that these antibiotics compete for ribosomal binding sites and so inhibit the function of messenger RNA in protein synthesis.

THE CLINICAL importance of chloramphenicol and tetracycline has resulted in many investigations of their biochemical mode of action (1–4) and is generally ascribed to the inhibition of protein synthesis which has been principally observed under conditions of complete inhibition of growth. A more complete understanding of the action of these antibiotics could be realized if the kinetics of bacterial generation was more completely elucidated in antibiotic concentrations less than those that result in complete growth inhibition, *i.e.*, subinhibitory concentrations.

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Previous reports from this laboratory (5–8) have established methods for the determination of population growth rates of *Escherichia coli* by viable and/or total cell count methods in the presence of these antibiotics either alone or in combination and have demonstrated the linear dependency of such growth rates upon antibiotic concentration. This was a general inhibition of growth rate at 37.5° rather than kill superimposed on an uninhibited growth at subinhibitory antibiotic concentrations. At higher antibiotic concentrations a definite organism kill occurred where kill was defined as a loss of the ability to produce colonies when a diluted sample was incubated on an agar plate.

Fassin *et al.* (9) have reported some work on the temperature dependency of chloramphenicol action using inhibitory and subinhibitory antibiotic concentrations. A more complete kinetic knowledge of the effect of temperature on the kinetics of antibiotic inhibited *E. coli* growth and the derived apparent heat of activation, ΔH, of inhibition are needed to gain insight into the energy requirements of such inhibition.

A lag period in *E. coli* growth after removal of the bacteria from a medium containing inhibitory concentrations of chloramphenicol has been indicated (10). Since the subinhibitory concentrations of chloramphenicol are thought to stimulate RNA synthesis more than higher concentrations (11, 12), a more detailed knowledge of the reversibility of the inhibition of population growth rates at these concentrations is necessary for the formulation of a kinetic mechanism of action.

EXPERIMENTAL

Organism.—All experiments were carried out with *E. coli* strain B/r, a strain that had been employed in previous experiments in this laboratory (5–8).

Medium.—Peptone broth (U.S.P. XVII) was used as the culture broth and combined with agar for colony counts.

Materials.—Assayed samples of tetracycline hydrochloride were supplied by courtesy of The Upjohn Co., Kalamazoo, Mich., and chloramphenicol by courtesy of Parke, Davis and Co., Detroit, Mich.

Temperature Studies.—A broth culture was allowed to grow for 12 hr. at 37.5°, diluted into fresh broth, and the growth rate determined by turbidimetric measurements with a Klett-Summerson colorimeter. When exponential growth had been established, sufficient inoculum to achieve a concentration of 10^6 *E. coli*/ml. was added to fresh broth to make 20-ml. replicate cultures. The cultures were maintained at either 25, 30, 31, 34, 35, 37, 37.5, 41, 43, or 45° on different days in constant-temperature water baths equipped with a shaker. Sufficient antibiotic was added after 90 min. to the cultures to achieve concentrations of 0.0, 1.04, 2.08, 3.12, or 4.16×10^{-7} *M* tetracycline or 1.17, 2.33, 3.50, or 4.66×10^{-6} *M* chloramphenicol.

The number of *E. coli*/ml., N, present in the cultures was determined by a viable (colony) count method and/or a total count method at appropriately spaced time intervals (5–8). For total counts, the Coulter counter (Coulter Electronics, Hialeah, Fla.) was used. Apparent specific growth rate constants, $k_{app.}$ in sec.$^{-1}$, were obtained from the least squares slopes of a plot of log N *versus* time in accordance with the apparent first-order expression,

$$\log N = (k_{app.}/2.303)t + \log N_0 \quad \text{(Eq. 1)}$$

where N_0 is the number of *E. coli*/ml. at $t = 0$, and t is in sec. Regression analysis was performed by an IBM 720 digital computer.

Reversibility Studies.—Three replicate culture flasks were inoculated with sufficient *E. coli* to yield initial concentrations of 10^6 *E. coli*/ml.

Sufficient antibiotic to achieve concentrations of 0.00 and 4.16×10^{-7} *M* tetracycline and 4.66×10^{-6} *M* chloramphenicol was added to the cultures after 90 min. of growth. After 200 min. of growth, a tenfold dilution of the cultures was achieved by adding 2 ml. of the cultures to 18 ml. of fresh broth. The number of *E. coli* present in the cultures was determined by total counts at approximately equally spaced time intervals.

Three replicate culture flasks were inoculated with sufficient *E. coli* to yield an initial concentration of 10^6 *E. coli*/ml. Sufficient antibiotic to achieve concentrations of 0.00 and 1.04×10^{-7} *M* tetracycline and 1.17×10^{-6} *M* chloramphenicol was added after 90 min. of growth. Final concentrations of 0.00 and 1.04×10^{-7} *M* tetracycline and 4.16×10^{-6} *M* chloramphenicol were achieved with the addition of more antibiotic after 150 min. of growth. The number of *E. coli* present in the cultures was determined at appropriately spaced time intervals by total counts.

Viable Count Method.—One milliliter of appropriately diluted (with 0.85% saline) 0.5-ml. samples of the culture was pipeted onto each of 5 replicate agar plates within 15 min. of sampling. Previous experiments (7) have shown the necessity of completing plating within this time interval.

Total Count Method.—One-half milliliter samples of culture were diluted with 0.86% saline which had been previously filtered through a double thickness of type HA Millipore filter paper. A drop of formaldehyde was added, and the total number of organisms was counted with a model A Coulter counter equipped with a 30-μ orifice. The conditions were: an aperture current of 5, a gain of 6, and a threshold setting of 10. When necessary, formaldehyde-inhibited culture aliquots were stored in the freezer for periods not longer than 4 hr. before counting. This procedure does not materially affect total counts (7).

RESULTS

Temperature Studies.—Apparent first-order generation rate constants as per Eq. 1 for the growth of *E. coli* in the presence of tetracycline and chloramphenicol at various temperatures are given in Table I. When the tetracycline concentration is

TABLE I.—APPARENT FIRST-ORDER RATE CONSTANTS (10^4 k IN sec.$^{-1}$) FOR *E. coli* GROWTH AT VARIOUS TEMPERATURES

	10^6 [Chloramphenicol]					10^7 [Tetracycline]			
° C.	0.00	1.17	2.33	3.50	4.66	1.04	2.08	3.12	4.16
25.0[a]	1.18	1.13	0.91	0.70	0.52	1.13	0.80	0.56	0.42
28.0[b]	1.74	1.69	1.31	0.90	0.63	1.78	1.18	0.88	0.63
30.0[a]	2.37	2.18	...	1.32	0.96	2.08	1.44	1.14	0.68
31.0[b]	2.39	2.18	1.57	1.12	0.80	2.17	1.48	1.09	0.74
34.0[b]	3.11	2.69	1.93	1.24	0.53	2.79	1.95	1.38	0.68
37.5[c]	4.88	3.62	2.60	1.46	0.64	3.95	3.01	2.24	1.58
39.0[a]	5.36[d]	4.10[d]	2.53	1.94	1.84	5.14[d]	2.99[d]	2.50[d]	...
41.0[b]	4.60	3.97	2.60	1.35	0.72	3.85	3.06[d]	...	2.01
41.0[a]	5.03	...	2.87	1.53	...	4.19	3.15[d]	3.10[d]	2.09[d]
43.0[b]	4.08	3.54	2.52[d]	1.40	0.62	3.87	3.04	2.49	1.96
43.0[a]	4.89[d]	4.23[d]	2.70[d]	1.55[d]	0.75[d]	4.43[d]	3.45[d]	3.04[d]	2.19[d]
45.0[b]	2.07	1.40	1.16	0.66	0.52	1.56	1.63	1.48	1.15
45.0[a]	2.19	1.56	1.16	0.89	0.47	1.80	1.95	1.57	0.88

[a] Derived from viable counts by colony counter. [b] Derived from total counts by Coulter counter. [c] Averaged from several total and viable studies. [d] The 95% confidence limits of these k values were ±0.06–0.10. All others were ±0.02–0.03.

below 4.16×10^{-7} M (0.2 mcg./ml.) or the chloramphenicol concentration is below 4.66×10^{-6} M (1.5 mcg./ml.), a net growth is observed; *i.e.*, $k > 0$. A coincidence of total and viable cell counts was observed during this reduced but exponential growth of the population in the presence of chloramphenicol. A typical example of such plots is given in Fig. 1. The mode of action at 37.5° has been shown to be an inhibition of population growth rate rather than kill superimposed on growth in similar experiments for tetracycline (7). Thus, the mode of action appears to be independent of the temperature range studied and similar for both chloramphenicol and tetracycline (Table I).

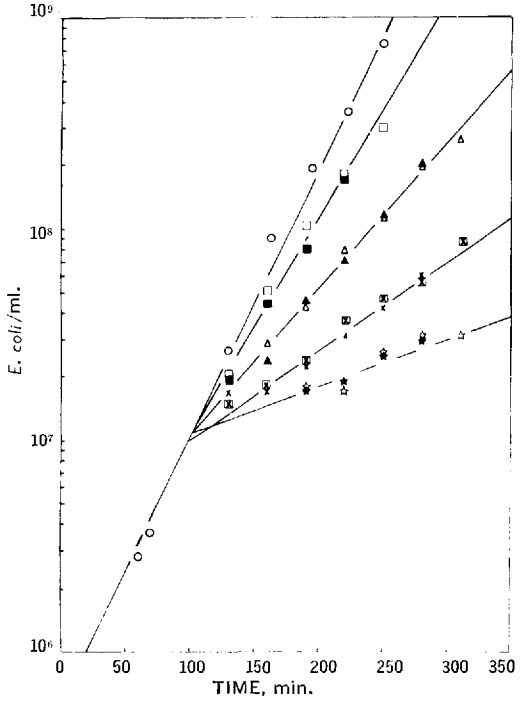

Fig. 1.—Example of coincidence of viable (open symbols) and total (solid symbols) counts for the logarithmic growth of *E. coli* in the presence of various concentrations of chloramphenicol at 41°. Key: ○, 0.00×10^{-6} M chloramphenicol; ■ and □, 1.17×10^{-6} M chloramphenicol; ▲ and △, 2.33×10^{-6} M chloramphenicol; × and ⊠, 3.50×10^{-6} M chloramphenicol; ★ and ☆, 4.66×10^{-6} M chloramphenicol.

It has been shown (5–8) that the specific growth rate constants obtained at 37.5° are linearly dependent upon the concentrations of tetracycline or chloramphenicol, A,

$$k_{app.} = k_0 - k_a A,\ k > 0 \qquad \text{(Eq. 2)}$$

where k_0 is the population growth rate constant in the absence of antibiotic. The inhibitory coefficient, k_a, may be obtained from plots of generation rate constants *versus* antibiotic concentration (Fig. 2). This linear relationship was found to hold over the entire temperature range studied, and the inhibitory coefficients are summarized in Table II.

The Arrhenius equation for the dependence of reaction rate constants upon temperature is

$$\log k = \log P - (\Delta H/2.303R)\ (1/T) \qquad \text{(Eq. 3)}$$

where R is 1.987 cal./mole and T is in degrees Kelvin. A typical Arrhenius plot for *E. coli* growth in the absence of antibiotics is shown in Fig. 3. The heat of activation is 20.8 ± 1.6 Kcal./mole for the linear portion. A similar temperature dependence is exhibited by the inhibitory coefficients, k_a, of Eq. 2. The heats of activation for k_a are 20.6 ± 1.5 Kcal./mole for tetracycline HCl and 23.7 ± 0.4 Kcal./

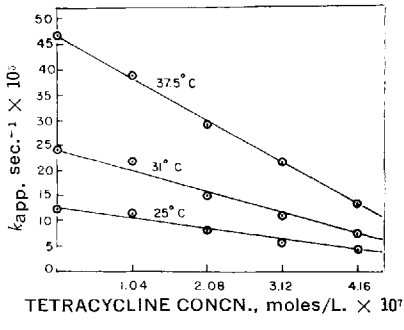

Fig. 2.—Typical example of the dependence of the apparent first-order generation rate constant for *E. coli* growth on antibiotic concentrations.

TABLE II.—APPARENT INHIBITORY CONSTANTS, k_a, FOR *E. coli* AT VARIOUS TEMPERATURES, k IN sec.$^{-1}$

° C.	25.0[a]	28.0[b]	30.0[a]	31.0[b]	34.0[b]	37.5[c]	41.0[b]	41.0[a]	43.0[b]	43.0[a]	45.0[b]	45.0[a]
Chloramphenicol	15	26	32	36	57	85	89	89	83	84	27	32
Tetracycline	180	280	330	390	550	790	660	660	600	640	200	310

[a] Derived from viable counts by colony counter. [b] Derived from total counts by Coulter counter. [c] Averaged from several total and viable studies.

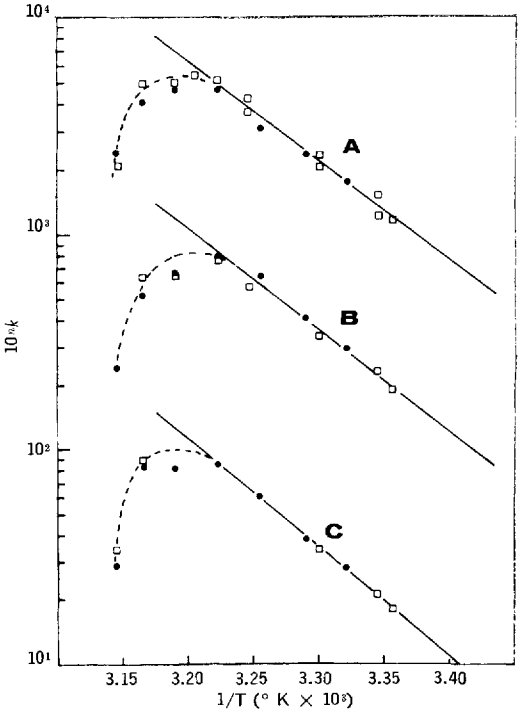

Fig. 3.—Arrhenius plots for the apparent first-order growth of *E. coli* and the inhibitory rate constants of such growth by tetracycline and chloramphenicol as determined from both total (solid symbols) and viable (open symbols) counts. Key: A, antibiotic free, $n = -8.0$; B, tetracycline inhibited, $n = 0$; C, chloramphenicol inhibited, $n = 0$.

mole for chloramphenicol. The coincidence of total and viable count methods are clearly demonstrated in the plots.

Reversibility.—Evidence that the inhibition of growth rates caused by these antibiotics is reversible in the subinhibitory range is shown in Fig. 4 for the chloramphenicol case. Cultures inhibited by the antibiotics, chloramphenicol and tetracycline, revert to population growth rates coincident with those found in the presence of very small concentrations of these antibiotics when they are diluted into fresh broth (Table III). Alternatively (Fig. 5), cultures inhibited by low concentrations of these antibiotics may be further inhibited by the addition of more antibiotic to give growth rates with the predictable rate constants (Table IV). Therefore, the inhibition of the growth rates caused by these antibiotics in the subinhibitory concentrations under study is reversible, at least to the same extent as for cultures in antibiotic-free media on dilution.

DISCUSSION

An operational model for the interference of antibiotic with protein synthesis in the individual cell can be constructed

$$A \rightleftarrows A' + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} (A'R) \qquad \text{(Eq. 4)}$$
$$K_1 \qquad K_2$$

A basic postulate is that the concentration of the antibiotic A in the media is readily partitioned into the cell with an equilibrium constant K_1, where A′ is the antibiotic concentration within the cell. There are R unreacted receptor sites within the cell

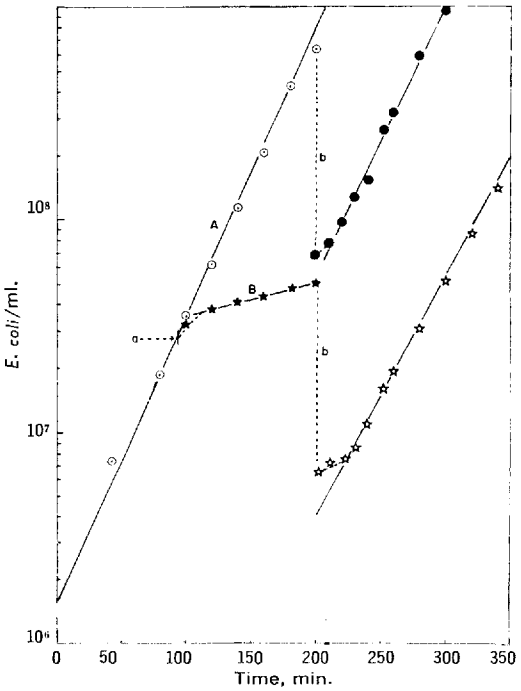

Fig. 4.—Example of reversibility of *E. coli* growth rate on dilution of chloramphenicol concentration. Curve A represents the logarithmic growth phase of an antibiotic-free culture. At time *a*, 4.66×10^{-6} moles/L. chloramphenicol are added to maintain the apparent first-order growth rate represented by line B. At time b, both the antibiotic-free culture, A, and the chloramphenicol culture, B, are diluted tenfold to demonstrate rapid reversibility of culture growth rates on dilution of antibiotic concentration in the subinhibitory antibiotic concentration range. Key: ⊙ = 0, chloramphenicol; ● = 0, chloramphenicol; ★ = 4.66×10^{-6} moles/L. chloramphenicol; ☆ = 4.66×10^{-7} moles/L. chloramphenicol.

TABLE III.—THE EFFECT OF DILUTION ON THE APPARENT GENERATION CONSTANTS, $10^4\ k$, OF *E. coli* CULTURES INHIBITED BY ANTIBIOTICS AT 37.5°

Before Dilution			After Tenfold Dilution		
$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$	$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$
0.0	4.88	4.90 ± 0.06	0.0	4.88	4.88 ± 0.10
46.6 (chloramphenicol)	0.92	0.73 ± 0.09	4.66 (chloramphenicol)	4.48	4.18 ± 0.03
4.16 (tetracycline)	1.60	0.80 ± 0.04	0.42 (tetracycline)	4.55	4.82 ± 0.03

[a] The calculations from $k = k_0 - k_a$ [antibiotic]. [b] 95% confidence limits of rate constant included.

that when interacted with the antibiotic in a reversible equilibrium, K_2, inhibit protein synthesis.

The subsequent development will consider that the rate of protein synthesis is proportional to unbound receptor sites. In order to account for the lack of direct proportionality between the rates of protein synthesis and the rates of reproduction of microorganisms, it will be necessary to introduce the concept that a minimum rate of protein synthesis is necessary for microbial generation. The rate of population increase will then be considered as proportional to this net rate of protein synthesis and to the number of organisms in the balanced-growth growth culture. Although total protein synthesis will be considered in the model, it is plausible that the diminution in reproductive rates may involve selective inhibition in the synthesis of specific proteins. The development will then be evaluated against the experimental information of this paper and the data available in the literature.

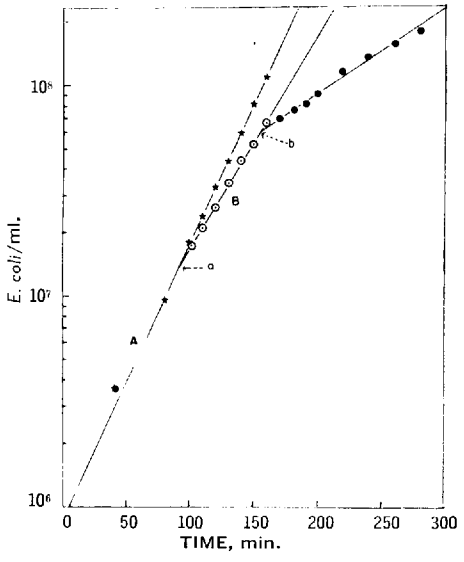

Fig. 5.—Example of rapid dependence of apparent generation rate constants of *E. coli* growth on increased amounts of antibiotic concentration. At times a and b, amounts of tetracycline were added to adjust the antibiotic concentrations to subinhibitory antibiotic concentrations specified. Key: ★ = 0, tetracycline; ⊙ = 1.04×10^{-7} moles/L.; ● = 4.16×10^{-7} moles/L.

The simplest postulate to make is that if Θ is the fraction of receptor sites reacted with antibiotic, the rate of protein synthesis, dP/dt, in a bacterium is proportional to the fraction, $1 - \Theta$, of receptor sites that are free.

$$dP/dt = k_P\,(1 - \Theta) \qquad \text{(Eq. 5)}$$

An implicit assumption in this postulate is that the numbers of receptor sites in a single bacterium are constant and independent of the mass of the cell or a time dependency of protein synthesis. Possible cases where this is not assumed are considered in the *Appendix*.

The equilibrium constant, K_2, for the antibiotic-site interaction of Eq. 4 may be defined by

$$K_2 = k_2/k_1 = (A'R)/[R_T - (A'R)](A') = (A'R)/(R)(A') \qquad \text{(Eq. 6)}$$

where (A'R) is the number of reacted sites, R is the number of unreacted sites, and R_T is the total number of sites whether reacted or unreacted. Thus, one may define the fraction, Θ, of sites reacted with antibiotic on appropriate rearrangement of Eq. 6 as

$$\Theta = (A'R)/R_T = K_2A'/(1 + K_2A') \qquad \text{(Eq. 7)}$$

Thus, Eq. 5 becomes

$$dP/dt = k_p - k_pK_2A'/(1 + K_2A') \qquad \text{(Eq. 8)}$$

where k_p is the steady-state rate of protein synthesis in the absence of antibiotic.

The concentration of antibiotic, A', in the cell may be related to the concentration, A, in the media by

$$A' = K_1A \qquad \text{(Eq. 9)}$$

It follows that the rate of protein synthesis by a

TABLE IV.—THE EFFECT OF ANTIBIOTIC ADDITION ON APPARENT GENERATION RATE CONSTANTS OF *E. coli* CULTURES INHIBITED BY ANTIBIOTICS AT 37.5°

Before Addition			After Addition		
$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$	$10^7\ M$ Antibiotic	Calcd. $10^4\ k^a$	Actual $10^4\ k^b$
0.00	4.88	4.90 ± 0.06	0.00	4.88	...
11.7 (chloramphenicol)	3.87	3.98 ± 0.11	46.6 (chloramphenicol)	0.92	1.05 ± 0.03
1.04 (tetracycline)	4.06	3.80 ± 0.07	41.6 (tetracycline)	1.60	1.48 ± 0.05

[a] Calculated from $k = k_0 - k_a$ [antibiotic]. [b] 95% confidence limits of rate constant included.

single bacterium is related to the antibiotic concentration A in the media by

$$dP/dt = k_p - k_pK_1K_2A/(1 + K_1K_2A) \quad \text{(Eq. 10)}$$

This rate of protein synthesis is associated with the generation time for a single organism, but not necessarily directly since it is reasonable to assume that a large fraction of the protein synthesized is consumed in sustaining processes for the maintenance of vitality and that a certain excess is necessary for reproduction.

If the minimum rate of protein synthesis necessary for reproduction division is k_p', the net rate of protein synthesis that results in reproductive division of a single cell is

$$dP'/dt = dP/dt - k_p' \quad \text{(Eq. 11)}$$

which in the absence of antibiotic is $k_p - k_p'$.

If it is postulated that the rate of population increase of N organisms is proportional to this net rate of protein synthesis and to the number of organisms in the balanced growth culture, it follows that

$$dN/dt = q(dP/dt - k_p')N \quad \text{(Eq. 12)}$$

where the proportionality constant q has dimensions of bacteria per unit of protein. Equation 10 may be substituted into Eq. 12.

$$dN/dt = [q(k_p - k_p') - qk_pK_1K_2A/(1 + K_1K_2A)]N \quad \text{(Eq. 13}a\text{)}$$

$$= [k_0 - k_aA/(1 + k_bA)]N \quad \text{(Eq. 13}b\text{)}$$

$$= k_{app.}N \quad \text{(Eq. 13}c\text{)}$$

where

$$k_a = k_b(k_0 + qk'_p) \quad \text{(Eq. 14)}$$

where the apparent first-order generation constant, $k_{app.}$, is observed in the presence of a constant antibiotic concentration, A. The inconsistency of derived equations with experiment when the need for a net rate of protein synthesis is ignored is considered in the *Appendix*.

When

$$k_bA \ll 1 \quad \text{(Eq. 15)}$$

i.e., when complete inhibition of microbial generation is effected by reaction of only a small fraction of total receptor sites, Eq. 13*b* reduces to

$$dN/dt = [k_0 - k_aA]N = k_{app.}N \quad \text{(Eq. 16)}$$

or

$$N = N_0e^{k_{app.}t} \quad \text{(Eq. 17)}$$

or

$$\log N = \log N_0 + (k_{app.}/2.303)t \quad \text{(Eq. 18)}$$

where

$$k_{app.} = k_0 - k_aA \quad \text{(Eq. 19)}$$

which is equivalent to Eq. 2.

In accordance with Eq. 18, the plots of the logarithm of numbers of organisms against time for any concentration of antibiotic that permits a net growth should give a straight line and is confirmed in the typical data for chloramphenicol plotted in Fig. 1.

The apparent first-order generation rate constants, $k_{app.}$, are obtainable from the slopes of such linear plots and are given in Table I for the several temperatures studied and can be obtained with a high order of precision.

If inhibition of the population growth rate is the mechanism for subinhibitory concentrations of the antibiotic rather than kill superimposed on normal growth, viable and total counts of *E. coli* with time should be coincident and the derived rate constants, $k_{app.}$, should be coincident for both total and viable count data. This has been shown to be true in the case of tetracycline (7) at one temperature and is now shown to be so for chloramphenicol and tetracycline for the several temperatures (Fig. 3 and Tables I and II). The coincidence of the plots of total and viable counts for *E. coli* population growth in the presence of graded concentrations of chloramphenicol are apparent from Fig. 1. The coincidence of the calculated $k_{app.}$ values from both count methods at various temperatures is apparent from Table I.

If the contingency of Eq. 15 is valid, it is predicted that a plot of the apparent first-order population growth rate constant in the presence of the antibiotics tetracycline and chloramphenicol should be reasonably linear when plotted against the concentration of the antibiotics. This has been shown to be the case for tetracycline and chloramphenicol (5) at one temperature and is demonstrated by the typical plots in Fig. 2 for several temperatures. The data of Table II include the apparent k_a values obtained from the slopes of such plots.

The proposed model is subject to test by the consistency of experiment with the several hypotheses. The reversible equilibria K_1 and K_2 of Eq. 4 should be quickly established. Literature evidence is confirmatory but has not been obtained at subinhibitory concentrations. The evidence in Fig. 4 and Tables III and IV clearly shows that addition and dilution of chloramphenicol concentrations in media containing a reproducing *E. coli* culture causes rapid changes in apparent population growth rates that are consistent with their linear dependence on antibiotic concentration (Eqs. 2 and 19). The rate transitions are no slower than that which occurs in diluting a control without antibiotic. Similar studies (Fig. 5, Tables III and IV) demonstrate the same phenomena for tetracycline.

Binding should have small energy requirements since it does not involve covalent bonding. The heat of activation, ΔH_a, of the population growth of *E. coli* can be estimated from the negative slope of logarithm of the generation rate constant, $\log k_0$, in the absence of antibiotic against the reciprocal of the absolute temperature, $1/T$ (Fig. 3) in accordance with the logarithmic Arrhenius expression of Eq. 3. The temperature for maximum *E. coli* growth rates can be noted from the maxima of the curves in Fig. 3.

When the derived inhibitory coefficients, k_a (Eq. 19) as given in Table II, are similarly treated, the ΔH_a values for the tetracycline and chloramphenicol inhibitory coefficients are the same as for the population growth rate constants of *E. coli* as can be observed from the parallelism of the slopes of Fig. 3. The consistency of total and viable counts with the Arrhenius relation can be also observed in Fig. 3. The parallelism of the Arrhenius

plots for the inhibitory coefficients and the growth rate constant in the absence of antibiotics is strongly indicative that the k_a is related to the k_0 as is indicated in Eqs. 13*a–c* and 13*A–C*. For example, consider

$$\log k_a = \log K_1K_2k_0 = \log k_0 + \log K_1 + \log K_2 = \log \text{constant} - \{[(\Delta H_a)_{k_0} + (\Delta H_a)_{K_1} + (\Delta H_a)_{K_2}]/2.303R\}\,(1/T) \quad \text{(Eq. 20)}$$

since each of the logarithmic values can be described by an Arrhenius expression similar to Eq. 3. Since, the heat of activation for k_a is within 1–2 Kcal./mole of that for k_0, the energies of partition and binding for K_1 and K_2 must be extremely small as would be expected if this model reflected reality.

A linear equation can be derived for the general case of Eq. 13 where the simplifying premise of Eq. 15 is not necessary and

$$1/(k_0 - k_{\text{app.}}) = (1/k_a)(1/\text{A}) + k_b/k_a \quad \text{(Eq. 21)}$$

When the reciprocal of the experimentally observed differences between the generation rate constants in the absence, k_0, and presence, $k_{\text{app.}}$, of antibiotics is plotted against the reciprocal of the antibiotic concentration, A, for the various data of Table I and previous data (7), reasonable linearity is obtained with intercepts passing close to or through the origin. This is to be expected if Eq. 19 is true. In several cases, a finite intercept could be ascertained whose reciprocal was of the order of magnitude $k_a/k_b > 10k_0$.

If the assumption were valid that the population growth was directly proportional to the rate of protein synthesis (Eqs. 13*A–15a*, *Appendix*) k_a/k_b should equal k_0. (See Eq. 15*a*, *Appendix*, Eq. 18.) This is not so. The postulate that population growth rate is proportional to a net rate of protein synthesis (Eqs. 11–14) is consistent with the observed facts where

$$k_a/k_b = k_0 + qk_p' > k_0 \quad \text{(Eq. 22)}$$

The fact that $k_a/k_b > 10k_0$ implies that it is only necessary to affect less than about 10% of the available protein synthesizing sites to completely inhibit population increase.

The interesting observation that cell-free preparations demonstrate chloramphenicol inhibition of protein synthesis regardless of whether they are derived from chloramphenicol resistant microorganism strains (13) is strongly indicative that the basic difference among strains is in the process by which the compound reaches the site of action or in the permeability of the cells. In this model, this is directly related to the magnitude of K_1 in Eq. 4.

Most of the data available on substrate incorporation on bacterial synthesis are for superinhibitory concentrations of chloramphenicol and tetracycline (11–15) where this refers to concentrations in excess of those minima necessary for complete inhibition of population increase. There are some data that can be interpolated from the literature, however, that are confirmatory when one considers that in the authors' *E. coli*, B/r system about 2–2.5 mcg./ml. of chloramphenicol completely inhibits generation at 37.5°. Chloramphenicol at 100 mcg./ml. inhibits 20–60% of various amino acid incorporation in *Staphylococcus aureus* (11). For *E. coli*, strain B, there is 50% inhibition of $^{35}SO_4$ incorporation and no inhibition of [^{14}C] uracil incorporation at 2 mcg./ml. of chloramphenicol (15). There is less than 20% inhibition of ammonia assimilation and oxygen consumption in the presence of about 2.5 mcg./ml. of chloramphenicol (12).

In cell-free systems of several *E. coli*, 2.5 mcg./ml. of chloramphenicol inhibited amino acid incorporation 30–50% and at 1.3 mcg./ml., the inhibition was 18–35% (13). This is optimum efficiency since partition of chloramphenicol (K_1 in Eq. 4) into the microorganism does not completely reflect the concentration of the antibiotic in the media and the actual inhibition of protein syntheses in the bacteriostatic region of chloramphenicol action, *i.e.*, 1.5 mcg./ml., will be much less than this 35%. The only data available in the appropriate concentration region for tetracycline are the demonstrations of 10–25% inhibition of amino acid incorporation in cell-free systems for various strains of *E. coli* at 2.5 mcg./ml. (13). In this case the authors have complete bacteriostasis at less than 0.2 mcg./ml. of tetracycline which certainly would correspond to a very low value of inhibition of protein synthesis.

These values are consistent with the necessary model that only a small fraction of protein synthesis inhibition is concomitant with bacteriostasis.

The most rational hypothesis to account for the action of chloramphenicol and tetracycline is that they inhibit the function of messenger RNA by blocking its attachment to ribosomes through competition for ribosomal binding sites (3). It has also been noted that the variation in chloramphenicol binding by ribosomes is in agreement with the ability of the antibiotic to inhibit protein synthesis in cell-free systems (16). These observations and data are consistent with the proposed model, the binding of the receptor sites on the ribosomes by these antibiotics to lower protein synthesis.

Although significant increases in both total and viable numbers of bacteria in cultures of *E. coli*, B/r, have been reported in the presence of "bacteriostatic concentrations" (these concentrations of 50 mcg./ml. are really superinhibitory) of chloramphenicol (17), no such significant increases were observed in the studies reported here in the subinhibitory concentration ranges of chloramphenicol up to the 2.5% mcg./ml. where total inhibition of population growth rate was observed. This was also true for tetracycline in comparable subinhibitory concentrations in these and previous studies (7). A change in the overt mechanism of action of these antibiotics in the sub- and superinhibitory ranges is indicated; from a truly bacteriostatic agent functionally dependent on the first power of chloramphenicol and tetracycline concentration where inhibition is additive on a kinetic basis (5, 8), to a bactericidal agent with a more complex functional dependency on these antibiotic concentrations (5, 6).

The proposed model explains the bacteriostatic effects and the functional dependencies on the premise of diminution of protein synthesis below the critical level necessary for generation; the bactericidal effects may be explained by the further inhibition of protein synthesis below that minimum necessary for survival and for regaining of generation capabilities.

APPENDIX

Variation of Receptor Sites with Time.—An implicit assumption in the postulated Eq. 5 is that the numbers of receptor sites in a single bacterium are constant and independent of the mass of the cell or a time dependency of protein synthesis. This is not necessarily so. The rate constant k_p of Eq. 5 includes the intrinsic synthesizing activity $(k_p)_0$ associated with each site and the numbers, R, of such sites so that

$$k_p = (k_p)_0 R \qquad \text{(Eq. 5}a\text{)}$$

If R at a time, t, is R_0, and the numbers of such sites exponentially increase with time with a rate constant, k_r, then

$$k_p = (k_p)_0 R_0 e^{k_r t} \qquad \text{(Eq. 5}b\text{)}$$

A possible simplifying assumption, is that proliferation of such synthesizing sites is associated with protein synthesis and proceeds at the same rate so that if $k_p \sim k_r$, the result is the transcendental equation

$$k_p = (k_p)_0 R_0 e^{k_p t} \qquad \text{(Eq. 5}c\text{)}$$

An alternate modification which was considered later in the development of the model was that a constant rate of protein synthesis is needed to sustain metabolic processes. Thus, only the excess rate characterized by $k_p - k_p'$ can be utilized in manufacturing synthesizing sites so that Eq. 5c may be modified to

$$k_p = (k_p)_0 R_0 e^{(k_p - k_p')t} \qquad \text{(Eq. 5}d\text{)}$$

where $k_p = (k_p)_0$ immediately after division at $t = 0$ of the generation time and k_p is a maximum at $t = t_{max.}$, the generation time for a single cell under the stated conditions. Since balanced growth cultures are being considered, a weighted mean k_p for the entire culture would be approximately constant averaged over all the organisms in the statistically distributed phases of growth between $t = 0$ and $t = t_{max.}$ If the increase in the numbers of sites is reasonably linear with time, Eq. 5b may be given as

$$k_p = (k_p)_0 (R_0 + k_r t) \qquad \text{(Eq. 5}B\text{)}$$

Nonpostulation of a Minimum Rate of Protein Synthesis for Reproduction Division.—If the hypothesis of a minimum rate of protein synthesis necessary for reproduction is not made, it is considered only that the rate of population increase of N organisms is directly proportional to the rate of protein synthesis in a single cell and the number of organisms. Then

$$dN/dt = [q'k_p - q'k_p K_1 K_2 A/(1 + K_1 K_2 A)]\ N \qquad \text{(Eq. 13}A\text{)}$$

$$= [k_0 - k_a A/(1 + k_b A)]N \qquad \text{(Eq. 13}B\text{)}$$

$$= k_{app.} N \qquad \text{(Eq. 13}C\text{)}$$

where

$$k_a = k_b k_0 \qquad \text{(Eq. 14}a\text{)}$$

It follows from Eqs. 13B, 13C, and 14a that

$$1/(k_0 - k_{app.}) = (1/k'_b k_0)(1/A) + 1/k_0 \qquad \text{(Eq. 15}a\text{)}$$

Thus, when the reciprocal of the experimentally observed difference between the generation rate constants in the absence, k_0, and presence, $k_{app.}$, of antibiotics are plotted against the reciprocal of the antibiotic concentration, A, the intercept of the presumed straight line should be $1/k_0$. It was shown that this is not the case, that the reciprocal of the intercept significantly exceeds the experimental k_0 value.

Thus, the hypothesis that the generation rate is proportional to a net rate of protein synthesis above that minimum necessary for maintenance of viability is preferable.

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Effects of Shear Processing and Thermal Exposure on the Viscosity-Stability of Polymer Solutions

By DAVID R. POWELL, JAMES SWARBRICK, and GILBERT S. BANKER

The effects of high-shear processing and thermal exposure on the molecular weight and solution viscosity stability of selected polymers were studied. Aqueous solutions of two molecular weight grades of hydroxyethyl cellulose (HEC) were subjected to high-shear rates using a high-shear mixer and two colloid mills. Extremely rigorous shearing conditions were required to alter the viscosity. The viscosity stability of the two grades of HEC was studied under prolonged thermal treatment at 70°. Viscosity degradation was found to follow a first-order reaction rate, the viscosity half-life for the higher grade being considerably less than that for the lower molecular weight grade. Number-average molecular weights for the HEC's were determined by the osmotic pressure technique and related to viscosity-average molecular weights through intrinsic viscosity data. The correlation between the two types of molecular weights was high, implying that bond-scissions and hydrolysis were the cause of viscosity degradation.

THE PHYSICAL nature of most pharmaceutical dispersed systems necessitates the use of viscosity imparting agents or dispersing aids to reduce phase separation tendencies, thereby enhancing medicament distribution. Virtually all such agents are polymeric in structure and fall under the general categories of natural gums, resins, and synthetic polymers. The latter group finds the most application in present-day suspension technology. The viscosity imparted to a system by the presence of these solvated polymeric agents is dependent on their spacial configurations in colloidal solution which is primarily a function of molecular weight, and to some extent, the degree of substitution or branching along the polymer chains.

Various physicochemical forces are capable of randomly rupturing the long chain structure of high molecular weight polymers (1). Two physical forces which may initiate depolymerization and subsequent viscosity loss in high polymer systems are mechanical shear and thermal energy. Studies (2–4) have shown that mechanical depolymerization of polymers in solution may occur and that this phenomenon is difficult to quantify. In most instances, the degradation reactions approximate the first-order rate law. Factors contributing to this mechanical phenomenon include (*a*) shear rate, (*b*) the average molecular weight of the polymer, (*c*) polymer–solvent interactions, and (*d*) the stereochemistry of the polymer and solvent. With polymer systems containing suspended particles, the concentration and particle size characteristics of the suspended material may affect the total shear within the system to provide an added factor influencing mechanical depolymerization (5).

The effect of temperature upon the viscosity stability of aqueous solutions of several hydrophilic polymers has been studied (6–8). While some workers (9, 10) suggest that viscosity reduction with prolonged heating is due to actual depolymerization or hydrolysis, Caldwell and Watters (11) contest the occurrence of any structural changes. A permanent viscosity decrease of a polymer solution should not be taken as absolute evidence of depolymerization, since this effect may be due to deaggregation and/or desolvation of the polymer.

This study was initiated to investigate the viscosity stability of certain hydrophilic polymers under conditions allied with the manufacture and production of pharmaceutical dispersions.

EXPERIMENTAL

Polymer Selection and Solution Preparation.—Two molecular weight grades of hydroxyethyl cellulose,[1] HEC-250G and HEC-250H, were used in this investigation. According to the manufacturer, the approximate molecular weights are 80,000 and 210,000, respectively; however, the method by which these values were obtained was not stated. These polymers were used as received from the manufacturer. The moisture content was determined and used in calculating the necessary weight of material to be used for preparing the required solutions.

Aqueous solutions (w/v) were prepared by dispersing the finely powdered polymers in distilled water at 5° with the aid of a magnetic stirrer. Stirring was then continued until the dispersions reached room temperature and solution had occurred. Generally 4–8 hr. were required. The solutions were made up to volume and allowed to hydrate for a minimum of 24 hr. before being used.

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[1] Natrosol 250G and 250H, Hercules Powder Co., Wilmington, Del.

TABLE I.—MILLING OPERATIONS ON HEC SOLUTIONS

Viscosity Grade	Concn., %, w/v	Vol. Milled, ml.	Power Equip.	r.p.m. and Gap	Shear Rate,[a] sec.$^{-1}$	Shear Time, min.
250H	1.0	1000	EHM[b]	8500–9000	...	5.5
250H	1.0	1000	EHM	11,000–11,500	...	5.0
250H	1.0	850	MGCM[c]	18,000 0.001 in.	1.88×10^6	90[e]
250H	1.0	1500	THCM[d]	11,000 0.002 in.	6.87×10^5	One pass
250G	2.0	900	EHM	8650–8950	...	5.0
250G	4.0	900	MGCM	18,000 0.002 in.	9.44×10^5	12.0

[a] The approximate shear rate for colloid mills was determined by the following equation given by Kostenbauder *et al.* (12): S = rotor peripheral velocity/rotor-stator clearance. [b] Eppenbach Homo mixer, model 1-L, Gifford Wood Co., New York, N. Y. [c] Manton-Gaulin colloid mill, model 2B, Manton-Gaulin Manufacturing Co., Everett, Mass. [d] Tri-Homo colloid mill, Tri-Homo Corp., Salem, Mass. [e] The temperature of the solution was maintained at 20°.

Viscosity Analysis.—Intrinsic viscosities were determined for both grades of HEC using a Cannon-Fenske No. 200[2] capillary viscometer at a constant temperature of 25 ± 0.01°. The viscometer was standardized with a 20-ml. sample of distilled water, and the mean solvent efflux time was determined. Efflux times for 20-ml. samples of five dilute solutions of HEC-250G (0.04–0.2 Gm./100 ml.) and HEC-250H (0.01–0.1 Gm./100 ml.) were determined and the respective relative viscosities (η rel.) calculated. By converting (η rel.) to reduced specific viscosities (η sp/c), and plotting these values against concentration, the intrinsic viscosities were obtained by extrapolation to infinite dilution.

Pseudoplastic rheograms of the HEC solutions at 25° were obtained using a Ferranti-Shirley[3] plate and cone viscometer. The instrument was calibrated with N.B.S. oil M[4] using the 2.75-in. diameter cone throughout.

Molecular Weight Determination.—Number-average molecular weights for both grades of HEC were determined in water at 25 ± 0.01° using a Stabin-Immergut[5] bi-membrane osmometer. Regenerated cellulose films No. 300[6] were used as the semipermeable membranes. Five different concentrations of each polymer were used to determine osmotic pressures which were corrected for membrane asymmetry. Corrected pressures were converted to reduced osmotic pressures which were plotted against concentration. The graphs were extrapolated to infinite dilution and the molecular weights were calculated by the following equation:

$$M\bar{n} = \frac{RT}{(\pi/c)_{c=0}} \qquad \text{(Eq. 1)}$$

where $M\bar{n}$ = number-average molecular weight,
R = gas constant (L. Atm. mole^{-1} degree^{-1}),
T = absolute temperature,
$(\pi/c)_{c=0}$ = reduced osmotic pressure at infinite dilution.

Shear Depolymerization.—Aqueous polymer stock solutions were subjected to varying degrees of agitation and hydrodynamic shear imparted by the use of power equipment common to the pharmaceutical industry (Table I.)

Thermal Depolymerization.—The effects of thermal exposure on the viscosity and molecular weight of 2.0% HEC-250G and 1.0% HEC-250H in solution were investigated. The viscosity stability of solutions of the polymer samples at room temperature was also observed.

Six 135-ml. samples of each polymer stock solution were placed individually in 8-oz. dark glass bottles which were then tightly sealed and placed in a dry air oven at 70 ± 1°. Samples were periodically removed from the oven over a 12–13-day interval and assayed viscometrically. A 1000-ml. sample of HEC-250G (2.0%) was treated at 70° for 48 hr. In addition to assaying this sample viscometrically, the number-average molecular weight was determined.

RESULTS

Mechanical Shear Effects.—Viscosity and molecular weight loss were indicated in only the 90-min. milling operation where a 1.0% HEC-250H solution was allowed to recycle through the rotor-stator mechanism of the Manton-Gaulin colloid mill (Table I).

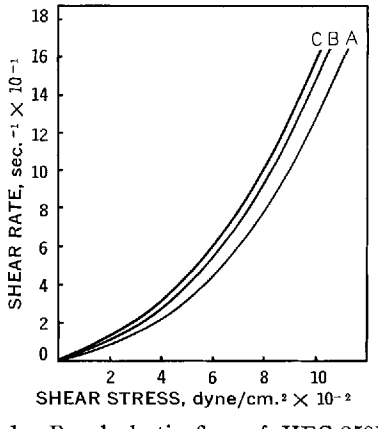

Fig. 1.—Pseudoplastic flow of HEC-250H (1% w/v) milled for 90 min. in the Manton-Gaulin colloid mill. Key: A, initial; B, 30, 45, and 60-min. samples; C, 75 and 90-min. samples.

[2] Cannon Instrument Co., State College, Pa.
[3] Ferranti-Shirley Co., Manchester, England.
[4] National Bureau of Standards, Washington, D. C.
[5] J. V. Stabin, Brooklyn, N. Y.
[6] FMC Corp., Fredericksburg, Va.

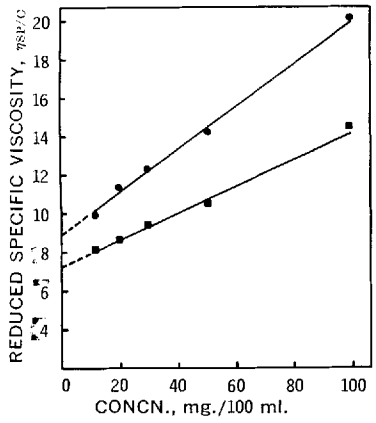

Fig. 2.—Reduced specific viscosity of HEC-250H before and after 90 min. of milling. Key: ●, initial $[\eta]$ = 8.7; ■, after 90 min. $[\eta]$ = 7.5.

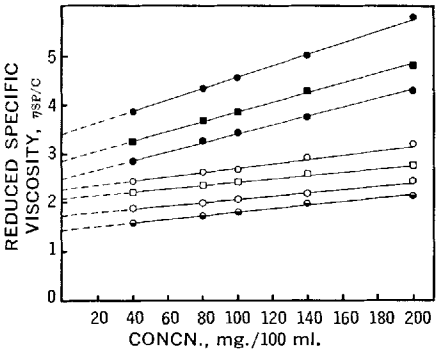

Fig. 3.—Reduced specific viscosity of HEC-250G solutions (2% w/v) thermally degraded at 70°. Key: ●, initial; ■, 24 hr.; closed hexagon, 48 hr.; ○, 94.5 hr.; □, 144 hr.; ⬡, 192 hr.; ◒, 300 hr.

Samples were removed after 30 min. and at 15-min. intervals thereafter and evaluated on the Ferranti-Shirley viscometer (Fig. 1). The reduction in shear stress at a shear rate of 1.66×10^3 sec.$^{-1}$ was 6% for the 30, 45, and 60-min. samples and 9.1% for the 75 and 90-min. samples. The bulk solution remaining after 90 min. was used to determine intrinsic viscosity (Fig. 2). The reduction in intrinsic viscosity was 15%.

Thermal Exposure Effects.—Preliminary studies indicated that the intrinsic viscosity of HEC-250G (2.0%) did not vary over an 18-day period at room temperature. Therefore, any change in the intrinsic viscosity of HEC solutions at elevated temperatures would be solely a function of the heat conditions used as opposed to any spontaneous viscosity change at room temperature.

The effects of temperature with time on the viscosity of HEC are given in Figs. 3 and 4. The method of least squares was used to obtain the slopes and intrinsic viscosities which are given in Table II. The slopes were approximately proportional to the square of their respective intrinsic viscosities, conforming to the equation derived by Huggins (13):

$$\eta_{sp/c} = [\eta]_{c=0} + K'[\eta]_{c=0}\, C \qquad \text{(Eq. 2)}$$

where $\eta_{sp/c}$ = reduced specific viscosity,
$[\eta]_{c=0}$ = intrinsic viscosity,
C = concentration (gramarity),
K' = constant for a homologous series of polymers in a given solvent.

The mean K' value for 35 reduced specific viscosities for HEC-250G was found to equal 1.236 ± 0.430. For the 35 reduced specific viscosities of HEC-250H, the mean K' value was found to equal 1.241 ± 0.458. The fair agreement between mean K' values indicated the validity of the viscosity data obtained. The over-all constant for HEC in water is 1.238 ± 0.444.

Pseudoplastic rheograms for thermally degraded HEC-250G and HEC-250H as a function of time are shown in Figs. 5 and 6, respectively. In each case, the pseudoplasticity of the material diminished with a trend toward Newtonian flow. There was no change in the rheograms of the samples stored at room temperature over the 300-hr. storage.

Molecular Weight–Intrinsic Viscosity Correlation.—The number-average molecular weights determined by osmotic pressure analysis for HEC-250G and HEC-250H were 4.73×10^4 and 1.325×10^5, respectively. The intrinsic viscosities of HEC were related to the corresponding number-average molecular weights by the Kuhn-Houwink (14) empirical equation. Thus,

$$[\eta]_{c=0} = KM^a \qquad \text{(Eq. 3)}$$

where $[\eta]_{c=0}$ = intrinsic viscosity,
M = molecular weight,
K and a = polymer–solvent interaction constants.

The constants K and a, valid only for linear polymers, were found to equal 1.445×10^{-4} and 0.9338, respectively. For HEC, therefore, the Kuhn-Houwink equation becomes:

$$\log[\eta]_{c=0} = 0.9338 (\log M\bar{n}) + \log 1.445 \times 10^{-4} \qquad \text{(Eq. 4)}$$

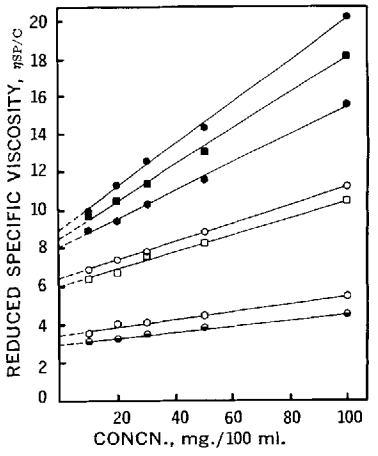

Fig. 4.—Reduced specific viscosity of HEC-250H solutions (1% w/v) thermally degraded at 70°. Key: ●, initial; ■, 24 hr.; closed hexagon, 48 hr.; ○, 96 hr.; □, 147.5 hr.; ⬡, 233 hr.; ◒, 306 hr.

TABLE II.—VISCOSITY DATA OF HEC SOLUTIONS TREATED AT 70°

HEC-250G, 2.0%			HEC-250H, 1.0%		
hr., 70°	$[\eta]_c = 0$	Slope	hr., 70°	$[\eta]_c = 0$	Slope
0	3.35	11.2	0	8.85	112.7
24	2.90	10.0	24	8.56	94.9
48	2.55	9.1	48	8.07	74.7
94.5	2.30	4.3	96	6.36	47.7
144	2.05	3.3	147.5	6.02	44.0
192	1.75	3.4	233	3.65	19.0
300	1.40	4.0	306	3.25	12.7

The validity of the interaction constants was tested by determining the intrinsic viscosity of an HEC-250G solution maintained at 70° for 48 hr. The molecular weight, calculated using Eq. 4, was 4.15×10^4; the observed molecular weight from osmotic pressure analysis was 4.199×10^4. The interaction constants, K and a, for HEC in water were, therefore, assumed to be valid.

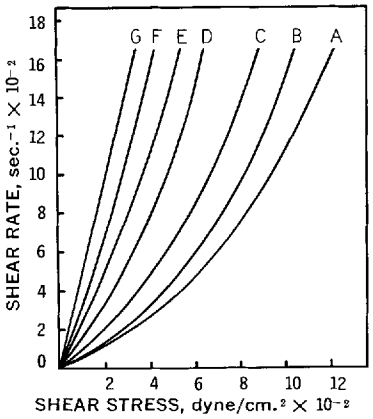

Fig. 5.—Pseudoplastic rheograms of 2% w/v HEC-250G solutions thermally degraded at 70°. Key: A, initial; B, 24 hr.; C, 48 hr.; D, 94.5 hr.; E, 144 hr.; F, 192 hr.; G, 300 hr.

Degradation Rate and Viscosity Half-Life.—The thermal viscosity degradation of HEC in water was found to best approximate a first-order reaction rate (Fig. 7). Viscosity degradation parameters are given in Table III along with the linear correlation coefficients.

The reaction rate constant for the degradation of HEC-250H is approximately 1.5 times greater than that of HEC-250G. This would indicate that the higher molecular weight 250H is more prone to thermal degradation than 250G, even though the concentration of HEC-250H was half that of HEC-250G and the initial pseudoplastic rheograms were nearly identical (Figs. 5 and 6).

DISCUSSION

The mechanical degradation study indicates that the two molecular weight grades of HEC are capable of withstanding intensive hydrodynamic shear rates. Only under the most rigorous conditions where a shear rate of 1.88×10^6 sec.$^{-1}$ was maintained for 90 min. was HEC-250H (1.0%) found to degrade. Analysis showed that a 15% reduction in molecular weight and intrinsic viscosity had been affected. These findings are in accordance with other investigations (2, 3), where it was noted that mechanical shear had little effect on polymers, in this case noncellulosics, of molecular weights less than 5×10^5.

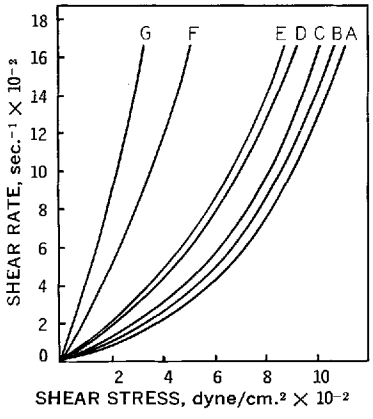

Fig. 6.—Pseudoplastic rheograms of 1% w/v HEC-250H solutions thermally degraded at 70°. Key: A, initial; B, 24 hr.; C, 48 hr.; D, 96 hr.; E, 147.5 hr.; F, 233 hr.; G, 306 hr.

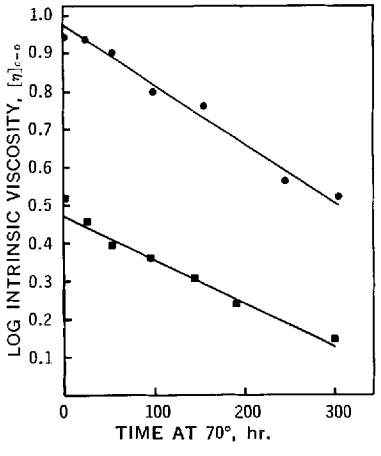

Fig. 7.—Degradation rate curves of aqueous HEC solutions at 70°. Key: ●, HEC-250H (1% w/v); ■, HEC-250G (2% w/v).

TABLE III.—VISCOSITY DEGRADATION DATA FOR HEC SOLUTIONS TREATED AT 70°

Mol. Wt. Grade	Reaction Rate Constant, hr.$^{-1}$	Slope	Viscosity Half-Life, hr.	Linear Correlation Coefficient
HEC-250G	2.58×10^{-3}	-1.12×10^{-3}	268.5	0.992
HEC-250H	3.64×10^{-3}	-1.58×10^{-3}	190.8	0.974

The mechanism associated with the thermal degradation and subsequent reduction in viscosity and molecular weight of HEC solutions is believed to be random hydrolysis of the glycosidal linkages. This proposed mechanism is in accordance with other degradation studies (6, 9, 10) involving cellulose derivatives. Hydrolytic cleavage of hydroxyethyl side chains or pyranose rings may also occur but is not likely to cause the marked viscosity changes found in this study. The extensive release of hydroxyethyl units would decrease the polymer solubility by conversion to cellulose; however, no turbidity was noted in the degraded samples. Rupture of the pyranose rings would not effectively alter chain length or molecular weight.

The relative viscosity stability and degradation rates of the two molecular weight grades of HEC at 70° indicates that when viscosity control is necessary, it is advantageous to employ a higher concentration of lower molecular weight polymer. It was shown that the pseudoplasticity of a 2% HEC-250G solution was nearly identical to that of a 1% HEC-250H solution, but that the viscosity half-life of the lower molecular weight 250G was 1.5 times greater than the 250H.

SUMMARY

A study was undertaken to determine the viscosity stability of two molecular weight grades of HEC. Polymers in aqueous solutions were subjected to processing conditions allied with the manufacture of pharmaceutical dispersed systems.

The intrinsic viscosity, pseudoplasticity, and molecular weight of two grades of HEC were not affected by the more moderate shearing operations used. In one case, under extremely rigorous shearing conditions, the higher molecular weight grade (HEC-250H) showed a significant viscosity and molecular weight reduction.

It was hypothesized that the decrease in viscosity at 70° was due to hydrolysis of glycosidal linkages; this was supported by number-average molecular weight analysis.

Good correlation between intrinsic viscosity and number-average molecular weight enabled calculation of the interaction constants in the Kuhn-Houwink equation. The constant of the Huggins equation relating dilute viscometry slope to the square of intrinsic viscosity was also calculated.

Thermally induced degradation of HEC was found to approximate a first-order rate law with high linear correlation.

The viscosity degradation rate for 1% HEC-250H was approximately 1.5 times that of 2% HEC-250G. This indicated that the higher molecular weight grade was more prone to thermal degradation than the lower molecular weight grade.

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Spectrophotometric and Chemical Studies of 5-Mercaptouracil, 5-Mercaptodeoxyuridine, and Their *S*-Substituted Derivatives

By THOMAS J. BARDOS and THOMAS I. KALMAN

5-Mercaptouracil (I) and 5-mercaptodeoxyuridine (II), structural analogs of thymine and thymidine, respectively, are effective growth inhibitors in various biological systems and under study as potential antineoplastic and antiviral agents. Both compounds were found to be extremely unstable in aqueous solutions as they undergo rapid autoxidation to the corresponding disulfides. Determination of the ultraviolet spectra and pKa values of the thiols was possible only by special techniques, using dithiothreitol (DTT) as a "protecting" agent. Both I and II have very low pKa's, and their anionic forms show characteristic absorption maxima in the 330-mμ region. These results are discussed in comparison with the spectra and ionization equilibria of related compounds including some new *S*-substituted derivatives of I and II. DTT was found to be also a uniquely suitable reagent for the preparation of pure I and II by stoichiometric reduction of the corresponding disulfides. A special technique was developed for the quantitative determination of the free thiols.

A STRUCTURAL analog of thymine (1), 5-mercaptouracil (I), has been under biological and preclinical investigation during the last few years as an experimental antineoplastic agent (2). Its 2′-deoxyriboside (II) was recently synthesized enzymatically (3) and chemically (4), and it was shown to have high inhibitory activity in various biological test systems (3). Both compounds, however, undergo rapid autoxidation in aqueous solution, and this property has presented a major problem in their preparation and biological testing. In fact, the autoxidation of I in dilute aqueous buffer solutions proceeds so rapidly that the ultraviolet spectrum originally reported (1) for this compound was actually that of the corresponding disulfide (III). In view of the continuing interest in the biological activities and possible chemotherapeutic applications of these compounds, a careful study was undertaken to determine their correct spectra and dissociation constants, and to establish the conditions of their stability to autoxidation.[1] This study was greatly aided by two recent developments, *i.e.*, (*a*) the availability of Cleland's reagent (5), dithiothreitol (DTT), and (*b*) the excellent method of Klotz and Carver (6) for the determination of sulfhydryl groups.

EXPERIMENTAL

Materials.—5-Mercaptouracil (I) and 5-uracilyl-disulfide (III) were prepared by previously described methods (1, 7). Both compounds were purified by repeated crystallizations from water, and the free thiol content of each preparation was determined by the titrimetric method described below. 5-Mercaptodeoxyuridine disulfide[2] (IV) was a sample of the enzymic preparation (3). The synthesis of 5-methylmercaptouracil (V) and 5-acetylmercaptouracil (VI) will be reported.[3]

Reagents.—DTT (dithiothreitol, Cleland's reagent)[4] and glutathione (reduced)[5] were used as indicated. Mersalyl acid (*o*-{[3-(hydroxymercury)-2-methoxypropyl]carbamyl}-phenoxyacetic acid)[6] was dissolved at 10^{-3} *M* concentration in 0.2 *M* phosphate buffer, pH 6.3, containing 8×10^{-3} *M* NaCl. Indicator dye, pyridine-2-azo-*p*-dimethylaniline,[7] was dissolved in absolute EtOH and used at 10^{-3} *M* concentration. Both the mercurial and the dye solutions were stored in the refrigerator and freshly prepared after 3 days.

Buffers.—The following buffers were used: between pH 3 and 5, 0.025 *M* acetate buffers; between pH 5 and 8, 0.015 *M* phosphate buffers; between pH 8 and 12, Sorensen's glycine buffers. Below 3 and above 12, dilute HCl and NaOH solutions were used, respectively.

Ultraviolet Absorption and pKa Determinations.—Stock solutions of compounds having sulfhydryl groups (I, II) were prepared in the following manner to prevent their rapid air oxidation. I was dissolved in 5×10^{-4} *N* HCl (0.250 mg./ml.) in the presence of 1.0 mg./ml. of DTT. The disulfide (IV) was reduced to II at 0.500 mg./ml. concentration (pH 7–9) by the addition of DTT (1 mg./ml.), then the pH was adjusted to 3–4 with HCl. Stock solutions of the other compounds were freshly prepared in distilled water, except for the disulfide (III) which was dissolved in 0.01 *N* NaOH.

A Leeds and Northrup pH indicator was used for the pH measurements.

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[1] A study of the kinetics of autoxidation and its dependence on several variables will be presented in a subsequent publication.

[2] The authors are grateful to Mrs. K. Baranski for a sample of this compound.

[3] The authors thank Mr. M. Kotick for the preparation of these compounds.

[4] Purchased from Calbiochem, Inc., Los Angeles, Calif.

[5] Purchased from Nutritional Biochemicals Corp., Cleveland, Ohio.

[6] Purchased from Winthrop Laboratories, New York, N. Y.

[7] Purchased from Sigma Chemical Co., St. Louis, Mo.

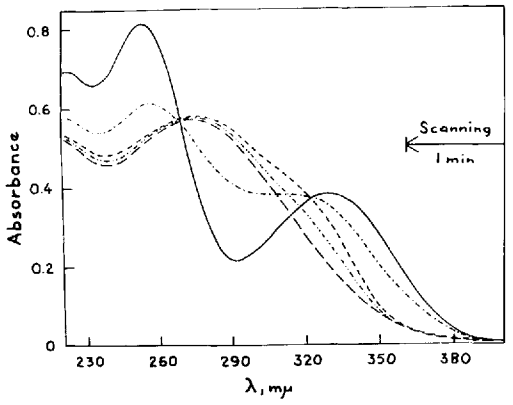


Fig. 1.—Change of ultraviolet absorption spectrum of 8.68×10^{-5} *M* solution of 5-mercaptouracil (I) at pH 7.4 (in 0.05 *M* phosphate buffer). Time lapse: from the dilution of the stock solution with the buffer until the beginning of the recording. Key: – · – · – ·, 2 min.; – – – –, 8 min.; — · · — · ·, 16 min.; — — —, 60 min.; ——, stabilized control (contains DTT). The scanning speed is indicated in the graph.

The ultraviolet absorption curves were obtained on a Beckman DB recording spectrophotometer, but a Gilford model 2000 photometer was used for the accurate determination of the $\lambda_{max.}$, $\lambda_{min.}$, ϵ, and pKa values. Aliquots of stock solutions were pipeted and diluted with buffers directly in the cells (10 mm. light path) to a final concentration of 12.5 or 25 mcg./ml.

Absorbance values measured at fixed wavelengths when plotted against pH gave the pKa values as midpoints of the curves. These pKa values were compared to the calculated ones obtained by the following formula (after Gage) (8):

if $A(a) < A(b)$:

$$\text{pKa} = \text{pHx} + \log \frac{A(b) - A(x)}{A(x) - A(a)}$$

if $A(a) > A(b)$:

$$\text{pKa} = \text{pHx} - \log \frac{A(a) - A(x)}{A(x) - A(b)}$$

where $A(a)$ is the absorbance value of the acidic (unionized) form, $A(b)$ is the value of the basic (ionized) species, and $A(x)$ is the value measured at a pH close to the apparent pKa. The pKa values obtained from the curves and by calculation agreed within 0.1 pH unit.

Reduction of the Disulfide with DTT.—The disulfide (III) (recrystallized from H_2O), 65 mg., and DTT, 130 mg., were dissolved in 30 ml. of 10^{-2} *N* NaOH and brought to pH 8. After a few minutes of stirring under N_2, the solution was acidified with HCl to pH 1.6, then 10 ml. of absolute ethanol was added, and the product was allowed to crystallize at −5 to −10°. The crystals were washed with 1:3 ethanol–water mixture (adjusted to pH 2 with hydrochloric acid), followed by absolute ethanol and, finally, by a few drops of acetone, then they were dried *in vacuo* at 110°. The infrared spectrum of the product showed a sharp peak at 2550 cm.$^{-1}$, characteristic for the S-H bond, and quantitative sulfhydryl group determination by the method described below proved that the reduction of the disulfide to the free thiol was complete.

Sulfhydryl-Group Determination.—Essentially, the method of Klotz and Carver (6) was employed with some modifications. An excess of the mercurial was immediately added in order to effect rapid and quantitative combination with the very unstable mercapto compounds. This was followed by the addition of a known excess of glutathione which was then titrated with additional portions of the mercurial in the described manner. Weighed samples were dissolved in 10^{-2} *M* HCl under N_2, from which aliquots were pipeted into a 18 × 150 mm. test tube. After 5 ml. of 10^{-3} *M* mersalyl acid was added and mixed, 0.1 ml. of 5×10^{-2} *M* glutathione and 0.6 ml. of indicator were added. The mixture was titrated with 10^{-3} *M* mersalyl acid, the color change was followed with a Bausch & Lomb photometer at 550 mμ, and the end point of the titration was determined as described (6).

RESULTS AND DISCUSSION

Figure 1 shows the rapid change of the absorption spectrum of I in a dilute aqueous solution (8.68×10^{-5} *M*) buffered at a neutral pH. Even when the spectrum was taken immediately after dissolving the compound in the buffer, it already showed a vast shift in comparison to the spectrum of a "stabilized" solution of I (*i.e.*, a freshly prepared solution containing DTT, see below) and within 16 min. became nearly identical with that of the disulfide (III). The same process was observed to occur at a comparable rate under alkaline conditions, while acid pH decreased the rate of oxidation, and the compound appeared to be relatively stable at pH < 3.7. Thus, solutions of I in 5×10^{-4} *N* hydrochloric acid did not show appreciable change within several hours and could be used as stock solutions in the spectrophotometric studies. (See *Experimental.*)

DTT was found to be an effective protecting

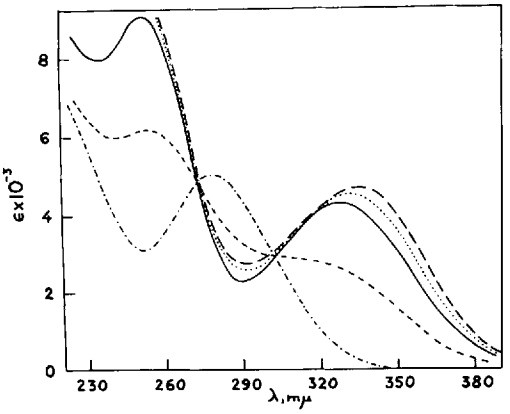


Fig. 2.—Ultraviolet absorption spectra of 5-mercaptouracil (I) stabilized by the addition of DTT (see text) at various pH values. Key: – · – ·–, pH = 2.0; – – – – –, pH = 5.3; ——, pH = 7.7; . . . . . , pH = 10.6; — — —, pH = 11.8.

TABLE I.—SPECTROPHOTOMETRICALLY DETERMINED, APPARENT pKa VALUES[a]

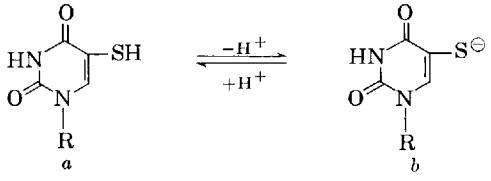
Compd.	pKa_1	pKa_2	pKa_3
I	5.3	10.6	>13
II	5.0	10.5	..
III	8.0	>13	..
IV	8.1	>13	..
V	8.5	>13	..
VI	>8[b]	...	..

[a] Limits of accuracy: ±0.05 pH units. [b] Hydrolysis occurs at pH > 8.

agent by virtue of its low redox-potential (5). This compound not only prevented the air oxidation of I, but it was also capable of reducing, rapidly and quantitatively, the disulfides (III and IV) to the corresponding thiols (I and II), respectively. Thus, in the presence of excess DTT, it was possible to determine the ultraviolet absorption spectra of the free thiols at various pH values and to estimate their dissociation constants by spectrophotometric methods. (See *Experimental.*) The ultraviolet absorption of DTT itself interfered with the spectra of I and II only at pH > 9, and then only in the range below 260 mμ. The spectra of I at various pH values in the presence of DTT are shown in Fig. 2; in the pH 2-7 range, two isosbestic points appear, at 273 and 302 mμ.

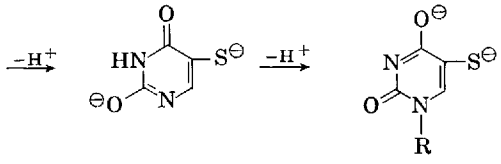
The apparent pKa values of I and its derivatives are given in Table I. The pKa_1 values corresponding to the first acid dissociation equilibria of I (5.3) and II (5.0), respectively, are considerably lower than the pKa of thiophenol (7.76) (9). 5-Nitrouracil has a similarly low pKa_1 as I, and in the case of the former this is due to the powerful electron-attracting inductive and conjugative effects of the nitro group both of which promote the ionization of the N-1 hydrogen (10). The relatively small, electron-releasing conjugative effect of the divalent sulfur (11) is essentially inoperative from the 5-position of the uracil nucleus (*meta* to both ring-nitrogens) (12) and the inductive (−I) effect of the C → S bond prevails. This would cause only a moderate increase in the acidity of the N-1, or N-3 hydrogen, corresponding to lowering of the pKa_1 value only by about 1 unit, as seen by comparison of the pKa_1 values (8.0–8.5) of the *S*-substituted derivatives, III, IV, V, and VI (Table I), with that of uracil (9.45) (10). Therefore, the much lower pKa_1 values of I and II clearly correspond to the dissociation of the sulfhydryl group, and the high acidity of this group must be attributed to the electron-attracting effect of the uracil nucleus. This effect seems to be slightly larger in the case of II. (Scheme I.)

Table II summarizes the spectrophotometric data of compounds I–V. The free thiols (I and II) are readily distinguished from their disulfides and other *S*-substituted derivatives by their characteristically large bathochromic shifts in neutral or basic solutions (*i.e.*, in their ionized forms). Compound II shows slightly higher wavelength and greater intensity absorption than I in both the "neutral" (*a*) (pH 2) and "mono-anionic" (*b*) (pH 7.7) form.



I, R = H
II, R = deoxyribosyl

Scheme I

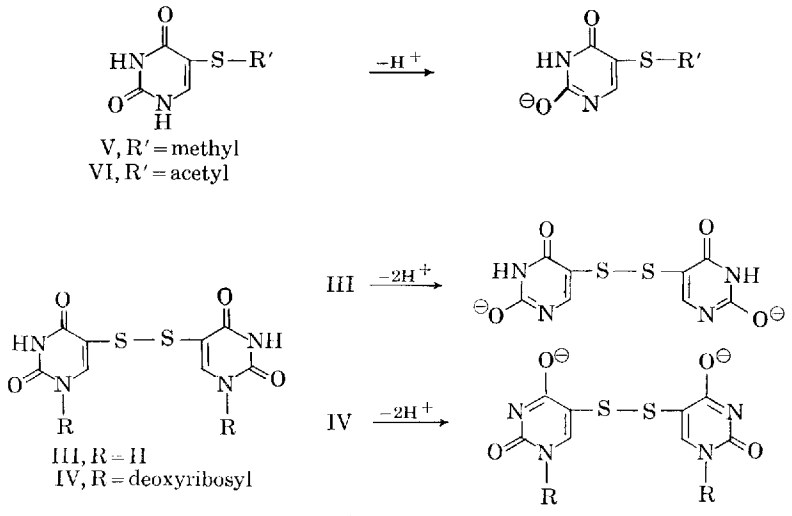


Scheme II

TABLE II.—ULTRAVIOLET ABSORPTION DATA

Compd.	pH	$\lambda_{max.}$[a]	$\epsilon \times 10^{-3}$[b]	$\lambda_{min.}$[a]	$\epsilon \times 10^{-3}$[b]
I	2.0	280	5.0	253.5	2.9
	7.7	253.5	9.4	239	8.3
		329.5	4.5	291	2.1
	11.8	(250–260)[c]	...[c]	...[c]	...[c]
		336	4.9	295	2.5
II	2.0	284	6.4	254	3.0
	7.7	253.5	9.4	242.5	8.4
		334	5.0	292	2.2
	11.8	(250–260)[c]	...[c]	...[c]	...[c]
		319.5	4.5	291	3.5
III	2.0	272	14.9	234	11.1
	10.6	292	18.5	241	9.0
IV	2.0	270.5	16.9	226	10.0
	7.0	270	16.5	231	10.7
	11.8	268	13.4	254	12.8
V	2.0	227 (infl.)	6.3		
		270 (infl.)	4.7		
	10.6	233.5	7.3	263	3.6
		294	7.7		
VI	2.0	269.5	7.4	242	3.8

[a] Absorption maxima and minima, wavelength in mμ. [b] Molar absorptivities at given wavelength. [c] Presence of DTT prevents correct reading below 260 mμ.



Scheme III

Ionization of the second proton (pH 11.8 in Table II) increases both the wavelength and the intensity of the absorption band in the 330 mμ region in the case of I, and decreases both values in the case of II; however, the dianions of I and II have of necessity different structures. (Scheme II.)

The *S*-substituted derivatives, III, V, and VI, show much smaller acid-base shifts in their ultraviolet spectra than the free thiols and rather resemble thymine in their spectral behavior (10), indicating that their first ionization involves the N-1 hydrogen. The disulfide (IV), in which both the sulfhydryl group and the N-1 position are blocked, shows no acid-base shift, being in this respect similar to 1-methyl-uracil (10) or thymidine (13). (Scheme III.)

Since the ionizations of the symmetrical disulfides (III and IV) probably proceed in two steps, *i.e.*, giving rise to intermediate structures in which only one of the pyrimidines is ionized, the spectra of these compounds do not give real "isosbestic points" (Fig. 3), and their pKa_1 values actually represent averages of *two* pKa's which are very close and cannot be clearly distinguished from each other.

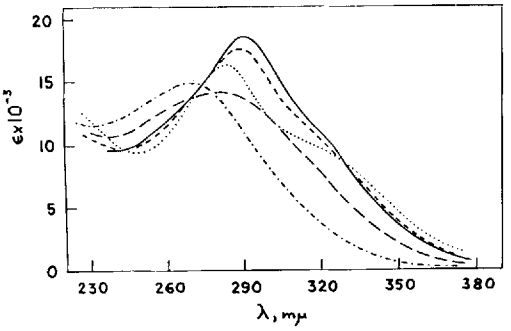


Fig. 3.—Ultraviolet absorption spectra of 5-uracilyl-disulfide (III) at various pH values. Key: -·-·-·, pH = 2.0; ———, pH = 7.7; ——, pH = 10.6; - - - - - -, pH = 13.0; . . . . . , pH = 14.0.

DTT was found to be a useful reducing agent for the preparative conversion of the disulfide (III) to the free thiol (I). (See *Experimental.*) This conversion was accomplished previously by a much less convenient procedure (using zinc and sulfuric acid) (1, 7), which cannot be applied to the reduction of the deoxyriboside (IV).

For the determination of the free thiol content of various preparations, the method of Klotz and Carver (6) was employed in a somewhat simplified form, without the elaborate precautions recommended for the exclusion of air. This, however, gave variable results in the case of 5-mercaptouracil since a considerable portion of the sample oxidized during the titration procedure. Glutathione, on the other hand, was found to be quite stable under the same conditions. Taking advantage of this fact, an "indirect method" was developed. (See *Experimental.*) The results[8] are given in Table III.

TABLE III.—SULFHYDRYL GROUP ANALYSIS

Compd.	Free SH/S × 100[a] Direct[b]	Indirect[c]
I[d]	55–85	98
I[e]	. . .	98–100
III	<2	<2
Glutathione	100	100
DTT	. . .	98–100

[a] Per cent of total sulfur present in the form of free sulfhydryl-group. [b] As determined by direct titration with mersalyl acid (6). [c] As determined by the modified, indirect procedure. (See *Experimental.*) [d] Recrystallized and stored in the desiccator for a period of over 12 months. [e] Prepared from III by reduction with DTT. (See *Experimental.*)

The results indicate that I is quite stable for a period of at least 1 year when stored in solid dry state. It is also seen from Table III that the reduction of the disulfide (III) to the free thiol (I) by DTT has proceeded in a quantitative manner.

[8] The authors acknowledge the technical assistance of Mr. Peter Forgach in these determinations.

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Absorption, Distribution, and Elimination of a Long-Acting Vitamin B_{12} Preparation

By KNUD KRISTENSEN* and TAGE HANSEN

The prolonged effect of various long-acting vitamin B_{12} preparations has been examined on rabbits regarding absorption, distribution, and elimination after intramuscular injection.

RECENT investigations (13) have shown that patients with pernicious anemia have considerably greater requirements for vitamin B_{12} than have previously been assumed. More recently, there has been considerable interest in preparations that can meet the requirements more adequately and in a more satisfactory way than the aqueous solutions of vitamin B_{12} used previously. The authors have investigated the characteristics of various preparations made on the basis of various principles. A preparation containing cyanocobalamin–tannin complex suspended in aluminum monostearate oil gel[1] was studied in detail.

The clinical value of this preparation is reflected in papers by Bastrup-Madsen *et al.* (4, 5), Schwartz *et al.* (19, 20), Meulengracht (14, 15), an editorial (8), Nielsen and Vedsø (16), Schrumpf (18), and Gough *et al.* (10).

EXPERIMENTAL

Materials and Methods

Preparations A–G were investigated (Table I). After intramuscular injection (i.m.) in rabbits, the following were investigated: (*a*) liberation of vitamin $B_{12}{}^{60}Co$ from the site of injection; (*b*) absorption of vitamin $B_{12}{}^{60}Co$ by the liver; and (*c*) excretion of vitamin $B_{12}{}^{60}Co$ in urine and feces.

For preparations F and G the investigations were supplemented by radioactivity counts and microbiological determinations of vitamin B_{12} in liver, kidney, and femoral muscle about 3 months after the start of trial.

Preparations

A number of preparations were made using vitamin B_{12} labeled with ^{60}Co. Their composition is shown in Table I. The products were prepared according to methods described in a British patent (6).

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Preparations F and G correspond to a marketed suspension of cyanocobalamin–tannin complex in aluminum monostearate oil gel,[1] except that labeled vitamin B_{12} (^{60}Co) was used instead of ordinary cyanocobalamin. Spectrophotometric and microbiological checks were made on the preparations. The radiochemical purity of the labeled compounds was confirmed by paper chromatography and subsequent counts and by microbiological determinations of the vitamin B_{12} activity on agar plates with *Lactobacillus leichmannii*, as described by Winsten and Eigen (23), among others.

Animal Material.—Twelve white male rabbits were used. The initial weights were from 2.7 to 3.0 Kg. The rabbits were anesthetized with sodium amobarbital (30 mg./Kg.) supplemented by ether during the radioactivity counts.

Standards and Methods of Measurement.—The radioactivities above the site of injection and above the liver were determined on a scintillation detector shielded by 5 cm. of lead with a 30-mm. round opening upon which the object to be measured was placed. Feces and organs were homogenized before

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* Present address: Danish National Health Service, Copenhagen, Denmark.
[1] Marketed as Betolvex by Dumex Ltd., Copenhagen, Denmark.

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Absorption, Distribution, and Elimination of a Long-Acting Vitamin B_{12} Preparation

By KNUD KRISTENSEN* and TAGE HANSEN

The prolonged effect of various long-acting vitamin B_{12} preparations has been examined on rabbits regarding absorption, distribution, and elimination after intramuscular injection.

RECENT investigations (13) have shown that patients with pernicious anemia have considerably greater requirements for vitamin B_{12} than have previously been assumed. More recently, there has been considerable interest in preparations that can meet the requirements more adequately and in a more satisfactory way than the aqueous solutions of vitamin B_{12} used previously. The authors have investigated the characteristics of various preparations made on the basis of various principles. A preparation containing cyanocobalamin–tannin complex suspended in aluminum monostearate oil gel[1] was studied in detail.

The clinical value of this preparation is reflected in papers by Bastrup-Madsen *et al.* (4, 5), Schwartz *et al.* (19, 20), Meulengracht (14, 15), an editorial (8), Nielsen and Vedsø (16), Schrumpf (18), and Gough *et al.* (10).

EXPERIMENTAL

Materials and Methods

Preparations A–G were investigated (Table I). After intramuscular injection (i.m.) in rabbits, the following were investigated: (*a*) liberation of vitamin $B_{12}{}^{60}Co$ from the site of injection; (*b*) absorption of vitamin $B_{12}{}^{60}Co$ by the liver; and (*c*) excretion of vitamin $B_{12}{}^{60}Co$ in urine and feces.

For preparations F and G the investigations were supplemented by radioactivity counts and microbiological determinations of vitamin B_{12} in liver, kidney, and femoral muscle about 3 months after the start of trial.

Preparations

A number of preparations were made using vitamin B_{12} labeled with ^{60}Co. Their composition is shown in Table I. The products were prepared according to methods described in a British patent (6).

The distribution and excretion of vitamin B_{12} after parenteral administration of preparation B and of aqueous solutions of cyanocobalamin were investigated in rats and in healthy subjects by Davis *et al.* (7), Thompson and Hecht (22), Astudillo *et al.* (3), and Glass *et al.* (9), but no records have been found of investigations on patients with pernicious anemia. The authors have not investigated any preparations of vitamin B_{12} suspended in oil or vitamin B_{12} suspended in 2% monostearate oil gel; the latter was described by Arnold *et al.* (1, 2), Sobell *et al.* (21), and Heinrich and Gabbe (11). It does not appear to possess any retarded action of interest for clinical use.

Preparations F and G correspond to a marketed suspension of cyanocobalamin–tannin complex in aluminum monostearate oil gel,[1] except that labeled vitamin B_{12} (^{60}Co) was used instead of ordinary cyanocobalamin. Spectrophotometric and microbiological checks were made on the preparations. The radiochemical purity of the labeled compounds was confirmed by paper chromatography and subsequent counts and by microbiological determinations of the vitamin B_{12} activity on agar plates with *Lactobacillus leichmannii*, as described by Winsten and Eigen (23), among others.

Animal Material.—Twelve white male rabbits were used. The initial weights were from 2.7 to 3.0 Kg. The rabbits were anesthetized with sodium amobarbital (30 mg./Kg.) supplemented by ether during the radioactivity counts.

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TABLE I.—DOSAGE SCHEDULE OF VARIOUS VITAMIN B_{12} PREPARATIONS

Prepn.	Form of Vitamin B_{12}	Vehicle	Vitamin B_{12} Concn., mcg./ml.	B_{12}-^{60}Co Concn., μc./ml.	Quantity Injected, ml.
A	Cyanocobalamin	Aqueous 0.9% NaCl soln.	500	0.18	0.30
B[a]	B_{12}–zinc–tannate	Aqueous suspension	500	0.18	0.30
C	B_{12}–zinc–tannate	Suspended in 2% aluminum monostearate oil gel	500	0.5	0.25
D[b]	B_{12}–tannate	Suspended in 2% aluminum monostearate oil gel	500	0.5	0.25
E	B_{12}–tannate	Suspended in sesame oil	500	0.5	0.25
F[b]	B_{12}–tannate	Suspended in 2% aluminum monostearate oil gel	1000	1.0	0.50
G[b]	B_{12}–tannate	Suspended in 2% aluminum monostearate oil gel	1000	1.0	0.50

[a] Trademarked preparation is Depinar. [b] Trademarked preparation is Betolvex.

counting. A scintillation detector with a well-type crystal was used for counting radioactivity in urine and liquid samples of feces and organs.

The microbiological vitamin B_{12} activity was determined by the *L. leichmannii* method (12) as modified by Noer (17).

Before and after each measurement the scintillation detector was checked against a standard and corrected accordingly.

In order to determine the absolute content of radioactive vitamin B_{12} in samples of urine, feces, and organs, a known quantity of the injection preparation in question was added after the samples had been counted. Thus, the counts of the samples and of the standards were carried out under the same conditions.

Trial Methods.—Blank values were determined on urine and feces collected for 24 hr. from the rabbits in specially constructed metabolism cages. After being anesthetized, the rabbits were strapped to the counting table with the right femoral muscle above the detector.

After the background had been counted, the preparation to be tested was injected intramuscularly into the right femoral muscle directly above the detector. The doses employed are shown in Table I. Immediately afterward, the radioactivity at the site of injection was screened at various sites around the injection site, and the counts were carried out at the site of highest activity. Immediately afterward, the radioactivity in the hepatic region was determined in the manner described above.

Counts of the activity at the site of injection and in the hepatic region were carried out at irregular intervals for 3 months, and their relative values, as percentages of the initial count, were calculated.

During the first 3–4 days after the injection, urine and feces were collected as 24-hr. samples.

In the tests made with preparations F and G, the rabbits were killed with ether after the test period of about 3 months; liver, kidney, and femoral muscle were dissected free, and their contents of radioactive material were determined. In addition to the organs mentioned above, the spleen, heart, bladder (containing urine), small intestine (duodenum, jejunum, ileum), large intestine (cecum, including vermiform appendix, colon, rectum), and contents of both large and small intestine were collected from rabbit *3*. (See Table II.) Liver, kidneys, and muscle from the right femur were taken from the control rabbit, which had been kept on the same diet as the test animals for 3 months, in order to obtain a basis of comparison and a standard for the content of vitamin B_{12}. A microbiological determination of vitamin B_{12} was carried out on all homogenates of liver, kidney, and femoral muscle.

RESULTS

Measurement Above the Site of Injection.—Figure 1 shows the increase of prolonged effect obtained by combining the different principles. It will be seen that a clearly prolonged effect is only obtained by suspending a vitamin B_{12} compound in

TABLE II.—PERCENTAGE RADIOACTIVITY ABOVE SITE OF INJECTION

Rabbit	Prepn.	Std.	Days After Injection 2	3	4	7	9	10	11	15	18	21	25	28	30
1	F	100	69.8		65.7				60				47.9		
2	F	100		76.2			68.2					52.7			
3	G	100	86.5					85		81.5				72	
4	G	100		61.1		55.1			50		40.5				34.6

TABLE II.—(*Continued*)

Rabbit	Prepn.	Std.	Days After Injection 37	39	44	46	53	59	66	78	87	95
1	F	100		34			22.7			9.7		
2	F	100	38.9						19.9		7.6	
3	G	100			54.6							
4	G	100				25.5		19.4				4.1

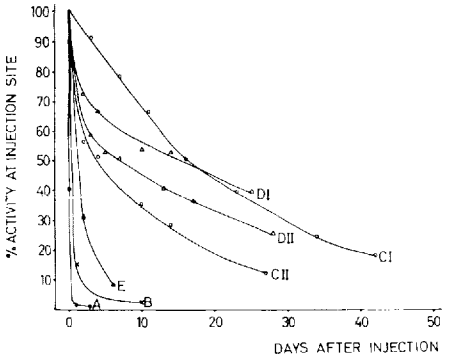

Fig. 1.—Absorption of radioactive vitamin B_{12} from the site of injection. Key: A, aqueous solution of vitamin B_{12}; B, aqueous suspension of B_{12}–zinc–tannate; C_I and C_{II}, B_{12}–zinc–tannate suspended in a 2% aluminum monostearate oil gel; D_I and D_{II}, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel; E, B_{12}–tannate suspended in oil.

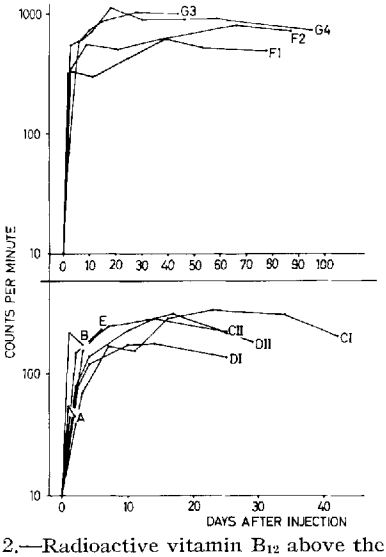

Fig. 2.—Radioactive vitamin B_{12} above the liver. Key: (bottom) A, aqueous vitamin B_{12} solution; B, aqueous suspension of B_{12}–zinc–tannate; C_I and C_{II}, B_{12}–zinc–tannate suspended in a 2% aluminum monostearate oil gel; D_I and D_{II}, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel. E, B_{12}–tannate suspended in oil. Key: (top) F_1 and F_2, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel; G_3 and G_4, B_{12}–tannate suspended in a 2% aluminum monostearate oil gel.

aluminum monostearate oil gel. Table II shows the prolonged effect of two preparations of vitamin B_{12}–tannate in aluminum monostearate oil gel (F and G). Rabbit *3*, which died during narcosis about 1.5 months after the beginning of the investigation, differed from the other three. During the period of investigation the rabbit did not move about much. This could have been the reason for the small liberation of vitamin B_{12} from its intramuscular depot.

Measurement Above the Liver.—Figure 2 gives an impression of the storage of vitamin B_{12} in the liver, obtained by combining the different principles. The graphs for preparations F and G show considerably higher counts because of the larger amount of radioactivity injected (see Table I).

The counts shown in Fig. 2 were obtained by deducting the value expressing a scattered radiation from the depot of the femoral muscle from the counts corrected for background. The value of scattered radiation decreases in proportion to the activity at the site of injection; accordingly, the correction will be:

(corr. counts per minute above liver immediately after injection) × (% activity at site of injection on the day in question) divided by 100.

Because of the somewhat inaccurate method of measurement, only the shapes of the graphs, not the absolute values should be compared.

Excretion in Urine.—Table III shows the difference in excretion in urine after administering the various preparations. The difference in urinary excretion between preparation D and preparations F and G may be explained partly by the larger quantity of F and G injected, and partly by a minute amount of free vitamin B_{12} in the preparations.

Excretion in Feces.—The excretion in the feces was <1%/24 hr.

Content of Vitamin B_{12} in Liver, Kidneys, and Femoral Muscle.—At the time when the rabbits were killed the vitamin B_{12} contents of liver, kidneys, and femoral muscle were measured, both by radioactivity measurements and by microbiological determinations. Table IV shows the results. The counts immediately show that the content of vitamin B_{12} in liver and kidneys of rabbits treated with preparations F and G has increased compared with that of the control rabbit. The content of vitamin B_{12} in the right femur is due to vitamin B_{12} not yet liberated from the site of injection, and a reasonable agreement with the results obtained by radioactivity measurements above the site of injection was found. The difference between vitamin B_{12} measured microbiologically and vitamin B_{12} determined by radioactivity gives us the level of vitamin B_{12} in the rabbits before the injection and is of the same order of magnitude as those obtained microbiologically from the control rabbit.

Radioactive Vitamin B_{12} Content in Other Organs and Tissues (Rabbit 3).—*Spleen.*—No measurable vitamin $B_{12}{}^{60}Co$ (<1% of dose administered).

Heart.—No measurable vitamin $B_{12}{}^{60}Co$ (< 1% of dose administered).

Contents of Intestine.—Less than 1% of dose administered.

TABLE III.—URINARY EXCRETION OF VARIOUS VITAMIN B_{12} PREPARATIONS

Rabbit	Prepn.	1st. 24 hr., %	2nd. 24 hr., %	3rd. 24 hr., %	4th. 24 hr., %
	A	60	<5	<2	...
	B	10–15	<5	<2	...
	C	<1	<1	<1	...
	D	<1	<1	<1	...
	E	19	11	2	...
1	F	7	6	<1	<1
2	F	13	1	<1	<1
3	G	2	4	<1	<1
4	G	6	1	<1	<1

TABLE IV.—VITAMIN B_{12} CONTENTS OF LIVER, KIDNEYS, AND RIGHT FEMORAL MUSCLE

Prepn.	F		G		
Rabbit	1	2	3	4	5 (Control)
Days after inj.	78	87	44	95	...
Liver					
Weight, Gm.	184	151	71	168	124
Vitamin B_{12}, microbiologically, total, mcg.	66.8	65.3	35.2	81.5	30.1
Total vitamin B_{12} radioactivity, mcg.	28.9	23.6	23.9	49.1	...
Kidneys					
Weight, Gm.	13.7	13.9	13.5	14.8	19.3
Vitamin B_{12}, microbiologically, total, mcg.	6.2	7.5	6.0	7.4	4.2
Total vitamin B_{12} radioactivity, mcg.	3.2	3.0	2.5	4.4	...
Right Femoral Muscle					
Weight, Gm.	101	126	98	131	112
Vitamin B_{12}, microbiologically, total, mcg.	67	26	...	30	8
Total vitamin B_{12} radioactivity, mcg.	60	23	275	24	...
Radioactivity above site of injection, % (see Table II)	12% of dose 9.7	4.6% of dose 7.6	55% of dose 54.6	4.8% of dose 4.1	...

Small Intestine (*Jejunum, Duodenum, Ileum*).—Less than 1% of dose administered.

Large Intestine (*Cecum, Including Vermiform Appendix, Colon, Rectum*).—Less than 1% of dose administered.

Bladder (*Containing Urine*).—Less than 1% of dose administered.

DISCUSSION

These investigations on rabbits clearly reveal that preparations of B_{12}–tannate or B_{12}–zinc–tannate in a 2% aluminum monostearate oil suspension release vitamin B_{12} from an intramuscular depot over a much longer period of time than does B_{12}–zinc–tannate in an aqueous suspension or B_{12}–tannate in an oil suspension or vitamin B_{12} in a water solution (Fig. 1).

Vitamin B_{12} preparations in a 2% aluminum monostearate oil suspension can release vitamin B_{12} for the body for more than 1 month compared with a few days for the other types of preparations investigated.

By this prolonged release one avoids the urinary excretion that always follows injections of vitamin B_{12} in excess of serum vitamin B_{12} binding capacities. This also means that vitamin B_{12} is available for storage in vitamin B_{12} depots over a longer period of time (Fig. 2). Due to the techniques used, only the shapes of the curves are comparable. A more detailed study of 2 different vitamin B_{12} preparations in a 2% aluminum monostearate oil suspension showed vitamin B_{12} was released from the intramuscular depots for more than 2 months, with some of the injected material still left at the injection site as vitamin B_{12} as determined by microbiological methods. Table IV shows there is good agreement between direct measurements above the site of injection and measurements of the dissected femoral muscle.

Furthermore, it is shown (Table IV) that the released vitamin B_{12} was deposited in the liver and kidneys, as could be expected, and that the liver and kidneys from rabbits injected with B_{12}–tannate in a 2% aluminum monostearate oil suspension contained a considerably greater amount of vitamin B_{12} in comparison with the control.

SUMMARY

By investigations carried out on rabbits, the properties of various vitamin B_{12} preparations with prolonged effect are demonstrated, including the liberation from intramuscular depots and the storage in the natural depots of the body. After intramuscular injection of B_{12}–tannate in aluminum monostearate oil gel the observations were: (*a*) a prolonged liberation of vitamin B_{12} from the depot injected into the muscle; (*b*) an increased vitamin B_{12} activity in liver and kidneys, taken as a sign of storage in the natural depots of the body; and (*c*) minimum excretion in urine.

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Mechanism of Action of Starch as a Tablet Disintegrant I

Factors that Affect the Swelling of Starch Grains at 37°

By JAMES T. INGRAM and WERNER LOWENTHAL

A study was initiated to determine if starch grains swell at 37° and what environmental conditions may influence swelling. To determine the extent of swelling which starch undergoes in various media, individual grain dimensions were measured microscopically. Full factorial experiments were conducted, and analyses of variance were calculated to determine whether significant differences in the mean grain sizes could be demonstrated when environmental conditions were changed. Potato, corn, and amioca starches and moisture content showed significant swelling in distilled water and simulated gastric fluid U.S.P. This was postulated as due to initial size difference of the grains being maintained during the experiment. Variation in pH had very little effect on swelling; however, evidence was obtained to show that the less acid medium produced more swelling than the media of lower pH. Salts did affect swelling, and results indicated that salts of polyvalent cations produced more swelling than the salts of monovalent cations. Analysis of the effect of time on swelling indicated that any swelling of the starch grains is apparently instantaneous.

IT IS DESIRABLE that a tablet, when used, disintegrate as rapidly as possible. Ideally, the process of tableting should not alter the therapeutic action of a drug or the time in which this action is produced. The same effect should be produced in essentially the same time when a tablet is swallowed as when the drug is taken in powder form.

The rate at which a physiological effect is produced from a drug taken orally is dependent upon the rate of absorption from the gastrointestinal tract. Before a drug in tablet form may be absorbed, it must first be released from the tablet by disintegration of the tablet. The usefulness of a tablet, therefore, arises almost wholly from its ability to disintegrate upon contact with liquid.

Compressed tablets are ordinarily manufactured containing substances to accelerate or aid their disintegration. Cornstarch has long been the standard disintegrant for compressed tablets. The mechanism by which starch functions as a tablet disintegrant has been assumed to be that the starch grains swell when in contact with moisture, causing the tablet to break open.

Knowledge of the exact mechanism of action of starch as a tablet disintegrant would be useful in the development of more efficient disintegrating agents.

DISCUSSION

Review of Pertinent Literature.—Little work has been reported on the exact mechanism of action of starch as a disintegrating agent. After an extensive literature search, all references which had been located state that starch acts as a disintegrant by swelling, but no data or proof are presented or referred to indicate that starch swells sufficiently *in vivo* or *in vitro* to cause disintegration (1, 2).

Curlin (3) tested the disintegration of aspirin tablets in cold water and found by microscopic examination that the starch grains were not swollen after disintegration.

Billups and Cooper (4) reviewed the proposed theories explaining the mechanism of action of tablet disintegration. They indicate that the most widely held view is that disintegration is caused by absorption of water by the disintegrating agent and development of pressure within the tablet by swelling or expansion of the disintegrating agent. In their study on the correlation of water absorption with tablet disintegration time, they concluded that while water absorption is a common qualitative characteristic of many disintegrating agents, it is not a quantitative measure of their effectiveness. Disintegrating agents with the highest rates of water absorption did not produce the fastest disintegration times. Some evidence supported the view that disintegrating agents with the highest rates of water absorption produced the longest disintegration times. Their results also showed that the amount of water absorbed by cornstarch, dried to a constant weight, after exposure to 98% relative humidity, was only 0.5% w/w in 50 min.

Crossland and Favor (5) employing a viscous water-binding dispersion medium (sodium alginate and high viscosity type carboxymethylcellulose), showed by means of viscosity measurements the stages of swelling. In the starch–alginate–water system, the first indications of swelling are observed at about 55°.

Leach, McCowen, and Schoch (6) evaluated swelling of various starches over a range of pasting temperatures (about 50–95°) and found that the swelling pattern was greatly influenced by the species of starch. Cornstarch showed a limited two-stage swelling, whereas potato starch underwent very

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rapid and unrestricted swelling at about 55°. The swelling of the grains was determined from the weighing of starch after submersion in water and correcting the value for solubles.

Hellman, Boesch, and Melvin (7) compared swelling of starch grains to water absorbed from atmospheres of different moisture content by following the dimensional changes of individual starch grains microscopically at a temperature of 25.10° over a period of 8–24 hr. At 100% relative humidity cornstarch showed a 9.1% increase in diameter over the diameter of vacuum dried starch. The corresponding increases for other starches examined under similar conditions were 12.7% for potato starch, 28.4% for tapioca starch, and 22.7% for waxy cornstarch.

Variation in the effect of different salt solutions on initial gelatinization of starches was shown by Sandstedt, Kempf, and Abbott (8). They showed that salt solutions change not only the temperature of initial gelatinization but also the initial temperature of each stage of gelatinization, length of transition period between stages, and rate or amount of gelatinization in each stage of the entire course of gelatinization. These changes were not correlated with each other. Their data indicated that each change in the course of gelatinization was due to a different property of the salt.

Abbott *et al.* (9) determined *in vitro* disintegration time of several commercial tablets in simulated gastric juice and human gastric juice and showed that disintegration is prolonged in human gastric juice. There is some correlation between prolongation of disintegration and gastric mucoid content. A high mucoid content in gastric juice may increase the rate of disintegration more than 16 times. *In vitro* disintegration is prolonged in simulated gastric juice if the tablets are first exposed to mucoidal material from human gastric juice.

EXPERIMENTAL

This study was planned to determine if starch grains swell at 37° and what environmental conditions may influence the swelling of starch grains at 37°. The following variables were studied: (*a*) time, (*b*) pH, (*c*) effect of ions and ionic concentration, (*d*) starch species, and (*e*) simulated gastric fluid U.S.P. (SGF). Evanson and DeKay (10), in a study of tablet disintegration, reported inconsistent results when distilled water was compared with artificial gastric juice with respect to time. For this reason distilled water was included as a submersion medium in this study to allow comparisons with other submersion media.

Procedure.—To determine the extent of swelling, if any, which starch undergoes in various media, microscopic examination was used and individual grain dimensions were measured. For the measurements of corn and amioca starches, an oil immersion 97× objective and 5× ocular micrometer were used. For the measurements of potato starch, a 43× objective and a 5× ocular micrometer were used, and measurements were mathematically converted to 485 magnification. Owing to the spherical and ellipsoidal shapes of the grains, the apparent grain length changed with position of focus. To achieve uniformity, each grain was measured at that depth of focus producing maximum diameter. The starch samples were slurried in the appropriate media at 37° and maintained at that temperature until a sample was removed at the appropriate time, placed on a slide, and covered with a cover slip. One hundred or 200-grain measurements were made from each slide. A mechanical stage was used and as the field of view was moved horizontally, each starch grain crossing the micrometer scale was measured. Each scale division on the micrometer represents 1.80 μ when using the 485 magnification.

Commercial grades of corn, potato, and amioca (waxy corn) starches were chosen for the initial investigation. To determine the effect of moisture content on swelling, both low and high moisture content forms of each starch were examined. For the high moisture content, the commercially available starches[1] were used. For the low moisture content, each starch was dried at 93° until a constant moisture content was obtained as determined on a moisture balance.[2] Moisture contents obtained were as follows: cornstarch, 2.25% w/w; potato starch, 2.25% w/w; and amioca starch, 2.88% w/w. In addition to these dried starches, a commercial redried cornstarch[3] (P-825) containing 1.5% maximum moisture was examined.

RESULTS AND DISCUSSION

In examining the factors influencing the swelling of starch grains, full factorial experiments were designed, and analyses of variance were calculated to determine whether significant differences in the grain sizes could be demonstrated when environmental conditions were changed. Preliminary experiments showed that the method of measurement was reproducible.

Distilled water and SGF were compared as submersion media by varying the type of starch, moisture content, and time in two 2 × 3 × 3 factorial designs. The mean grain sizes are shown in Table I for the distilled water and in Table II for SGF. In each experiment the only single effect producing a significant difference in grain size was the type of starch. In each case, the type of starch was significant at the 0.5% level and was shown by Duncan's multiple range tests (11) to be due to the potato starch. No significant difference was shown between corn and amioca starches.

The mean grain size of potato starch is approximately 29 μ while that of cornstarch is approximately 10.5 μ and that of amioca is approximately 9.5 μ. The large difference in mean grain sizes between the potato and the cornstarches would result in a larger error mean square term in the analyses of variance. This is due to the fact that the variances are proportional to the mean grain sizes. Because of this, the analyses of variance were recalculated omitting the potato starch data. This was done to obtain a more sensitive comparison between corn and amioca starches, whose mean grain sizes were more nearly the same. Moisture content and time became significant at the 2.5%

[1] Cornstarch and potato starch marketed by S. B. Penick and Co. as *Melojel (MJ) and amioca starch* marketed by National Starch and Chemical Corp.
[2] Cenco Moisture Balance, Central Scientific Co., catalog No. 26680-1.
[3] Marketed as Purity 825 by National Starch and Chemical Corp.

TABLE I.—EFFECT OF MOISTURE CONTENT AND TIME ON MEAN GRAIN SIZES OF VARIOUS STARCHES SUBMERSED IN DISTILLED WATER

		Mean Grain Sizes in Scale Divisions[a]		
		Time, min.		
Type	Moisture	0	5	30
Corn	High (MJ)	6.133	6.129	5.823
	Low (P-825)	5.676	5.919	5.652
Potato	High	16.354	14.863	11.948
	Low	16.243	12.935	14.679
Amioca	High	5.482	6.157	6.221
	Low	5.063	5.672	6.245

[a] One scale division = 1.8 μ.

TABLE II.—EFFECT OF MOISTURE CONTENT AND TIME ON MEAN GRAIN SIZES OF VARIOUS STARCHES SUBMERSED IN SGF

		Mean Grain Sizes in Scale Divisions[a]		
		Time, min.		
Type	Moisture	0	5	30
Corn	High (MJ)	6.133	6.408	6.221
	Low (P-825)	5.676	5.805	5.955
Potato	High	16.354	13.482	12.935
	Low	16.243	20.042	17.065
Amioca	High	5.482	6.388	6.467
	Low	5.063	6.320	6.225

[a] One scale division = 1.8 μ.

TABLE III.—EFFECT OF TIME OF SUBMERSION ON MEAN GRAIN SIZES OF VARIOUS LOW MOISTURE CONTENT STARCHES IN SGF

	Mean Grain Sizes in Scale Divisions[a]					
	Time, min.					
Type	0	5	30	60	120	180
Corn (dried)	5.020	5.475	5.050	5.185	5.620	5.620
Corn (P-825)	5.676	5.805	5.955	6.600	5.645	5.510
Potato (dried)	16.243	20.042	17.065	18.376	13.821	15.198

[a] One scale division = 1.8 μ.

level in the distilled water experiment and at the 10 and 5% levels, respectively, for the SGF experiment. Duncan's multiple range tests showed in each case that the difference in grain size was between unsubmerged starch (zero time) and starch which had been submerged. No significant difference in grain diameter was observed in the starches which had been submerged between 5 and 30 min. Preliminary experiments had shown that submersion time was not significant but no comparisons were made to unsubmerged starch.

Starch moisture content effect was probably significant because the initial size differences between the high and low moisture content of the starches were maintained throughout the experiment.

To investigate further the effect of moisture content and submersion time on swelling, a 3 × 6 factorial experiment was designed using three low moisture content starches and SGF as the submersion medium. The starches were slurried and the diameters measured at various time intervals up to 3 hr. The data are given in Table III. The analysis of variance showed that the only significant difference observed was in the type of starch. This was again shown by Duncan's multiple range test to be due to the potato starch; the two forms of cornstarch were not significantly different.

The analysis of variance was recalculated, omitting the potato starch, and a significant difference was shown between the two cornstarches at the 10% level. This difference is again most likely due to the initial differences in the grain sizes which was maintained up to 120 min.

A 3 × 6 factorial experiment was designed to determine the effect of pH and time of submersion on swelling. Commercially dried cornstarch[3] was used for this experiment. The mean grain sizes are presented in Table IV. The analysis of variance showed that pH was significant at the 10% level, and a breakdown of this effect showed that at a pH of 5.3 the swelling that occurred was significantly greater than that at pH 1.3 or 3.3. The significance of the swelling at pH 5.3 could be due to onset of starch hydrolysis. Starch will hydrolyze at an alkaline pH. Unsubmerged starch (zero time) was included in this experiment to allow better comparisons between pH and time, but no significant difference was demonstrated between the measurements at the various time intervals.

To investigate the effect of salts on swelling of untreated starch grains, a 2 × 2 × 3 × 4 factorial experiment was designed. Unsubmerged starch

TABLE IV.—EFFECT OF pH OF SUBMERSION MEDIUM ON MEAN GRAIN SIZES OF LOW MOISTURE CONTENT CORNSTARCH

	Mean Grain Sizes in Scale Divisions[a]		
	pH		
Time, min.	1.3	3.3	5.3
0	5.676	5.676	5.676
5	5.675	5.285	6.070
30	5.995	5.750	5.930
60	5.455	5.610	6.020
120	5.225	5.585	6.490
180	5.925	6.215	6.195

[a] One scale division = 1.8 μ.

TABLE V.—EFFECT OF SALT AND ITS CONCENTRATION IN SUBMERSION MEDIUM ON MEAN GRAIN SIZES OF CORN AND AMIOCA STARCHES

			Mean Grain Sizes in Scale Divisions[a]		
			Time, min.		
Type	Medium	Concn., %	0	5	30
Corn	Na_2SO_4	0.2	5.275	5.708	5.398
		0.9	5.275	5.851	5.652
	$MgCl_2$	0.2	5.275	6.439	5.835
		0.9	5.275	5.597	5.716
	$AlCl_3$	0.2	5.275	5.891	5.851
		0.9	5.275	5.338	5.796
	NaCl	0.2	5.275	5.664	5.323
		0.9	5.275	5.442	5.466
Amioca	Na_2SO_4	0.2	5.482	5.911	5.728
		0.9	5.482	5.633	6.177
	$MgCl_2$	0.2	5.482	6.511	6.348
		0.9	5.482	6.436	6.750
	$AlCl_3$	0.2	5.482	6.940	6.626
		0.9	5.482	6.034	6.773
	NaCl	0.2	5.482	6.078	6.269
		0.9	5.482	6.054	5.652

[a] One scale division = 1.8 μ.

was included as zero time in the experiment to allow comparisons between salt effect and time. Table V shows the variables investigated and the mean grain sizes. The analysis of variance showed that the single effects of type of starch, time submersed, and submersion medium, each produced a significant difference at the 0.5% level. Concentration of the salt in the submersion medium was not significant. Three-factor interactions were not significant. All statistical two-factor interactions involving time produced a significant difference in grain diameters at the 10% level or less. Duncan's multiple range tests showed in each case that the mean grain sizes of the unsubmerged starch (zero time) were significantly different from those of the submerged starch. Duncan's multiple range tests performed on the time-submersion medium interaction and on the single effect of submersion medium showed a significant difference at the 10% level between the salts with polyvalent cations ($MgCl_2$ and $AlCl_3$) and the salts with monovalent cations (Na_2SO_4 and NaCl), $MgCl_2$ and $AlCl_3$ producing greater diameter changes.

SUMMARY AND CONCLUSIONS

The literature pertaining to swelling of starch grains was reviewed, and a procedure for determining swelling of starch grains in various environmental conditions is described.

Corn and amioca starches and the moisture content of these starches showed a significant difference in grain sizes when submersed in distilled water or simulated gastric fluid U.S.P. This was postulated as due to the initial size differences of the starches. It was observed that initially high moisture content starches had larger mean grain sizes than low moisture content starches and that this difference was maintained throughout the time of the experiments. The initial differences in mean grain sizes of the corn and amioca starches was also maintained throughout the experiments.

The two cornstarches submersed 5 to 30 min. in simulated gastric fluid U.S.P. had greater increase in grain sizes over those submersed in distilled water.

Changes in pH had little effect on swelling. However, evidence was obtained to show that starch grains may swell more at a pH of 5.3 than in lower pH media.

Salts affected the swelling of starch grains, with polyvalent cationic salts ($MgCl_2$ and $AlCl_3$) producing more swelling than monovalent cationic salts (NaCl and Na_2SO_4). Ionic concentration did not show any effect on swelling.

There was no significant difference in swelling of starch grains demonstrated between the various time intervals. However, when unsubmerged starches were included in the analyses of variance, significant size differences were shown between the unsubmerged starches and the starches slurried for 5 min. and longer. No significant additional increase in mean grain size was found after 5-min. submersion, indicating any swelling that occurs is apparently instantaneous.

The swelling of the starch grains observed was in the order of 5 to 10% increase in mean grain size. At present, this does not seem to be a large enough change to cause tablets to rupture. Further work is in progress to determine other factors that may influence the swelling of starch grains, and to determine whether the observed increases in mean grain diameter are sufficient to rupture tablets.

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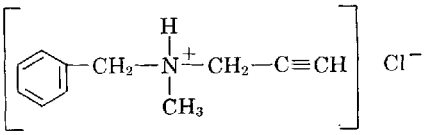
Qualitative and Quantitative Tests for Pargyline Hydrochloride

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

N-Methyl-*N*-(2-propynyl)-benzylamine hydrochloride; $C_{11}H_{13}N \cdot HCl$; mol. wt. 195.69. The structural formula of pargyline hydrochloride may be represented as



Physical Properties.—Pargyline hydrochloride occurs as a fine white crystalline powder having a characteristic odor, m.p. 158–162° (U.S.P., class I). It is very soluble in water and freely soluble in alcohol. Pargyline hydrochloride sublimes slowly when maintained at temperatures approaching its melting range.

Identity Tests.—A 1 in 2,500 solution of pargyline hydrochloride in 0.1 *N* hydrochloric acid exhibits ultraviolet absorbance maxima at about 251, 257, 262 [absorptivity (a) about 1.55], and 268 mμ, and absorbance minima at about 229, 253, 259, and 266 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of pargyline hydrochloride in potassium bromide, in a disk of about 0.82 mm. thickness is shown in Fig. 2.

Dissolve about 50 mg. of pargyline hydrochloride in 3 ml. of water, add ammonia T.S. until basic, and filter. Acidify the filtrate with diluted nitric acid and add 1 ml. of silver nitrate T.S.: a white precipitate forms, which is insoluble in diluted nitric acid, but soluble in ammonia T.S. (presence of chloride).

Purity Tests.—Determine the water content of pargyline hydrochloride by the titrimetric (Karl Fischer) method: not more than 1% is found.

Char about 1 Gm. of pargyline hydrochloride, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.2%.

Determine the heavy metals content of pargyline hydrochloride by the U.S.P. heavy metals test, method II: the heavy metals limit for pargyline hydrochloride is 20 p.p.m.

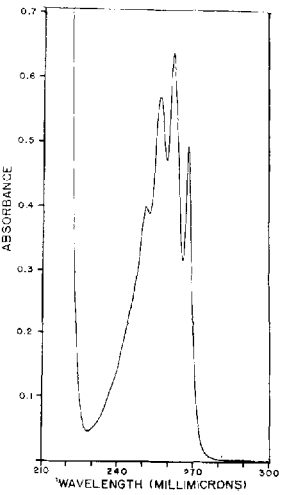


Fig. 1.—Ultraviolet absorption spectrum of pargyline hydrochloride in 0.1 *N* hydrochloric acid (400 mcg./ml.); Beckman model DK-2A spectrophotometer.

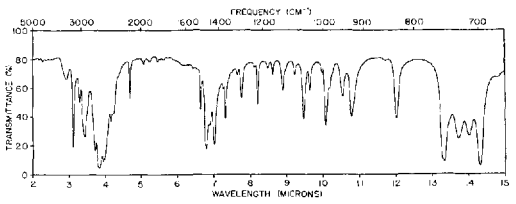


Fig. 2.—Infrared spectrum of pargyline hydrochloride in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

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Accepted for publication March 21, 1966.
Abbott Laboratories, North Chicago, Ill., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

A solution of pargyline hydrochloride in water (1 in 10) is colorless and clear, except for some mechanical impurities which may remain in suspension.

Assay.—*Acetylenic Group.*—Transfer about 200 mg. of pargyline hydrochloride, accurately weighed, to a tall-form 200-ml. beaker and dissolve in 20 ml. of alcohol. Add 25 ml. of 0.1 *N* alcoholic silver nitrate (prepared by dissolving 8.5 Gm. of silver nitrate in 20 ml. of water and diluting to 500 ml. with alcohol), 50 ml. of 0.2 *M* alcoholic trishydroxymethylaminomethane (THAM), and mix. Titrate the mixture potentiometrically with 0.1 *N* alcoholic potassium hydroxide using glass and calomel electrodes. Each milliliter of 0.1 *N* alcoholic potassium hydroxide is equivalent to 9.785 mg. of $C_{11}H_{13}N \cdot HCl$. The amount of pargyline hydrochloride found is not less than 98.0% and not more than 102.0%.

Amine Group.—Transfer about 400 mg. of pargyline hydrochloride, accurately weighed, to a tall-form 200-ml. beaker, and dissolve in 50 ml. of glacial acetic acid. Add 10 ml. of mercuric acetate T.S. and titrate potentiometrically with 0.1 *N* acetous perchloric acid. Alternatively, add 2 drops of crystal violet T.S. and titrate to a green end point.[1] Each milliliter of 0.1 *N* perchloric acid is equivalent to 19.57 mg. of $C_{11}H_{13}N \cdot HCl$. The amount of pargyline hydrochloride found is not less than 98.0% and not more than 102.0%.

DOSAGE FORMS OF PARGYLINE HYDROCHLORIDE

Pargyline Hydrochloride Tablets

Identity Tests.—Transfer to a 100-ml. volumetric flask an amount of powdered tablets equivalent to about 40 mg. of pargyline hydrochloride. Add about 50 ml. of 0.1 *N* hydrochloric acid and shake mechanically for 15 min. Dilute to volume with 0.1 *N* hydrochloric acid, mix, and filter: the filtrate exhibits ultraviolet absorbance maxima and minima at the same wavelengths as a solution of pargyline hydrochloride reference standard in the same medium.

Assay.—*Amine group.*—Weigh and finely powder not less than 20 tablets. Transfer to a 125-ml. separator an amount of powder, accurately weighed, equivalent to about 40 mg. of pargyline hydrochloride. Add 30 ml. of water and 1 ml. of sodium hydroxide T.S. and extract with four 20-ml. portions of chloroform. Wash the combined chloroform extracts with 10 ml. of water and discard the water phase. Filter the chloroform extract through a pledget of purified cotton into a tall-form 200-ml. beaker and wash the cotton with 10 ml. of chloroform. Add 25 ml. of acetonitrile and titrate potentiometrically with 0.01 *N* perchloric acid in dioxane. Each milliliter of 0.01 *N* perchloric acid is equivalent to 1.957 mg. of $C_{11}H_{13}N \cdot HCl$. The amount of pargyline hydrochloride found is not less than 90.0% and not more than 110.0% of the labeled amount.

[1] If the indicator method is used, perform a blank titration and make any necessary correction.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Pargyline hydrochloride[2] is a nonhydrazine monoamine oxidase inhibitor which acts as an antihypertensive.

Identity Tests.—The ultraviolet absorption spectrum of the extract obtained from commercial 10-mg. tablets compared qualitatively with that of Fig. 1 in the region of from 250–270 mμ. Extraneous absorbance, due to unidentified tablet constituents, was present throughout the ultraviolet range but did not obscure the distinctive features of the pargyline hydrochloride spectrum.

Quantitative Methods.—The assay based on the acetylenic group in pargyline hydrochloride is similar to the assays provided in the N.F. XII monographs on ethchlorvynol and ethinamate. In the present assay, however, the titration with 0.1 *N* alcoholic potassium hydroxide determines the bound hydrochloric acid in addition to the acid released on formation of the silver acetylide. The bound acid may be determined independently by titration of a sample to which no silver nitrate has been added. Titration of the sample without the addition of THAM buffer results in extreme darkening of the solution as the titration progresses. This effect, which is believed to be due to a darkening of the precipitated silver chloride as well as the deposition of some silver oxide, is minimized in the buffered medium. The assay of pargyline hydrochloride gave an average value of 100.6 ± 0.5%.[3]

Caution is to be observed in disposing of completed titration mixtures since silver acetylides are explosive when dry. The titration vessel should be flushed with water thoroughly prior to disposal. Occluded precipitates should be dissolved with nitric acid.

Attempts to employ this method for the direct determination of pargyline hydrochloride in the tablets proved unsuccessful. The voltage change in the vicinity of the end point was gradual, and the results obtained tended to be high (about 110% of label claim).

The nonaqueous titration of pargyline hydrochloride with perchloric acid gave an average value of 99.4 ± 0.1%.[3] With crystal violet indicator, the color change from turquoise to a definite green corresponded to the midpoint of the inflection in the potentiometric titration curve. Generally, the visual titration is not as advantageous as the potentiometric determination due to the fading of the indicator in the presence of the precipitate which forms during the titration. An additional drop of crystal violet T.S. as the end point is approached will aid in identifying the desired indicator change.

Extraction of the powdered tablets with chloroform followed by titration of the free base with perchloric acid gave an average value of 99.8 ± 0.4%[3] of the labeled amount of pargyline hydrochloride.

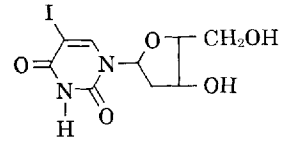
[2] Marketed as Eutonyl by Abbott Laboratories, North Chicago, Ill.

[3] Maximum deviation from the mean value.

Qualitative and Quantitative Tests for Idoxuridine

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

5-IODO-2′-DEOXYURIDINE; $C_9H_{11}IN_2O_4$; mol. wt. 354.10. The structural formula of idoxuridine may be represented as



Physical Properties.—Idoxuridine occurs as a white, odorless, crystalline powder. In a melting point capillary it darkens and then decomposes with evolution of iodine fumes at 180–185° (U.S.P., class I*a*). It is slightly soluble in water and in alcohol, and is practically insoluble in chloroform and in ether. A 1% solution in 1 *N* NaOH is dextrorotatory.

Identity Tests.—Heat about 100 mg. of idoxuridine in a porcelain crucible over a free flame: violet vapors of iodine are evolved.

Dissolve 100 mg. of idoxuridine in 100 ml. of a 1 in 4 solution of isopropylamine in methanol. To 5 ml. of this solution add 5 ml. of chloroform and 0.2 ml. of a freshly prepared 1 in 100 solution of cobaltous acetate in methanol: a violet to blue-violet color is produced.

A 1:50,000 solution of idoxuridine in 0.1 *N* sodium hydroxide exhibits an ultraviolet absorbance maximum at about 280 mμ [absorptivity (*a*) about 16.0] and a minimum at about 253 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of 0.5% dispersion of idoxuridine in potassium bromide, in a disk of about 0.82-mm. thickness, is shown in Fig. 2.

Purity Tests.—Char about 1 Gm. of idoxuridine, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.5%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of idoxuridine in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U.S.P. XVII heavy metals test, method I: the heavy metals limit for idoxuridine is 20 p.p.m.

Assay.—*Iodine.*—Transfer about 75 mg. of idoxuridine, accurately weighed, into a large nickel crucible. Add 1 Gm. of anhydrous potassium carbonate powder and mix thoroughly with the sample using a dry glass rod. Gently tap the crucible to compact the mixture. Overlay the mixture with 12 Gm. of anhydrous potassium carbonate powder and again tap the crucible to compact the material. Ignite the contents of the uncovered crucible for 30 min. at 525° in a muffle furnace preheated to that temperature. Cool the crucible and transfer the contents to a 400-ml. beaker, rinsing the crucible with several portions of water, and adding the rinsings to the beaker. Add about 50 ml. of water to the beaker, heat gently, and break up the melt with the aid of a stirring rod. Filter the suspension through paper,

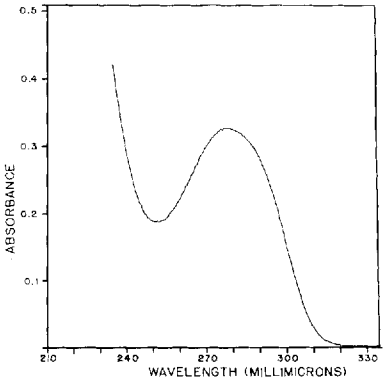


Fig. 1.—Ultraviolet absorption spectrum of idoxuridine in 0.1 *N* sodium hydroxide (20 mcg./ml.); Beckman model DK-2A spectrophotometer.

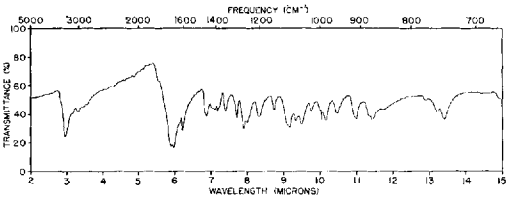


Fig. 2.—Infrared spectrum of idoxuridine in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

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Alcon Laboratories, Fort Worth, Tex., Allergan Pharmaceuticals, Santa Ana, Calif., and Smith Kline & French Laboratories, Philadelphia, Pa., have cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

collecting the filtrate in a 250-ml. volumetric flask. Wash the beaker and filter paper with hot water until near the mark. Cool to room temperature, dilute to volume with water, and mix well. Pipet a 50-ml. aliquot of this solution into a 500-ml. conical flask and add 150 ml. of water. Add methyl orange T.S., then add phosphoric acid to produce a definite pink color. Add 10 ml. of bromine T.S. (sufficient amount to impart a definite yellow color to the solution) and several glass beads and heat to boiling. Boil the solution until it is colorless, rinse the inside walls of the flask with water, and boil an additional 5 min. Stopper the flask loosely, and cool to room temperature in a cold water bath. Add 5 ml. of phenol solution (1 in 20), again rinse the walls of the flask, and allow to stand 5 min. Add 5 ml. of potassium iodide solution (1 in 10) and titrate immediately with 0.05 *N* sodium thiosulfate, adding 3 ml. of starch T.S. as the end point is neared. Perform a blank determination and make any necessary correction. Each milliliter of 0.05 *N* sodium thiosulfate is equivalent to 1.058 mg. of iodine (I). The amount of iodine found is not less than 34.7% and not more than 36.6% of the weight of the sample taken.

Idoxuridine.—Dissolve about 500 mg. of idoxuridine, accurately weighed, in 40 ml. of dimethylformamide which has been previously neutralized to thymol blue T.S. Titrate with 0.1 *N* sodium methoxide to a blue end point. Each milliliter of 0.1 *N* sodium methoxide is equivalent to 35.41 mg. of $C_9H_{11}IN_2O_5$. The amount of idoxuridine found is not less than 98.0% and not more than 101.0% of the weight of the sample taken.

DOSAGE FORMS OF IDOXURIDINE

Idoxuridine Ophthalmic Solution

A sterile solution of idoxuridine in water. The solution may be rendered isotonic by addition of appropriate agents.

Identity Tests.—Evaporate 5 ml. of idoxuridine ophthalmic solution to dryness with the aid of a stream of air, add 2 ml. of alcohol, and again evaporate to dryness. To the residue add 5 ml. of a 1 in 4 solution of isopropylamine in methanol. If the residue is not completely soluble in this solution, the use of a stirring rod will aid in dissolving the idoxuridine present. Add 5 ml. of chloroform and 0.2 ml. of a freshly prepared 1 in 100 solution of cobaltous acetate in methanol: a violet to blue-violet color is produced.

Purity Tests.—To about 10 ml. of idoxuridine ophthalmic solution add a few drops of starch T.S.: no blue color is produced (absence of free iodine).

Assay.—To 4 Gm. of purified siliceous earth in a 4-oz. glass mortar add 4 ml. of water and incorporate by kneading thoroughly with a flexible spatula blade until the mixture is fluffy. Transfer to a chromatographic tube (19 × 200 mm.) equipped with a Teflon stopcock, and tamp gently to compress the material to a uniform mass. Transfer 2 Gm. of purified siliceous earth to the glass mortar, add 2.0 ml. of idoxuridine ophthalmic solution, mix as before, and transfer to the tube without tamping. Transfer 2 Gm. of purified siliceous earth to the same mortar, add 2 ml. of water, mix well, transfer about half of the mixture of the tube, and tamp down gently until the column appears uniform. Transfer the remaining portion of the tube, tamp as before, and cover with a small pledget of glass wool. Elute with chloroform–*n*-butanol (5:1) at a flow rate of approximately 1 ml./min., discarding the first 15–20 ml. of eluant. Collect the next 80 ml. of eluant in a 100-ml. volumetric flask, dilute to volume with the eluting solvent, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of idoxuridine,[1] in the same medium, at a concentration of about 20 mcg./ml., in 1-cm. cells, at 292 and at 310 mμ, with a suitable spectrophotometer, using chloroform–*n*-butanol (5:1) as the blank. Calculate the quantity, in mg., of $C_9H_{11}IN_2O_5$ in the volume of ophthalmic solution taken by the formula $0.1C \times (A_{u292} - A_{u310})/(A_{s292} - A_{s310})$ in which C is the exact concentration of the standard solution, in mcg./ml., A_u is the absorbance of the sample solution, and A_s is the absorbance of the standard solution. The amount of idoxuridine is not less than 90% and not more than 110% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., have been used wherever feasible.

Idoxuridine,[2] synthesized by Prusoff (1), is an antiviral agent indicated in the treatment of corneal erosions caused by the herpes simplex virus. The effectiveness of idoxuridine in the treatment of herpes simplex keratitis has been attributed to its similarity of structure to thymidine; idoxuridine differs from thymidine only in the substitution of an iodine atom for a methyl group in the carbon-5 position of the uracil moiety. Administration of idoxuridine in the form of an ophthalmic solution results in a competition with thymidine for incorporation in the synthesis of deoxyribonucleic acid (DNA), the genetic material of herpes simplex virus which determines its ability to infect and reproduce. Incorporation of idoxuridine in the DNA synthesis results in the production of a "faulty" DNA, inhibiting reproduction of the invading virus.

Identity Tests.—Idoxuridine reacts with cobalt salts in nonaqueous alkaline medium to produce a colored complex. This color reaction is rather specific for compounds possessing the —CONHCO— or —CONHCS— group (2). Barbiturates, hydantoins, and xanthines are typical classes of compounds which produce colored complexes under similar conditions. The method may be adapted for quantitative determination of idoxuridine in the ophthalmic solution. The presence of isotonic agents such as sodium chloride which are relatively insoluble in the reaction solvents decreases the reproducibility of the method. The ultraviolet absorption spectrum may be run alternatively in acid solution with a maximum absorbance at about 288 mμ [absorptivity (a) about 21.5] and a minimum at about 248 mμ. These tests together

[1] Prepare a methanolic solution of idoxuridine in a concentration of 2 mg./ml. Dilute 1.0 ml. of this solution to 100.0 ml. with chloroform–*n*-butanol (5:1).

[2] Marketed as Dendrid by Alcon Laboratories, Fort Worth, Tex., as Herplex by Allergan Pharmaceuticals, Santa Ana, Calif., and as Stoxil by Smith Kline & French Laboratories, Philadelphia, Pa.

with the identity test based upon the evolution of iodine vapor and comparison of the infrared spectrum provide a satisfactory identification of idoxuridine.

Purity Tests.—Thin-layer or paper chromatographic procedures may be included for testing the purity of bulk idoxuridine. Comparison of idoxuridine to a reference standard is made by examination of developed chromatograms using an ultraviolet light and/or color producing reagents, *i.e.*, cysteine–sulfuric acid. The idoxuridine spot should be equivalent in position to the reference standard spot for idoxuridine, and no other spots at other positions should be visible. The spotting of control solutions containing the degradation products of idoxuridine (5-iodouracil, uracil, and deoxyuridine) will aid in detecting the position of extraneous spots on the chromatograms.

Quantitative Methods.—The quantitative determination of the iodine content of idoxuridine is similar to the official assay for sodium liothyronine (3) and gave an average value equivalent to 36.1 ± 0.1%[3] iodine. A rapid, precise measure of the iodine content may also be determined by the oxygen flask method (4–6). Nonaqueous titration of idoxuridine with sodium methoxide gave an average value of 99.8 ± 0.6%.[3] Azo violet indicator may also be used for the end point detection of the titration. Analysis of the sterile ophthalmic solutions by column partition chromatography was based on the procedure of Simpson and Zappala (7). Acid washed Celite 545 was used as the supporting phase without prior treatment. Celite 545 may be used as the adsorbant by prewashing an acidified column with organic solvents to remove extractable impurities. The organic solvents are then removed by oven drying. Incorporation of water or 0.1 *N* hydrochloric acid in the preparation of the Celite columns gave comparable results representing an average recovery of 97.4 ± 5.7%[3] of the theoretical amount of idoxuridine in the ophthalmic solutions.

The volume of eluting solvent included for the assay of idoxuridine ophthalmic solution gave quantitative recoveries with the Celite used. However, it should be noted that the elution rate for idoxuridine may vary from lot to lot of Celite which then necessitates a minor change in the volume of eluate collected. This should be demonstrated for each batch of Celite by a satisfactory recovery of a standard aqueous idoxuridine solution subjected to the column procedure.

[3] Maximum deviation from the mean value.

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(7) Simpson, C. A., and Zappala, A. F., *J. Pharm. Sci.*, **53**, 1201(1964).

Technical Articles

Automated Nephelometric Determination of Rat Liver Glycogen in Adrenal Steroid Bioassays

By WILLIAM F. BEYER

An automated procedure for the nephelometric determination of rat liver glycogen is described. Alkaline liver digests are mixed with 57.5 per cent alcohol and heated at 45°. Samples are analyzed at a rate of 60/hr. using an automatic sampler and analyzer (Technicon AutoAnalyzer) in conjunction with a commercially available fluorometer. The coefficient of variation for the automated procedure is approximately 1 per cent.

RAT LIVER glycogen is used as the criterion of response in the bioassay for endocrine principles of the adrenal cortex and also for synthetic steroids. The assay is based on the method of Pabst *et al.* (1) and is currently an official procedure for adrenal cortex injection as directed by N.F. XII (2). For the assay, livers of adrenalectomized rats, previously injected with test samples, are digested in hot 30% potassium hydroxide. After standing overnight at room temperature, the alkaline liver digests are diluted with water and glycogen is determined.

An automated procedure for glycogen has been described by Singer *et al.* (3) requiring manual deproteination with trichloroacetic acid. Glyco-

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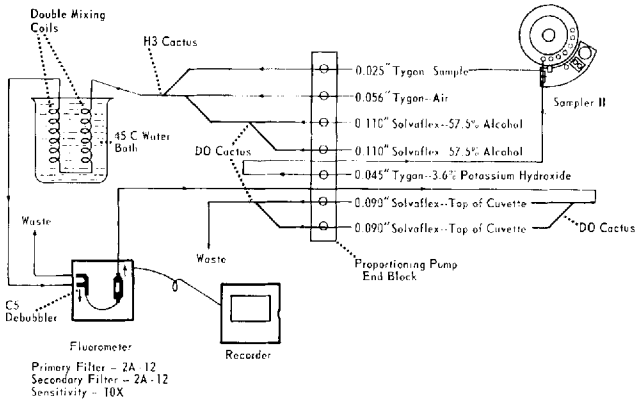

Fig. 1.—Manifold flow diagram for the automated determination of rat liver glycogen using an automatic sampler, proportioning pump, and recorder in conjunction with a commercially available fluorometer adapted for nephelometry.

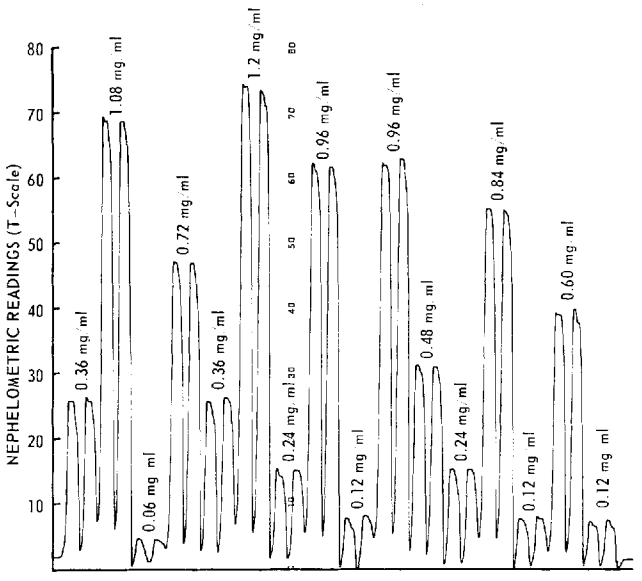

Fig. 2.—Recordings of various quantities of rat liver glycogen using automated nephelometry at a rate of 60 samples/hr.

gen determinations are then made automatically by measuring the intensity of the glycogen–iodine complex. This report gives details for an automated assay of rat liver glycogen using a fluorometer adapted for nephelometry. The method, based on a previously described procedure (4), depends upon the cloud produced by glycogen from alkaline liver digests in 55% alcohol. Technicon instruments and a commercially available fluorometer equipped with a square quartz flow cell are used in the analyses, permitting glycogen determinations at a rate of 60 samples/hr.

MATERIALS AND METHODS

Instrument and Equipment.—(*a*) Automatic sampler,[1] proportioning pump, flow through door for fluorometer, recorder, chart reader, double mixing coils, and assorted glass fittings and tubing. (*b*) Fluorometer[2] with Wratten 2A-12 primary and secondary filters and blue lamp, permitting measurements at approximately 510 mμ. (*c*) Square quartz flow cell, 3 mm. i.d. × 5-mm. o.d. with 12-mm. masked adapter insert.[3] (*d*) Water bath operated at 45°.[4]

Reagents.—(*a*) Potassium hydroxide, 3.6% and 30%. (*b*) Alcohol, 57.5%, (v/v). (*c*) Test rat liver digests. Adrenalectomized rats are injected with adrenal steroid preparations as directed by N.F. XII. Livers are removed, digested in 12 ml. of hot 30% potassium hydroxide, and diluted to 100 ml. with water after standing overnight at room temperature. (*d*) Pooled livers of adrenalectomized uninjected rats (blank pool). Prepared in the same manner as liver digests of test rats for each series of glycogen determinations. (*e*) Standard rat liver glycogen. Prepared according to van der Vies (5). (*f*) Standard solutions of rat liver glycogen. Fresh solutions of glycogen standard are prepared in the blank pool. For a standard curve, solutions in the range of 0.06 to 1.2 mg./ml. are adequate. To check instrumental and reagent changes, a 0.5-mg./ml. standard is used.

Method.—The sample line is placed in the blank

[1] AutoAnalyzer Sampler II, Technicon Controls, Chauncey, N. Y.
[2] Turner Fluorometer model III, Arthur H. Thomas, Philadelphia, Pa.
[3] Catalog numbers B16-63019 and A363-62140, American Instrument Co., Barrington, Ill.
[4] Tamson water bath, Witt Sales, Cincinnati, Ohio.

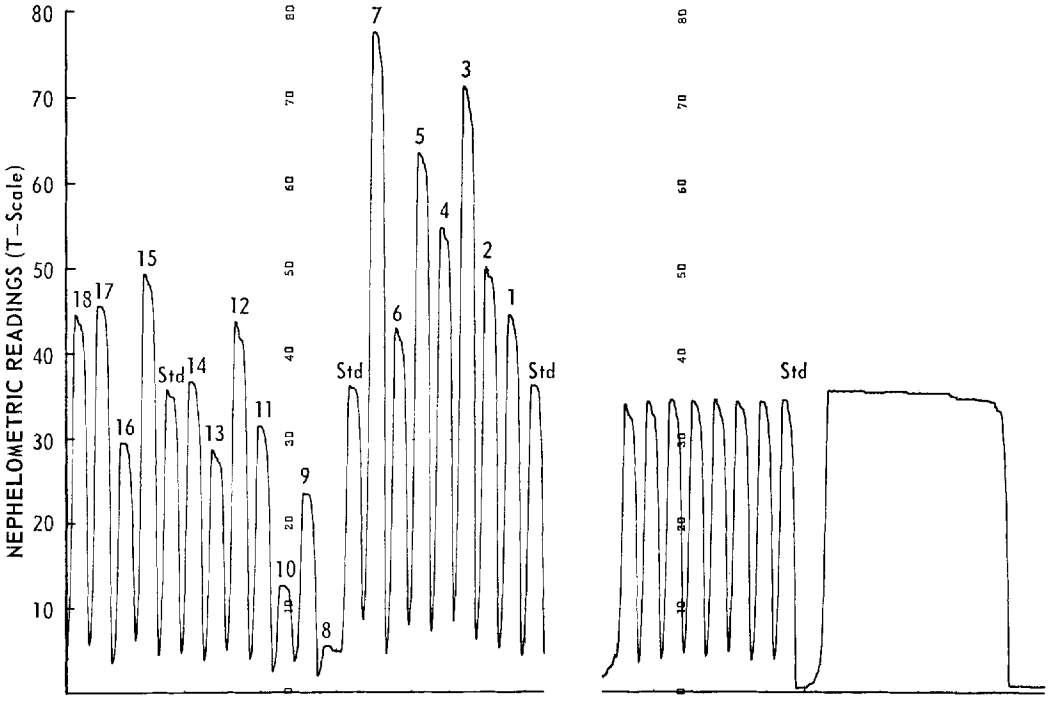

Fig. 3.—Automated nephelometric recordings of rat liver glycogen standard and representative recordings of unknown test samples. Key: numbered peaks, recordings of unknown test samples; Std, 0.5, mg./ml. standard glycogen dissolved in the blank liver pool.

TABLE I.—RECOVERY OF GLYCOGEN FROM RAT LIVER DIGEST USING THE AUTOMATED NEPHELOMETRIC PROCEDURE

Glycogen Present in Digest, mg./ml.	Glycogen Std. Added, mg./ml.	Theoretical Amt. Glycogen Present, mg./ml.	Glycogen Found, mg./ml.	Recovery, %[a]
0.055	0.430	0.485	0.480	99.0
0.111	0.430	0.541	0.547	101.1
0.221	0.430	0.651	0.648	99.5
0.332	0.430	0.762	0.761	99.9
0.443	0.430	0.873	0.873	100.0
0.553	0.430	0.983	0.972	98.9

[a] Average recovery = 99.7%.

pool and all other lines in proper reagents as shown in the manifold flow diagram of Fig. 1. Sensitivity of the fluorometer is positioned at 10× and 2A-12 filters are used for both primary and secondary filters. With all instruments operating, a zero base line is established. The 0.5-mg./ml. glycogen standard is placed in duplicate cups initially and singly thereafter at regular intervals following a group of test samples. The analyses are made at a rate of 60/hr. using 3.6% potassium hydroxide in the rinsing system of the automatic sampler.

Calculations.—The quantity of glycogen in each rat liver is calculated using the formula:

$$Gu = \frac{Nu}{Ns} \times Gs \times V$$

where, Gu = milligrams glycogen per liver; Gs = milligrams glycogen standard per milliliter of blank pool; Ns = nephelometric reading of glycogen standard on transmission scale; Nu = nephelometric reading of test sample on transmission scale; and V = milliliters of liver digest.

To correct for changes in the automated system average readings of standards on either side of a group of test samples are used with appropriate liver digests.

TABLE II.—PRECISION OF REPLICATE RAT LIVER GLYCOGEN DETERMINATIONS

Rat Liver No. 11 mg. Glycogen/Liver		Rat Liver No. 25 mg. Glycogen/Liver	
52	52	65	66
52	52.5	66.5	65.5
52.5	51.5	65.5	64.5
52	51.5	65	65.5
51.5	51	65.5	66.5
52	51.5	64	65.5
52	51.5	65	65
		65.5	66.5
Av. = 51.8		Av. = 65.4	
Coefficient of variation = 0.81%		Coefficient of variation = 1.07%	

TABLE III.—PROTOCOL OF A RAT LIVER GLYCOGEN DEPOSITION ASSAY FOR ADRENAL CORTEX EXTRACT[a]

mg. Glycogen/Individual Rat Liver							
U.S.P. Hydrocortisone Std.(F)				Adrenal Cortex Extract			
0.288 mg./Rat		0.48 mg./Rat		0.288 mg./Rat		0.48 mg./Rat	
A	M	A	M	A	M	A	M
32	32	55	54	16	13	53	54
27	29	60	61	46	46	53	54
26	25	66	63	31	31	52	55
29	30	61	60	38	40	53	52
56	57	55	57	33	32	53	52
58	57	59	58	46	46	52	52
50	51	44	43	36	38	54	54

	A	M
Potency, mg. F ≅ ml.	0.141	0.142
Log-confidence interval	0.2253	0.2338
95% Confidence limits, mg. F ≅ ml.	0.105–0.177	0.105–0.181
Test for parallelism of slopes (F)	0.0015	0.0710

[a] Liver glycogen of each rat was determined by both automated (A) and manual (M) procedures. Potency and validity tests were calculated as directed by N.F. XII.

TABLE IV.—STATISTICAL RESULTS OF RAT LIVER GLYCOGEN DEPOSITION ASSAYS OF ADRENAL CORTEX EXTRACTS

	Automated		Manual	
Prepn.	Potency, % of Theory	Log-Confidence Interval (L)	Potency, % of Theory	Log-Confidence Interval (L)
1	126	0.1757	122	0.1744
2	141	0.2253	142	0.2388
3	97	0.2758	94	0.3135
4	147	0.3281	152	0.3830
5	101	0.3005	106	0.2479
6	73	0.2236	73	0.2105
7	125	0.2117	125	0.2127

Alternatively, the chart reader and a standard curve for rat liver glycogen may be used. Liver glycogen values of test samples are adjusted in accordance with nephelometric readings of the 0.5-mg./ml. standard placed at regular intervals.

RESULTS AND DISCUSSION

Following preliminary experiments with various manifold systems and temperatures, optimum operating conditions and manifold design were determined. The 0.025-in. sample line (Fig. 1), delivering 0.23 ml./min., dilutes the 57.5% alcohol carried by two 0.110-in. lines at a rate of 6.78 ml./min., to 55%. This concentration of alcohol was found to effectively form the glycogen cloud.

Nephelometric responses to liver glycogen concentrations when plotted were linear over the range of 0.06 to 1.2 mg./ml. (6 to 120 mg./liver) and had a zero intercept. An upper limit was not established; however, the concentration of liver glycogen encountered in the bioassay of adrenal steroids normally does not exceed 120 mg./liver. Although glycogen values may vary by a factor of 10 in glycogen deposition assays, sample carry over with the automated procedure is minimal. Figure 2 shows that very similar readings are recorded for duplicate samples of glycogen standard even though preceded by a standard having either high or low nephelometric readings.

Figure 3 gives a typical recorder tracing of unknown test liver digests and glycogen standard showing the variation in liver glycogen that is encountered normally. A portion of the recording, extreme right, shows continuous sampling of the 0.5-mg./ml. glycogen standard and duplicate sampling of the same standard.

Recovery studies were carried out using the automated procedure on samples of glycogen standard added to varying quantities of test liver digest. Table I shows that satisfactory recovery was obtained, with results varying from 98.9–101.1% for the six samples tested.

The reproducibility of the procedure was determined by sampling repeatedly two test liver digests. Table II gives the results: a coefficient of variation of 0.81% was obtained for 14 analyses and 1.07% for 16 subsequent analyses carried out at a later date.

No significant differences in liver glycogen determinations occurred for the manual procedure (4) and the automated method. Table III gives the results of a representative adrenal cortex assay with glycogen determined by both methods on parallel samples of liver digests. It can be noted that only small differences occurred in individual glycogen values; consequently, statistical results are very similar. A comparison of seven additional liver glycogen deposition assays is shown in Table IV. In all cases, comparable potencies and log-confidence intervals were obtained, indicating that the automated procedure is suitable for rat liver glycogen determinations in the glycogen deposition assay of adrenal steroids.

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Analog Computer in Drug Dosage and Formulation Design

By EDWARD R. GARRETT and HOWARD J. LAMBERT

Methods have been described which utilize the analog computer as a laboratory aid in the preparation of drug formulations with improved therapeutic efficacy. Five basic dosage models were used to show the versatility of the computer and the variety of programs that are possible. These included single dosage, repetitive dosage, and three different types of sustained and delayed-release mechanisms. The analog computer may be used to predict which combination of existing formulas should be combined to give the desired response or may be used to provide specific information on the desirable physical characteristics of such formulas in advance of their manufacture. The major information needed for such predictions are the pharmacokinetic parameters specific to the drug in question. The analog computer provides an inexpensive means of predicting formulation requirements and may perform, in a matter of hours, work that might take weeks or months by normal laboratory screening methods.

IN AN ERA where both public opinion and federal legislation demand increased proof of drug efficacy and drug safety, the "art" of drug dosage and formulation design is being supplanted by the sophistications of advanced science and technology. Qualitative evaluations of drug potency and action are no longer adequate. Instead, the many transfer processes and reactions undergone by a drug during its release from the dosage forms and its passage through the body should be quantitatively described by mathematical models when reliable analytical data can be obtained. Models, originally formulated by Teorell (1), Dominguez (2), and others allow the description of a drug's action by listing certain numerical values obtained by mathematical treatment of experimental data. These values include rate constants for dissolution, absorption, tissue distribution, metabolism, and excretion. Such mathematical constants then become the focus of attention, an abstraction one level above the observed data (3). Also provided are quantifiable concepts that are extremely useful, such as metabolic half-lives and compartmental volumes of distribution which yield a pharmacokinetic profile or "fingerprint" for a drug. The work of Swintosky (4) on the drug sulfaethylthiadiazole is an excellent illustrative example. At best, however, the direct application of such analytical mathematical techniques is most approximate and difficult. The complexity of the mathematics involved leads to simplification of complex models and only sequential or parallel drug transferences are the models considered.

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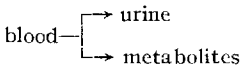
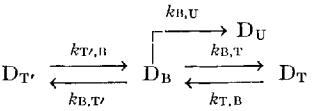
tablet → gastrointestinal tract →



DISCUSSION

The basic premises behind all such models include: (*a*) rapid equilibration of drug between blood and (*b*) other body tissues, fluids, or compartments.



The amount of drug in the blood (D_B) supposedly reflects and is proportional to the amount of drug in tissues T and T′ (D_T and $D_{T'}$) provided that the equilibration is extremely rapid. Furthermore, the rates of transfer must be invariant functions of the amounts of drug in each of these compartments (*i.e.*, first-order kinetics are followed). This implies that the compartmental sizes are limitless and the distribution model is independent of the magnitude of the dose. The slow rate-determining step must be either the elimination of the drug from the body or its metabolism, where these rates are proportional to the amount in the blood.

If both of these conditions are satisfied, then Eq. 1 will hold,

$$\log D_B = \frac{-k_e t}{2.303} + \text{constant} \qquad \text{(Eq. 1)}$$

and a plot of the logarithm of the blood concentration (D_B) *versus* time will result in a straight line with a slope of $-k_e/2.303$. In addition, the biological half-life, defined as that period of time needed for any blood concentration (after tissue equilibration is established) to reach one-half its value, will be constant for any blood level or dose chosen,

$$t_{0.5} = \frac{2.303 \log 2}{k_e} = \frac{0.693}{k_e} = \text{constant} \qquad \text{(Eq. 2)}$$

The k_e referred to here is the rate constant derived from the plotting of Eq. 1. It should be realized that this is not $k_{B,U}$ (the rate constant for urinary and/or metabolic elimination), but rather, is de-

pendent upon the ratios of other distribution rate constants (12).

$$k_e = \frac{k_{B,U}}{1 + \frac{k_{B,T}}{k_{T,B}} + \frac{k_{B,T'}}{k_{T',B}}} \quad \text{(Eq. 3)}$$

If the transfer of drug between one compartment, *e.g.*, T′, and the blood is an extremely slow process, for example, the slow release of thiopental from fatty tissues (5), or the deeper compartment for psicofuranine (13), this reaction then becomes rate determining and deviations from apparent first-order kinetics occur. A similar situation develops when a nonlinear Langmuir-type binding occurs, *e.g.*, the binding of sulfonamides to plasma proteins (6). When such phenomena occur, the "biological half-life" varies with the dose and loses its value as a descriptive entity since Eq. 2 no longer holds.

Another instance where the biological half-life concept may fall down is when one of the distribution compartments is of limited size. With increasing dose, the amount of drug stored in this compartment will increase until a saturation point is reached. At this point, nonlinear discontinuities will occur in the first-order kinetic distribution pattern.

These phenomena are difficultly described by available analytical mathematics. The advent of computers has provided a pharmaceutical research tool to handle such complex problems with efficiency and reliability.

The Analog Computer.—The analog computer is an extraordinary tool for the elucidation of the mechanisms and rates of transformations and distribution of drugs in *in vivo* and *in vitro* systems. It permits the determinations of models for absorption from the site of administration into the blood, diffusion into tissues, and other volumes of distribution (*i.e.*, lymph, cerebrospinal fluid, red blood cells, etc.), metabolic pathways, and excretion processes. Such determinations are carried out by programing the computer for any sequential, or parallel, changes in drug amount in the various depots of an organism. The "read-out" can be obtained as plotted curves of drug amount *versus* time for any selected compartment in the model chosen.

The theory and details of analog computer programing are presented in many excellent texts and brochures (7–12). In general, the analog computer can simulate postulated physiological models by a dynamic electrical network. Employing a set of linked integrators, the output from each integrator can represent the time-variable amount (drug concentration times the apparent volume of distribution) of the drug distributed in a particular biological compartment. The integrators are connected by rate setting potentiometers and summing amplifiers so as to simulate the distribution of the drug on the basis of the chosen model. Dosage is introduced by applying an initial condition voltage to either the "stomach" integrator or the "blood" integrator depending on whether oral or intravenous modes of administration are to be simulated.

Typical examples of such analog computer applications are found in the literature for the distribution of the nucleoside antibiotic psicofuranine (13) and for ^{47}Ca dynamics in the dog (14, 15). An elementary handbook for the practical application of analog computer methods to pharmacokinetics has also been presented (16).

Dosage Form Design.—A dosage form for oral administration has several prerequisites. (*a*) The dosage form (a tablet or a capsule) must disintegrate in the gastrointestinal tract and the powders or granules released must dissolve in the surrounding fluids. (*b*) The drug in solution must be absorbed by the gastrointestinal mucosa and be transferred into the blood. (*c*) If the blood level of the drug is proportional to its therapeutic response, the blood level achieved should be within the therapeutic concentration range for the desired duration of action. With many pharmacological agents, it is not necessarily true that blood levels directly reflect pharmacological activity. However, these discussions will be restricted to those cases where this is so. Blood levels in the subthreshold therapeutic range will result in ineffective biological activity, whereas concentrations above those eliciting the optimum response may yield toxic manifestations. For these reasons, drugs with a high therapeutic ratio (LD_{50}/ED_{50}) are normally used. (*d*) The duration of the therapeutic blood level should be optimum for the desired therapy. (*e*) The pharmacokinetic parameters which describe the absorption, distribution, metabolism, and excretion of the drug in the human should be known. They can be determined by definitely programed acute studies on intravenous and oral administration by methods which have been previously cited in the literature (13–15). This assumes that there is no change in the metabolic rate with continued administration as there is with barbiturates and many other drugs.

Theories of disintegration and dissolution have been covered in the recent pharmaceutical literature by Wagner (17), Higuchi (18), and others (19–21). Since this literature is both extensive and thorough, these theories will not be discussed herein.

The pH-partition theory of drug absorption through biological membranes has been discussed by Schanker in several review articles (22). The basic premises are that a drug will not be absorbed unless it is unionized and can be partitioned into a lipid-like membrane. These rules hold for drugs which are "passively" rather than "actively" transported in biological systems and permit estimates of ease of absorption. However, absorption rates can be quantified and used whether they are active or passive since, not withstanding the mechanism, they are largely concentration dependent.

Methods of prolonging blood levels, such as using enzyme poisons to slow drug metabolism or drugs to compete for excretory transport systems [*e.g.*, the use of probenecid to block the tubular secretion of penicillin (23)] have been used. These are objectionable both because of their lack of specificity of action and because of their tendency to alter the physiological *status quo*.

The study of the relationship between the physical and chemical properties of drugs and their administered dosage forms and the resultant biological effects has been called biopharmaceutics. The proper use of pharmacokinetic and biopharmaceutical techniques should achieve optimal therapeutic response from a given dose of drug.

Means by which such goals may be obtained are by systematic variation of the following factors: (*a*) the form of the drug (*i.e.*, free acid or base, ester, salt, complex, etc.); (*b*) the physical state (crystal or powder), particle size, and surface area; (*c*) the

presence or absence of other materials with the drug in the dosage form; (*d*) the type of dosage form in which the drug is administered; (*e*) the processes encountered during the manufacture of the dosage form. These alterations usually affect the absorption rate of the compound by controlling its rate of dissolution from the dosage form and into the gastrointestinal fluids. Since these methods alter only the time availability of the drug and not the biological system, they are obviously the methods of choice.

Application of Computer Techniques.—The purpose of this paper is to show how the analog computer may be programed to give information on how to improve the therapeutic efficiency of a drug formulation. Such information may be obtained in two ways. First, having a knowledge of the pharmacokinetics of the drug the proper dose and dissolution rate constants which will give optimum therapy may be ascertained. Second, using knowledge of the physical characteristics of available formulations, which combinations will provide the desired effect can be predicted.

With either or both of these methods, information can be provided faster, more accurately, and at lower cost than by the sole use of the *in vivo* and *in vitro* testing available. It must be stressed, however, that the ultimate test of therapeutic efficacy of drug formulations is in biological systems.

RESULTS AND DISCUSSION

The basic model used in these present computer studies on optimization of drug dosage forms was

$$GI \xrightarrow{k_{GI,B}} B \xrightarrow{k_{B,U}} U \qquad \text{(Eq. 4)}$$

where GI is the gastrointestinal compartment; B, the body compartment; U, the sum of excretory compartments (urine, metabolism, feces, etc.); $k_{GI,B}$ is the first-order rate constant for absorption of drug from the gastrointestinal tract into the blood; and $k_{B,U}$ is the sum of the first-order rate constants for loss (*i.e.*, glomerular filtration, metabolism, etc.) of the free, therapeutically active drug moiety from the body compartment.

This model was chosen for its simplicity since drug formulation rather than complex pharmacokinetics was being investigated. It should be remembered, however, that the computer can be used for any model where the kinetic patterns have been established. Indeed it may be only with the use of a computer that such complex models can be verified in the first place (13–15).

The assumptions that are inherent in the choice of this simple model are that (*a*) the sequential transfers from GI → B → U are by first-order processes and are therefore dependent on the drug amount in each compartment; (*b*) the drug used is nonionic in character and shows no variation in absorption due to pH changes along the length of the gastrointestinal tract; (*c*) the drug is completely absorbed; and (*d*) instantaneous equilibration of the drug occurs between all tissues permeable to the drug and the blood. At any time, therefore, the blood concentration of drug mirrors the concentrations in all drug-containing tissues.

The rate constants for absorption and elimination were held constant throughout the course of the experiment and had the following values:

$$k_{GI,B} = 4.60 \text{ hr.}^{-1}$$

$$k_{B,U} = 1.15 \text{ hr.}^{-1}$$

These values closely approximate those which may be calculated for an oral dose of 400,000 units of potassium penicillin V from the data of Holland *et al.* (24). The high absorption rate constant permits all (100%) of the dose to be absorbed in 1.5 hr., while the elimination rate constant allows the drug a biologic half-life of 0.6 hr.

A computer plot of the gastrointestinal, body, and urinary amounts of drug (labeled D_{GI}, D_B, and D_U, respectively) as fractions of an initial 10 units of drug given in solution or as a rapidly dissolving tablet, with time, is given in Fig. 1.

Certain clinical parameters are needed for proper design of dosage forms. For a given dose the threshold level for this drugs' action is considered to be 2.5 units of the total dose in the blood compartment with a maximum effect noted at 3.5 units (the upper and lower dashed lines of Fig. 1 demonstrate these values). It is interesting to note that only 14% of the dosage was used in the therapeutic range, 21% was expended in D_B levels above 3.5, and 65% at subthreshold levels.

The differential equations and analog computer program which describe the basic model are found in the *Appendix* under *Model I* (Eqs. 1*a*–1*c* and Fig. 2).

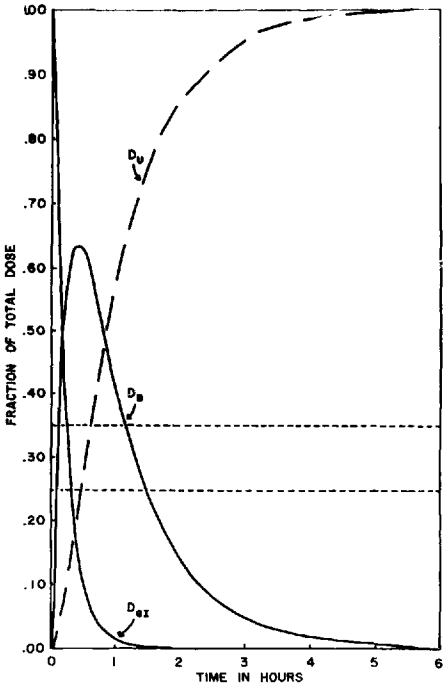


Fig. 1.—Analog computer plot of compartmental distribution of a single 10 unit dose of drug. D_{GI} = amount of drug in the gastrointestinal tract, D_B = amount of drug in the body, and D_U = amount of drug eliminated from the body. The interval between the dashed lines indicates the desired therapeutic range.

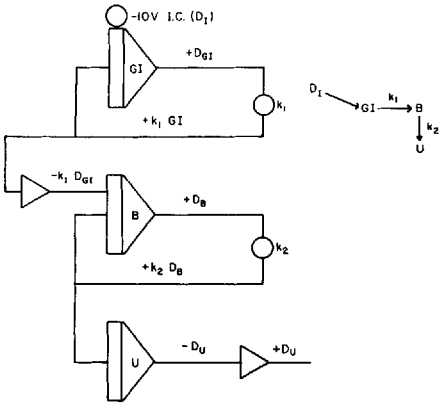


Fig. 2.—Analog computer program for a single 10-unit dose of drug administered in solution into the GI compartment (*Model I*).

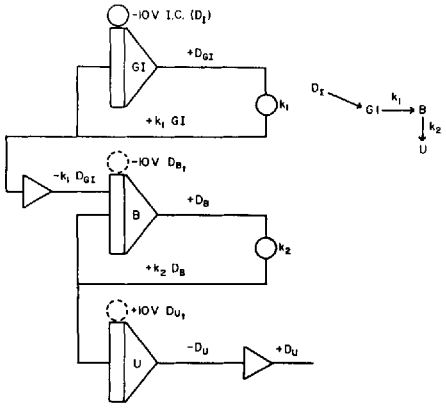


Fig. 4.—Analog computer program used for investigation of a multiple dosage regimen (5 units of drug every 1.2 hr.) (*Model II*).

A logical approach to maintain therapeutic levels with greater drug efficiency is multiple dose therapy. When a drug is administered on a multiple dose regimen with a constant dosage interval, the blood levels of the drug reach a steady state condition after several doses. This steady state is evidenced by the blood levels in a given dosage interval being essentially the same as those in the preceding and following intervals. For the authors' drug, the computer was dosed with half the original dose of Fig. 1 (5 units) at zero time followed by equal doses at consecutive 1.2 hr. intervals (2 half-lives) until 8.4 hr. The typical compartmental profile for such multiple dosing is seen in Fig. 3. The equations used for this approach are the same as those used for the basic model (*Model I*, Eqs. 1*a*–1*c*). The computer program involves only minor modifications of the one used for single dosing and is seen in *Model II* (*Appendix*) and Fig. 4. It is readily apparent that adjustments of dosage amounts and times are easily accomplished by slight modifications of the computer program to graphically evaluate optimum choices. In Fig. 3 therapeutic blood levels are maintained for the better part of 9 hr. The need for dosage form optimization is evident, however, to supplant the repetitive dosing regimen as well as to moderate the undesirable rising and falling of the body compartment levels.

Sustained and Delayed-Release Models.—The desirable qualities of oral sustained or delayed-release formulations have been listed by Rowland and Beckett (25) and include an initial and a maintenance dose to give and maintain blood concentrations of drug which elicit the desired therapeutic effects.

The benefits would be to reduce the frequency of drug administration compared with conventional dosage forms and to give a more uniform biological response with a reduced incidence and intensity of side effects.

In general, the total dose used in a sustained-release dosage form is the sum of the amount in the initially fast dissolving portion (D_I) and that present in the more slowly dissolving maintenance form

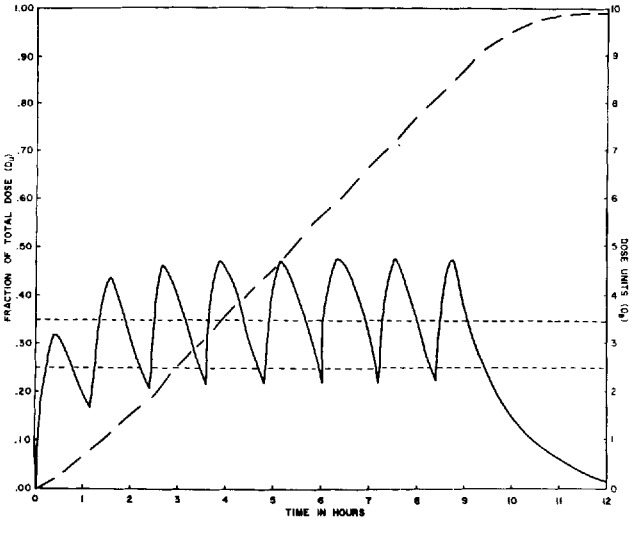


Fig. 3.—Compartmental levels of drug using a repetitive dosage regimen (5 units of drug every 1.2 hr.). The ordinates for blood level are given on the right in terms of units of drug. Key: – – – –, D_U; ———, D_B.

(D_M). As ideally postulated, the "loading" dose (D_I) will bring the drug amount in the body to its therapeutic level, while the D_M will maintain this level for the prescribed period of time.

The major prerequisite for the "loading" dose, D_I, is that it dissolve rapidly and completely. This end is normally achieved by either placing the drug in solution as an initial dose or formulating a conventional, fast disintegrating and dissolving tablet which, in essence, achieves the same goals. For the authors' purposes, D_I was placed on the computer as an initial condition on the GI compartment integrator (see *Appendix*). What this accomplishes is to have 100% of D_I in solution and ready for absorption at time zero.

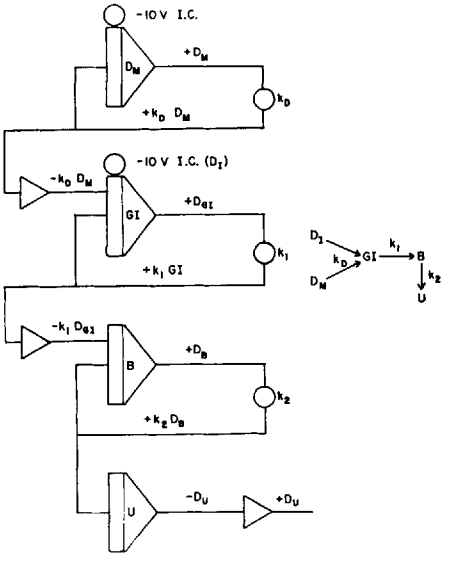


Fig. 5.—Analog computer program used for evaluation of first-order sustained-release dosage formulations (*Model III*).

The formulation and programing of the "maintenance" or slowly releasing form, D_M, is a problem of greater complexity. Three basic models for sustained and delayed-release formulations have been chosen.

First-Order Release.—Both Wiegand and Taylor (26) and Wagner (17) have shown that per cent released *in vitro versus* time data reported in the literature for the dissolution of many sustained-release preparations give linear pseudo (or apparent) first-order rates from about 0.5 hr. to the time the test was completed. Literature data subsequent to these papers have confirmed these observations and include: (*a*) drug embedded in an insoluble tablet matrix (27), (*b*) drug coated with waxy or polymeric materials (28), and (*c*) drug complexed with cation exchange resins (29). Since these preparations make up the predominant number of available sustained-release products, it was decided to investigate the programing of a first-order release rate for D_M as a method for prolonging the blood level of the drug under investigation.

The equations and program which describe this model are found in the *Appendix* under *Model III*, Eqs. 3*a*–3*d*, and Fig. 5.

An infinite number of first-order disappearances of D_M can be obtained by systematic alteration of the potentiometer corresponding to k_D (the rate constant for dissolution of D_M).

Using these k_D's and various combinations of D_I and D_M, the computer was then programed to determine the set of conditions which yield the maximum therapeutic benefit.

Figure 6 shows the type of sustained release obtained in a typical case with such a first-order loss from D_M. In this case, 10% of the total dose (which was twice the dose used in Fig. 3) is present as D_I, while 90% is D_M. The dissolution rate constant (k_D) for D_M was chosen as 0.375 hr.$^{-1}$.

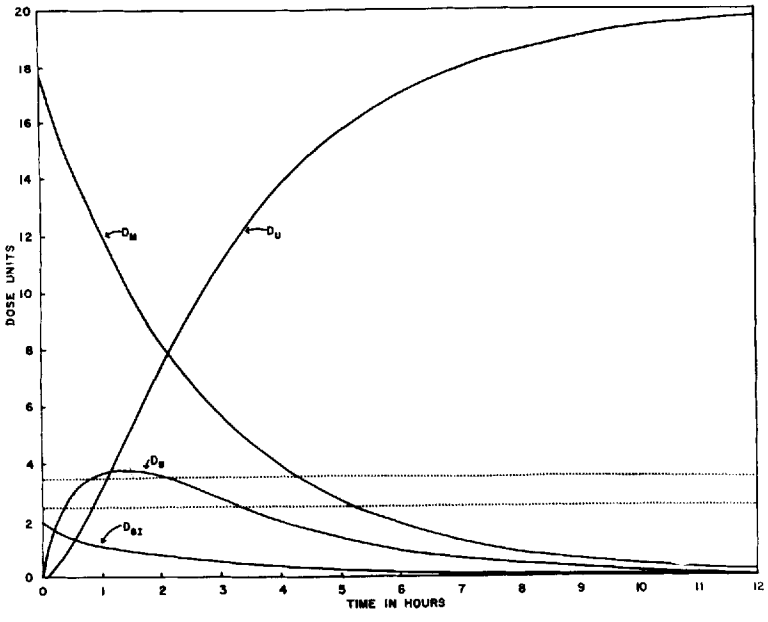


Fig. 6.—Effect of a first-order sustained-release maintenance dose (D_M) on compartmental drug levels. This formulation was programed to yield a drug-free system after 12 hr. D_I = 2 units; D_M = 18 units.

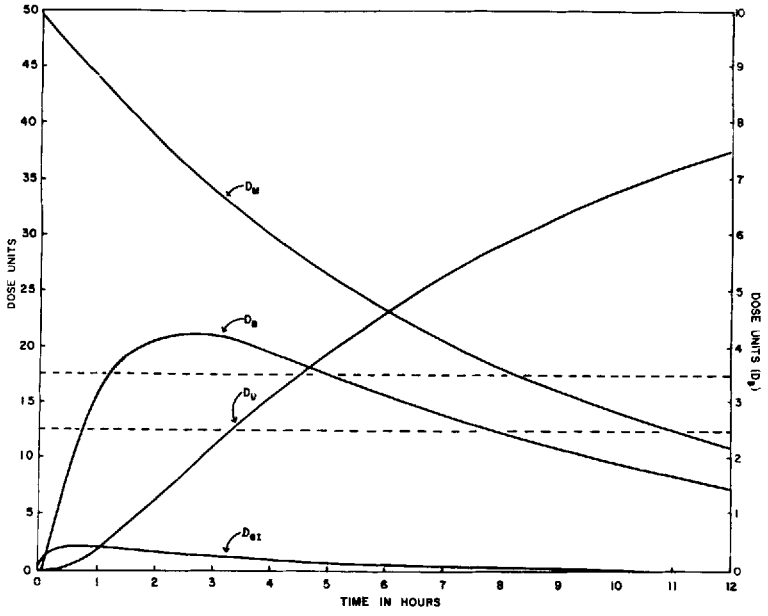


Fig. 7.—Effect of increasing amount of drug in D_M and decreasing k_D. No time limit was set here on the maintenance of drug in the body. The ordinates for blood level are given on the right. Key: D_M = 50 units; D_I = 0.

It would seem, at least for this drug, that a first-order mechanism for the release of the maintenance dose offers no significant improvement over multiple dosing. The optimum increase in blood level duration for twice the amount of drug is twice that obtained for the original dose. Other combinations of k_D, D_I, D_M lead to the same conclusions. When the dose or k_D is increased, drug levels in the body are increased. The only apparent advantage of such a sustained-release dosage design is the reduction in times of oral administration; there is no apparent increase in the efficient usage of the drug amounts. An illustration of this point is Fig. 7, where 5 times (50 units) the dose used in Fig. 1 is given with a k_D of 0.125 hr.$^{-1}$. The increased duration of blood level is only 5 times that observed for 10 units of drug.

Release from Coated Pellet Formulations.—Many companies now supply products in the form of a hard gelatin capsule containing round candy pellets upon which has been deposited a fixed amount of drug. Coated over the drug are one or more thicknesses of a waxy or polymeric coating, whose dissolution rate has been evaluated in both *in vitro* and *in vivo* systems.

When either the thickness or the type of coating substance is varied, it is possible to obtain several different populations of drug pellets. If the appearance of drug in solution is measured with time, the drug will only appear after the protective coating has been ruptured, dissolved, or digested away.

If the situation was ideal, and all members of a given population of pellets had exactly the same coating thickness, *i.e.*, the variance among pellets was zero, the drug in solution *versus* time plot should show a lag period (equal to that amount of time necessary to rupture, dissolve, or digest the protective coating) followed by a steep, almost instantaneous, appearance of all the drug from this population of pellets into solution. This would be a direct simulation of the repetitive dosage regimen we have previously considered in *Model II* and Fig. 3.

What is far more likely, however, is that a finite wide variance does indeed exist within a chosen population of pellets. This being the case, the dissolution of the population of pellets is not instantaneous, but rather normally distributed with mean $\bar{t}$ and variance, σ^2. Integration of the area under the normal curve, number of pellets ruptured, or releasing drug *versus* time, will give the amount of drug in solution. A plot of this integral *versus* time will yield a symmetrical sigmoid curve whose midpoint is $\bar{t}$.

In accordance with the authors' postulated model, the release of drug from the pellet types occurs at separated intervals with a Gaussian distribution of release about the mean of this interval. The amount of drug which is to be released at each interval is a function of the numbers of pellets so formulated. The release of drug from each pellet is relatively instantaneous; the release of total drug for that pellet type is normally distributed about the mean time of release of that pellet population. If a dosage form is produced with 100 or more populations of effective thicknesses with each containing equivalent total amounts of drug an over-all zero-order release can be simulated.

Figure 8 demonstrates a typical compartmental profile using a pellet-type formulation; the D_I = 5 dose units and the D_M consisted of 4 populations of pellets (each containing 5 units of drug) with different mean times of release. The standard deviation about these mean times of release was considered to be the same for each population in this case. The D_B and D_{GI} are plotted in terms of dose units in the respective compartment while D_U is shown on scale as 0.4 times the actual dosage units excreted.

Several studies in the literature (30–32) have demonstrated a simulation of t.i.d. dosing with coated pellet sustained release. Per cent release data for 5 bulk pellet groups, presented in one paper (30), support the normal distribution postulate.

The rather unique equations and programs used to simulate the normally distributed pellet popula-

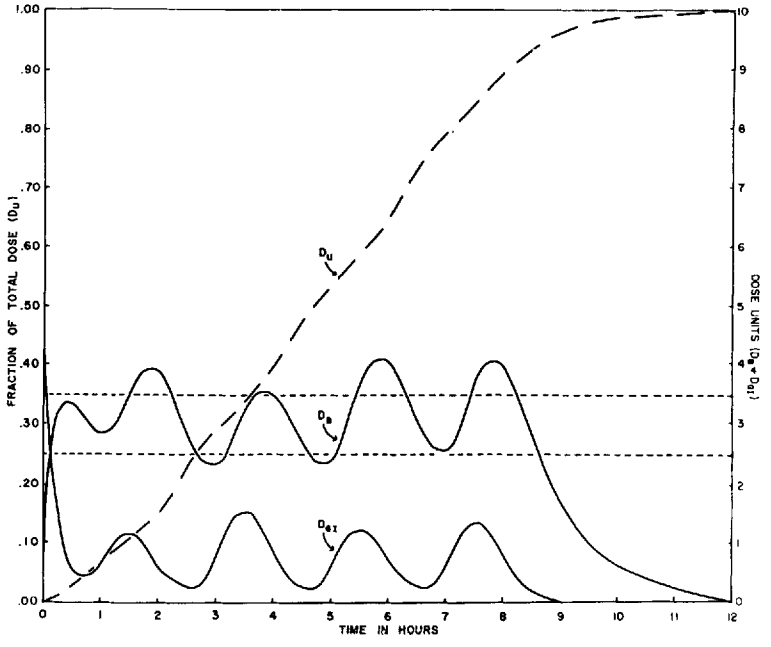


Fig. 8.—A simulated repetitive dosing compartmental profile obtained after a single dose of a coated pellet formulation. Four pellet populations, each containing 5 units of drug, with the same standard deviation but different mean times of dissolution were used in addition to 5 units of immediately dissolving D_I.

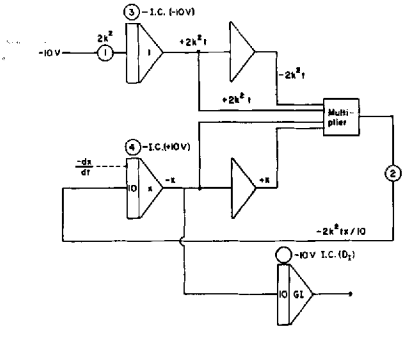


Fig. 9.—Analog computer program for the generation and integration of a Gaussian type distribution (coated pellet type of sustained release). The remainder of the program is the same as in Fig. 2 (*Model IV*).

tions (33) are found in the *Appendix* under *Model IV*, Eqs. 9–12, and Fig. 9, respectively.

Zero-Order Release.—The ideal mechanism to maintain a constant blood level is to release drug from D_M by zero-order kinetics (*i.e.*, at a constant rate independent of concentration).

To achieve optimum therapy, the rate of change of drug in the blood (D_B) with time should be zero. Since this rate is the difference between the rate of absorption and the rate of excretion

$$dD_B/dt = k_{GI,B}D_{GI} - k_{B,U}D_B = 0 \quad \text{(Eq. 5)}$$

it follows that the amount of drug in the gastrointestinal tract, D_{GI}, should be a constant for a desired blood level, D_B, since from Eq. 5,

$$D_{GI} = \frac{k_{B,U}}{k_{GI,B}} D_B \quad \text{(Eq. 6)}$$

It also follows that the amount of drug in the gastrointestinal tract, D_{GI}, should remain invariant with time

$$dD_{GI}/dt = dD_M/dt - k_{GI,B}D_{GI} = 0 \quad \text{(Eq. 7)}$$

From Eqs. 6 and 7 the rate of release from the maintenance dose should be

$$\frac{dD_M}{dt} = \frac{k_{B,U}}{k_{GI,B}} \cdot k_{GI,B}D_B = k_{B,U}D_B \quad \text{(Eq. 8)}$$

Thus, the rate of release from the maintenance dose D_M must be constant and equal to the rate of loss of drug from the blood at a blood level corresponding to D_B. This rate is given by Eq. 8.

Zero-order release may be feasible in *in vivo* situations when the drug has a limited solubility in the gastrointestinal fluids. Under the circumstances, the drug will initially dissolve at a rate proportional to the amount of drug remaining in the tablet until a saturated solution is formed in the gastrointestinal fluids. Afterward, the drug should dissolve at the same rate as it is absorbed with a net result of a constant dissolution rate.

Figure 10 demonstrates the compartmental profile for a formulation containing $D_I = 4$ units and $D_M = 6$ units (solid line) and $D_I = 4$ units, $D_M = 16$ units (dashed line). The solubility (D_S) was chosen at 4.0 drug units. The improvement in therapeutic effect over those conditions used in Fig. 1 can be observed here where the desired blood level has a duration of 1.9 hr. compared to the 1.4 hr. noted with a conventional dosage form.

The equations used to predict the k_D required for the D_M of Fig. 10 as well as those describing the model are found under *Model V* (*Appendix*). The computer program for zero-order release is Fig. 11.

APPENDIX

Model I.—*Computer Simulation of a Single, Rapidly Dissolving Dose.*—A single dose, in solution

$$D \longrightarrow GI \xrightarrow{k_{GI,B}} B \xrightarrow{k_{B,U}} U$$

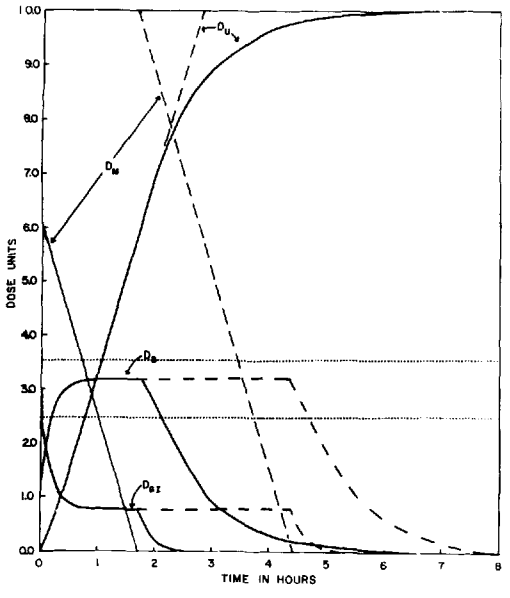


Fig. 10.—Effect of a zero-order sustained-release dosage form on compartmental drug levels. The solid lines are for a formulation containing $D_I = 4$ units and $D_M = 6$ units, while the dashed lines are for $D_I = 4$ units and $D_M = 16$ units of drug.

in the GI compartment at time zero is absorbed into, and lost from, the blood at a rate proportional to the residual drug concentration.

$$-\frac{dD_{GI}}{dt} = k_{GI,B}D_{GI} \qquad \text{(Eq. 1}a\text{)}$$

$$-\frac{dD_B}{dt} = k_{B,U}D_B - k_{GI,B}D_{GI} \qquad \text{(Eq. 1}b\text{)}$$

$$\frac{dD_U}{dt} = k_{B,U}D_B \qquad \text{(Eq. 1}c\text{)}$$

The computer program resulting from these equations is seen in Fig. 2.

Model II.—*Computer Simulation of Repetitive Dosing Regimen.*—One-half of the dose used in

$$D \longrightarrow GI \xrightarrow{k_{GI,B}} B \xrightarrow{k_{B,U}} U$$

Model I is administered at time zero followed by the same dose every 2 half-lives (1.2 hr.). The equations describing repetitive dosing are identical to Eqs. 1*a*–1*c*. The computer program, Fig. 4, is essentially the same, differing only by the presence of initial condition potentiometers on the blood and urine integrators. At the beginning of each dosage interval the computer is stopped and these potentiometers are used to place the amount of drug remaining from the previous dosing in each compartment. For example, referring to Fig. 3, the amount of drug placed on the blood potentiometer at $t = 1.2$ hr. ($t = 0$ for dose 2) was 1.7 dose units.

Model III.—*Computer Simulation of First-Order Sustained Release.*—Dosage forms:

$$\begin{matrix} D_I \longrightarrow \\ D_M \overset{k_D}{\longrightarrow} \end{matrix} GI \xrightarrow{k_{GI,B}} B \xrightarrow{k_{B,U}} U$$

A combination of immediately soluble D_I and slowly releasing D_M is given at time zero. The rate of release of D_M is proportional to the amount of D_M remaining in the dosage form.

$$-\frac{dD_M}{dt} = k_D D_M \qquad \text{(Eq. 3}a\text{)}$$

$$-\frac{dD_{GI}}{dt} = k_{GI,B}D_{GI} - k_D D_M \qquad \text{(Eq. 3}b\text{)}$$

$$-\frac{dD_B}{dt} = k_{B,U}D_B - k_{GI,B}D_{GI} \qquad \text{(Eq. 3}c\text{)}$$

$$\frac{dD_U}{dt} = k_{B,U}D_B \qquad \text{(Eq. 3}d\text{)}$$

The computer program used to predict the optimum D_M and k_D for sustained therapeutic blood levels is seen in Fig. 5.

Model IV.—*Computer Simulation of a Coated Pellet Type of Sustained Release.*—Several populations of pellets, whose dissolution pattern follows a normal distribution, are combined to yield, upon administration of a single dose, a compartmental profile similar to that obtained with repetitive dosing.

A unique method was found (33) for computer simulation of a normal distribution and is as follows.

The frequency distribution which describes the normal distribution (Gaussion errror function) is

$$f(\gamma) = \frac{1}{\sqrt{2\pi}\sigma} e^{-(\gamma-\mu)^2/2\sigma^2} \qquad \text{(Eq. 9)}$$

or

$$x = Ae^{-k^2t^2}$$

where

$$A = \frac{1}{\sqrt{2\pi}\sigma}, \; k = \frac{1}{\sqrt{2}\sigma}, \text{ and } t = \gamma - \mu \qquad \text{(Eq. 10)}$$

The first derivative of Eq. 10 is

$$\frac{dx}{dt} = -2Ak^2te^{-k^2t^2} \qquad \text{(Eq. 11)}$$

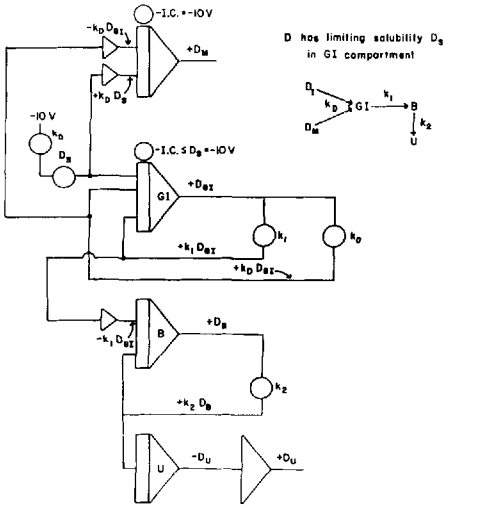


Fig. 11.—Analog computer program for evaluation of solubility limited, zero-order sustained-release dosage formulations (*Model V*).

Substituting Eq. 11 into Eq. 10 one obtains

$$\frac{dx}{dt} + 2k^2tx = 0 \qquad \text{(Eq. 12)}$$

The analog computer program for Eq. 12 is seen in Fig. 9. The plotted output of the x integrator gives the bell-shaped curve expected for the Gaussion error function. Integration of the area under this curve gives the amount of drug released into the gastrointestinal tract (D_{GI}). The remainder of the program (for D_{GI}, D_B, and D_U) is the same as in Fig. 2.

Model V.—*Computer Simulation of a Zero-Order Sustained-Release Mechanism.*—Let drug D have a

$$\begin{matrix} D_I \longrightarrow \\ D_M \text{---}\rfloor \end{matrix} GI \xrightarrow{k_{GI,B}} B \xrightarrow{k_{B,U}} U \qquad k_D$$

finite, limiting solubility (D_S) in the gastrointestinal tract. Let the initial dose, D_I, present as an initial condition in the GI compartment be equal to or less than D_S. As saturation is approached, the release of D_M will become constant.

The equations used for this program are

$$-\frac{dD_M}{dt} = k_D(D_S - D_{GI}) \qquad \text{(Eq. 4}a\text{)}$$

$$-\frac{dD_{GI}}{dt} = k_{GI,B}D_{GI} + k_DD_{GI} - k_DD_S \qquad \text{(Eq. 4}b\text{)}$$

$$-\frac{dD_B}{dt} = k_{B,U}D_B - k_{GI,B}D_{GI} \qquad \text{(Eq. 4}c\text{)}$$

$$\frac{dD_U}{dt} = k_{B,U}D_B \qquad \text{(Eq. 4}d\text{)}$$

The program corresponding to these equations is seen in Fig. 11.

The calculations used to predict the exact k_D required are as follows. Since Eq. 4*c*

$$-\frac{dD_B}{dt} = k_{B,U}D_B - k_{GI,B}D_{GI}$$

For a sustained blood level, $\frac{dD_B}{dt} = 0$, and the D_{GI} necessary is

$$D_{GI} = \frac{k_{B,U}}{k_{GI,B}} D_B \qquad \text{(Eq. 13)}$$

for D_{GI} to be constant, $dD_{GI}/dt = 0$ and, from Eq. 4*b*

$$k_{GI,B}D_{GI} = k_D(D_S - D_{GI}) \qquad \text{(Eq. 14)}$$

and, since D_S, D_{GI}, and $k_{GI,B}$ are known, the necessary k_D can be calculated

$$k_D = \frac{k_{GI,B} \cdot D_{GI}}{D_S - D_{GI}} \qquad \text{(Eq. 15)}$$

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Species Differences in Lipid and Endocrine Gland Responses to a Stilbene Derivative

By T. S. DANOWSKI, N. R. LIMAYE, R. E. COHN, B. J. GRIMES, J. V. NARDUZZI, and C. MOSES

A stilbene derivative, α-(*o*-anisyl)-β,β-diphenylacrylic acid, administered to healthy male adults did not affect, as it did in certain experimental animals, food intake, body weight, serum lipid levels, thyroid activity, adrenocortical function, or the other endocrine indices tested.

A DERIVATIVE of the stilbenes, α-(*o*-anisyl)-β,β-diphenylacrylic acid,[1] at dosages of 10 mg./Kg./day or higher, exerts a hypocholesterolemic effect in the mouse, rat, and dog, but not in the monkey (1). Studies in experimental animals have also demonstrated that ingestion of the compound is accompanied by an absolute or relative decrease in seminal vesicle and prostate weights and increases in pituitary, thyroid, and adrenal size. The adrenomegaly in such animals is associated with decreased adrenocortical function, judging from a delayed excretion of a water load, decreased tolerance to cold stress, and diminished corticogenesis *in vitro* in adrenals from rats pretreated with this derivative. Estrogenic effects have also been observed during such therapy and in the rat the compound has been found to inhibit the ovarian hypertrophy which follows unilateral ovariectomy. This derivative exerted a profound anorexigenic effect in the mouse, adult rat, and rabbit resulting in a marked loss of weight, but this was not evidenced in the dog or monkey. It has been suggested that the hypocholesterolemic effect in animals may be related to decreased food intake and that the compound probably exerts antithyroid, antiadrenocortical, and antigonadotropic actions (1).

The studies reported here indicate that in healthy adult males comparable dosages, up to 800 and 1600 mg./day for 1 month or longer, neither affected body weight, serum cholesterol, triglycerides, or other solutes, nor did it produce changes in indices of the thyroid, adrenocortical, adrenomedullary, or other endocrine gland function.

MATERIALS AND METHODS

The derivative of stilbene was administered *per os* to 14 healthy adult male prisoners in increasing dosages: 50 mg. daily for 3 weeks, followed by 100 mg. daily for 3 weeks, 200 mg. daily for 3 weeks, 400 mg. daily for 4 weeks, and 800 mg. daily for 4 weeks. In another group of four men 1600 mg. was administered daily for 4 weeks.

Blood and serum solutes (venous blood sugar and NPN) and serum CO_2, Cl, Na, K, albumin, globulin, calcium, inorganic phosphorus, uric acid (2), creatinine (3), total and α- and β-lipoprotein cholesterol and triglycerides (4–7), NEFA (8), and protein bound iodine (9) were measured in samples obtained at 6:30 to 7:00 a.m. during the fasting state on two occasions 1 week apart prior to therapy. The same studies were repeated at the end of each dosage period.

Urinary 17-ketosteroids (10), Porter-Silber chromogens (11), 11-desoxycortisol metabolites (12), pressor materials as measured by aortic strip assay (13), and gonadotropins (14) were determined in 24-hr. refrigerated specimens of urine prior to and during the administration of 400, 800, and 1600 mg. per day.

Plasma 17(OH) corticosteroids (15) were measured at weekly intervals prior to and during therapy in samples of venous blood obtained between 6:30 and 7:00 a.m.

The response of plasma 17(OH) corticosteroids to exogenous ACTH [80 units (Parke-Davis) in 500 ml. of 5% dextrose in water] administered by intravenous infusion during a 6-hr. period was examined before and during therapy with the compound at 400 mg./day for 4 weeks and 1600 mg./day for 4 weeks. The plasma corticosteroid levels were measured in samples of blood obtained just before the infusion and just at the end of the infusion.

The effects of intravenously administered rapidly acting insulin (0.1 unit/Kg. of body weight injected intravenously) upon venous blood sugar and serum inorganic phosphorus levels, were assessed prior to and during the 12th week of therapy when the dosage had reached 400 mg./day.

Certain hepatic indices (serum bilirubin, cephalin flocculation, thymol turbidity, alkaline phosphatase in addition to serum albumin, globulin, and lipids already cited) were obtained before and during therapy. Renal status was evaluated by means of routine urine analyses and creatinine clearance on two occasions prior to and two others during therapy. The hemoglobin and relative blood cell volume (hematocrit) were also measured on the above schedule.

RESULTS

The drug was taken without evoking any symptoms or signs. Body weights were of the same order of magnitude prior to and during therapy. Therapy

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[1] Supplied by Smith Kline & French Laboratories, Philadelphia, Pa., as SK&F 16046.

was not associated with any change in the venous blood sugar or NPN or the serum CO_2, Cl, Na, K, Ca, inorganic phosphorus, albumin, globulin, and uric acid. The serum total cholesterol and triglycerides and their respective α and β lipoprotein fractions remained unchanged. Serum PBI levels were not altered during therapy. The excretion of urinary 17-ketosteroids, Porter-Silber chromogens, and 11-desoxycortisol metabolites expressed in mg./day/1.0 Gm. of creatinine was essentially the same before and during ingestion of this compound. During therapy the fasting a.m. levels of plasma 17(OH) corticosteroids did not differ significantly from the values recorded during the control period.

The plasma 17(OH) corticosteroid responses to intravenous ACTH prior to and during administration of the compound were of the same order of magnitude. Therapy for 12 weeks was not accompanied by significant alteration in the hypoglycemic or hypophosphatemic effects of intravenously administered rapidly acting insulin. The urinary excretion of gonadotropins, urinary pressor activity (aortic strip assay), serum creatinine, and creatine clearances and routine urine analysis were unchanged at the end of the treatment period. Hepatic indices and the hematocrit and hemoglobin remained within the pretherapy range during ingestion of this agent.

DISCUSSION

None of the effects of this derivative of the stilbenes, variously observed in some but not all species of experimental animals tested (1), could be discerned in the trials in healthy male adults described here. Thus, anorexia, weight loss, and hypocholesterolemia (perhaps related to decreased food intake) did not occur. Also indices of pituitary, thyroid, adrenocortical, adrenomedullary, and gonadotropic and other endocrine gland function were not affected. Blood sugar responses to intravenous insulin were not changed. The compound did not alter the hepatic indices tested. In other words, effects of this stilbene derivative, observed in certain species of experimental animals, were not evident in healthy male adults receiving this compound in comparable dosages.

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Effects of Certain Preservatives on the Aging Characteristics of Acacia

By OSCAR E. ARAUJO

The rheological characteristics of acacia solutions of various concentrations were examined. Based on the results, a series of 25 per cent acacia solutions using five different preservatives and two combinations of preservatives were studied for a period of 1 year. Rheological, pH, and organoleptic data were obtained in order to describe the aging characteristics of the solutions. In each case, the results showed a reduction in pH and viscosity, which was most pronounced in the unpreserved control solution. Possible reasons for the decrease, as well as for the rate at which it occurred, are discussed.

ACACIA HAS been in use for at least 4000 years and yet comparatively little quantitative research appears in the literature (1). Curiously, in spite of the popularity enjoyed by this polysaccharide as a protective colloid and emulsifier in numerous pharmaceutical preparations, only a handful of findings pertaining to its aging characteristics are reported.

Taft and Malm (2) found that bacterial growth appeared in dilute solutions of gum arabic 36–48 hr. after preparation. Osborne and Lee (3) carried out experiments to establish the effect of aging upon preserved and unpreserved acacia mucilages. The preservative used was 0.2% benzoic acid. These investigators reported an initial rise and an eventual decrease in the viscosity of the mucilages. They further observed a considerably greater decrease in the viscosity of the unpreserved mucilage than of the preserved one. Joslin and Sperandio (4) reported that acacia mucilages prepared with boiling water exhibited a reduction in viscosity over a period of 8 weeks. However, the authors stated that this decrease in viscosity was less pronounced than the reduction occurring in those mucilages prepared with water at room temperature. They further noted that the acacia mucilages became slightly more acidic during the aging period.

Recently, Ory and Steiger-Trippi (5) reported the changes in viscosity observed after 1 month's

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storage in solutions containing 7.5% and 20% acacia. Four preservatives were used in concentrations of 2%: sorbic acid, phenyl mercuric borate, a mixture of the sodium salts of the ethyl and propyl esters of *p*-hydroxy benzoic acid, and an arylalkyl quaternary ammonium sulfate. The results indicated that a reduction in viscosity occurred in all solutions except those preserved with phenyl mercuric borate.

The above studies assumed that acacia solutions at the concentrations used behaved as Newtonian systems, and, therefore, the viscosity at only 1 rate of shear was necessary. It appeared reasonable to establish the experimental validity of such an assumption by measuring the viscosity of acacia solutions of various concentrations at several rates of shear. If the rheograms were to show Newtonian behavior, then acacia solutions of an arbitrary concentration would be studied further to determine the effect of a number of preservatives on their aging characteristics.

EXPERIMENTAL

Preparation of Solutions.—All acacia solutions were prepared by placing the proper volume of distilled water previously heated to 60° into a Waring blender. The accurately weighed gum arabic was then added to the surface of the water, permitted to hydrate momentarily, and then blended for 1 min. All percentages reported are on a weight to weight basis. The preservatives used in this study were dissolved in the warm water prior to the blending procedure. Six hours were permitted to elapse after the preparation of the solutions before any measurements were made. This was done to insure complete hydration of the gum and to allow any foam created by the mixing process to subside. All solutions were examined for a period of 1 year. U.S.P. grade acacia taken from the same lot was employed in order to eliminate source variations.

pH Measurements.—A Photovolt electronic pH meter model 110 was used to measure the pH of the solutions at convenient intervals of time.

Rheological Measurements.—A Stormer viscosimeter equipped with the modified cup and bob as suggested by Fischer (6) was used in this investigation. A weight hanger with various slotted weights provided the shearing stress. Calibration curves using castor oil as a standard were constructed periodically throughout the study. The viscosity of castor oil was obtained from standard tables. An average instrumental constant, K_v, was calculated from the data collected according to the expression

$$\eta = K_v \frac{\text{Gm.}}{\text{r.p.m.}} \qquad \text{(Eq. 1)}$$

where Gm. represents the shearing stress and r.p.m. the rate of shear. Once the constant, K_v, vas obtained, the apparent viscosity, η, of any system could be arrived at by means of this equation. All viscosity measurements were carried out at 20 ± 0.1°.

Organoleptic Observations.—A description of the appearance and the odor of the solutions was recorded periodically throughout the investigation.

Preservatives Used.—Five different preservatives were employed in the following concentrations: 0.2% benzoic acid, 0.2% methylparaben, 0.05% propylparaben, 0.5% chlorobutanol, and 0.01% benzalkonium chloride. Combinations of benzoic acid–propylparaben and benzoic acid–chlorobutanol in the above percentages were also used.

RESULTS AND DISCUSSION

Verification of Newtonian Characteristics.—Solutions containing 10, 12.5, 15, 20, 25, 30, 35, 40, and 45% acacia were prepared by the previously described method. The rheological data were obtained within 24 hr. from the time of preparation, thus obviating the addition of preservatives to the solutions. Figure 1 shows representative rheograms of the results. It was clearly seen that Newtonian behavior occurred at the lower concentrations as evidenced by the linearity of the plots. At concentrations of 40% acacia and above, pseudoplastic characteristics were observed, as denoted by a decrease in viscosity with increasing shearing stress.

The power law equation (7)

$$F^N = \eta' G \qquad \text{(Eq. 2)}$$

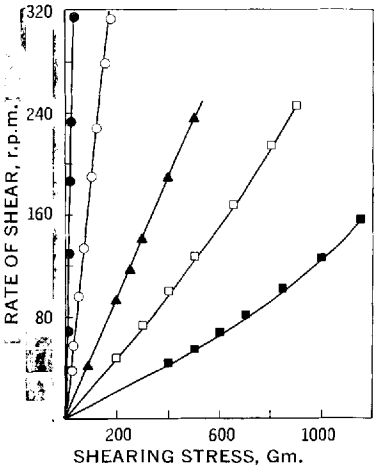

Fig. 1.—Rheograms of unpreserved acacia solutions of various concentrations. Key: ●, 10%; ○, 25%; ▲, 35%; □, 40%; ■, 45%.

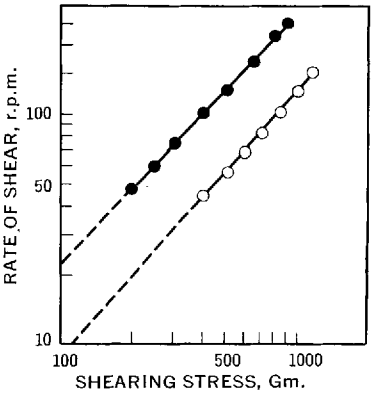

Fig. 2.—Log-log rheograms of unpreserved acacia solutions for calculation of index of pseudoplasticity. Key: ●, 40%; ○, 45%.

TABLE I.—pH OF 25% ACACIA SOLUTIONS

Preservative Used	Time Elapsed						
	0 Days	1 Wk.	2 Wk.	6 Wk.	3 Mo.	9 Mo.	1 Yr.
None	4.80	4.78	4.60	4.30	4.20	3.85	3.50
Benzoic acid	4.72	4.70	4.48	4.47	4.30	4.20	3.90
Benzoic acid–chlorobutanol	4.65	4.60	4.55	4.22	4.20	4.20	3.90
Benzoic acid–propylparaben	4.65	4.60	4.52	4.28	4.20	4.20	3.90
Propylparaben	4.79	4.60	4.65	4.35	4.32	4.25	4.00
Methylparaben	4.79	4.70	4.70	4.25	4.20	4.20	4.10
Chlorobutanol	4.80	4.78	4.62	4.34	4.30	4.25	4.00
Benzalkonium chloride	4.79	4.75	4.70	4.32	4.23	4.20	4.00

where F is the shearing stress in Gm., G is the rate of shear in r.p.m., η' is an indicator of viscosity, and N is an index of non-Newtonian behavior, has been used to describe pseudoplastic systems. This exponential expression in its logarithmic form

$$\log G = N \log F - \log \eta' \qquad \text{(Eq. 3)}$$

was applied to the rheological data of the solutions exhibiting pseudoplasticity (40 and 45% acacia). The resulting linear relationships are shown in Fig. 2. The value of N must be greater than unity if the system is pseudoplastic, and its magnitude is proportional to the degree of pseudoplasticity. The calculated value of N for the 40% acacia solution was 1.08 and that for the 45% solution was 1.15. Thus, the degree of pseudoplasticity appeared to increase with increasing concentrations of acacia. This was in line with the concept whereby linear polymers tend to orient themselves along the direction of flow when a shearing stress is applied. At low concentrations, this orientation probably occurs at minimal shearing stresses and the solution behaves as a Newtonian system, where the viscosity is constant. At higher concentrations, it is conceivable that low stresses are not sufficient to rearrange the greater number of polymers present, resulting in pseudoplastic behavior until the force is large enough to produce complete alignment.

Aging Studies—*General.*—It was apparent that it would be considerably easier to characterize a Newtonian system, which has a constant viscosity, than a pseudoplastic one. It was also felt that a solution containing enough acacia to support an appreciable amount of bacterial growth was desirable. With these criteria in mind, solutions containing 25% acacia were prepared, as before, for the aging studies conducted.

The previously mentioned preservatives were added in the amounts earlier stipulated. An unpreserved 25% acacia solution served as a control.

pH Considerations.—The change in pH of the solutions upon standing appeared to follow a definite pattern, as seen in Table I.

A sharp decrease in pH occurred in the first 6 weeks, ranging from 0.25 pH units for the benzoic acid preserved solutions to 0.54 units for the solution containing methylparaben. These values represented 30 and 78%, respectively, of the total drop in pH occurring after 1 year's storage time for each of these solutions. The mean drop in pH after 6 weeks for all solutions studied, expressed as a per cent of the decrease observed after 1 year on the shelf, was 53%.

These results seemed to indicate some manner of bacterial attack on the carbohydrate, with a consequent production of acid groups causing a pH lowering. The pH decreased rapidly at first, but after 6 weeks the medium either had become too acid to support the same rate of bacterial decomposition, or there had been a severe depletion of available substrates for bacterial action. The reduction in pH may also have resulted from spontaneous hydrolysis of certain ester linkages of the carbohydrate.

The over-all mean decrease in pH after 1 year for all the preserved solutions was 0.75 units, while that for the unpreserved solution was 1.30. This was expected since the latter should have supported the

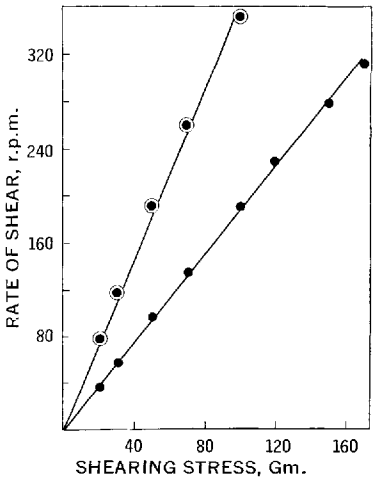

Fig. 3.—Rheograms of unpreserved 25% acacia solutions. Key: ●, 6 hr. after preparation; ◉, 1 year after preparation.

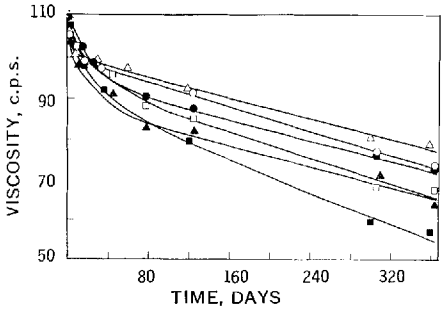

Fig. 4.—The viscosity stability of preserved and unpreserved acacia solutions. Key: △, 0.2% benzoic acid; ○, 0.2% methylparaben; ●, 0.05% propylparaben; □, 0.5% chlorobutanol; ▲, 0.01% benzalkonium chloride; ■, unpreserved.

greatest amount of bacterial decomposition and, consequently, the largest reduction in pH.

Organoleptic Qualities.—None of the solutions showed any visible growth in the first month of storage. Each initially exhibited its characteristic chemical odor, depending on the preservative used. The unpreserved solution had a typical musty odor of gum, which grew unpleasant after 10 days. The solution containing benzalkonium chloride, after only 1 week, gave rise to a pungent, disagreeable odor, which became increasingly offensive as time elapsed. Two months after preparation, mold growth appeared on both the unpreserved solution and the one containing benzalkonium chloride. There appeared to be gas released as the containers were opened, a common occurrence in many types of bacterial action.

After 1 year of shelf life, all the solutions exhibited a slight degree of turbidity and, as before, the unpreserved and benzalkonium chloride solutions gave evidence of the greatest amount of growth and most offensive odors. The reduction in pH of the solutions, due to the aforementioned bacterial attack, provided a good medium for mold growth most apparent in the unpreserved solution where the drop in pH was the greatest.

Rheological Observations.—It was assumed that 25% acacia solutions behaved as Newtonian systems throughout the entire study. This premise was verified by rheograms of the solutions constructed at various storage times. Figure 3 shows representative plots where the linear characteristics can be seen.

The changes in viscosity of the various solutions during the period studied are shown graphically in Fig 4. In order to avoid confusion, the plots for the solutions containing the combinations of preservatives were not included. The data collected, however, would place these graphs immediately below the plot for the solution containing benzoic acid.

A comparison of the rheological results with the pH observations previously discussed revealed certain similarities. First, a definite reduction in viscosity was observed in all solutions. Second, as expected, the unpreserved solution showed the greatest decrease in viscosity at the end of 1 year. Finally, a sharp decrease in viscosity occurred during the first 6 weeks on the shelf. The same rationale used in explaining the reduction in pH would apply here.

It is interesting to note that, unlike the pH observations, the viscosity drop after 6 weeks, expressed as a per cent of the total reduction over a 1-year period, ranged only from 30% for the solution containing methylparaben to 42% for the propylparaben preserved solution. Therefore, at least from a viscosity stability standpoint, it makes little difference whether the acacia solutions are preserved. It is obvious, however, as can be seen from the organoleptic qualities described, that other factors have to be considered.

The rate of reduction in viscosity became essentially linear for all solutions following the pronounced drop seen in the first 6 weeks. The degradation of most linear polymers has been reported to be primarily first order (8), while that of tragacanth has been described as zero order (9).

It was apparent from the results of this investigation that the viscosity stability curves for the initial 6-week period fit neither a first nor a zero-order process. It could be argued, however, that after 6 weeks the essentially linear nature of the data throughout the remaining shelf life suggests a zero-order degradation.

The results of this investigation appeared to indicate that, when all aging characteristics were considered, benzoic acid was the most effective preservative used. Furthermore, when benzoic acid was combined with two of the other preservatives used, little difference was seen in the behavior of the solutions.

SUMMARY

1. The rheograms of a series of acacia solutions ranging in concentration from 10–45% were constructed. Newtonian behavior was observed in all except the 40–45% solutions, which showed pseudoplastic characteristics.

2. A series of 25% acacia solutions using various preservatives were studied for a period of 1 year.

3. The pH of all these solutions decreased rapidly in the first 6 weeks, then tapered off. The largest reduction in pH occurred in the unpreserved control solution.

4. The appearance and odor of the solutions during a 1-year period were noted and various degrees of growth and turbidity were observed, along with a variety of fermented and unpleasant odors.

5. The viscosity of the solutions was calculated at various intervals of time, and viscosity–stability curves were shown. All the solutions revealed a decrease in viscosity with age. The benzoic acid preserved solution exhibited the smallest reduction, while the unpreserved solution revealed the greatest drop in viscosity.

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Mechanism of Action of Anticonvulsant Drugs III. Chlordiazepoxide

By JOHN H. MENNEAR and ALLAN D. RUDZIK

The anticonvulsant activity of chlordiazepoxide in mice was found to be antagonized by pretreatment with reserpine or Ro4-1284 but not by α-methyl dopa or α-methyl tyrosine. This antagonism of chlordiazepoxide by reserpine was reversed by the administration of α-methyl dopa, *d*-amphetamine, or 5-hydroxytryptophan. The failure of α-methyl tyrosine to antagonize chlordiazepoxide suggests that the anticonvulsant action of chlordiazepoxide is not mediated through the release of biogenic amines. Similarly, the reserpine antagonism of chlordiazepoxide appears to be mediated through some mechanism other than catecholamine depletion.

EARLIER communications from these laboratories have described the effects of various amine-depleting agents on the anticonvulsant properties of diphenylhydantoin (1) and acetazolamide (2). These reports have confirmed the findings of earlier workers (3, 4) that reserpine antagonizes the anticonvulsant activities of both diphenylhydantoin and acetazolamide. In addition, however, the authors have demonstrated that this action is not specific for reserpine, but that the benzoquinolizine derivatives, tetrabenazine and Ro4-1284[1] also antagonize both anticonvulsants. Furthermore, acetazolamide was found to be antagonized by all agents which deplete brain biogenic amines, whereas diphenylhydantoin was not. These results suggest that the anticonvulsant action of acetazolamide is mediated, in some manner, through brain amines. The action of diphenylhydantoin is probably not mediated through brain amine release.

The experiments reported in this communication were conducted to compare the action of chlordiazepoxide, which has been reported by Randall *et al.* (5) to possess anticonvulsant activity in mice, to the anticonvulsant activities of acetazolamide and diphenylhydantoin.

EXPERIMENTAL

Male albino mice (Harlan Industries) weighing 18–22 Gm. were used in all experiments. Each animal was used only once. Prior to experimentation the mice were housed in groups of 50 with free access to food and water. All drugs were administered intraperitoneally and doses and pretreatment times are shown in Tables I and II.

Maximal seizures were produced by the method of Swinyard *et al.* (6) employing a current of 50 ma. and 0.2-sec. duration delivered *via* corneal electrodes. The criterion for protection against maximal electroshock was abolition of the hind leg extensor component of the seizure.

The anticonvulsant potency of chlordiazepoxide was compared in groups of mice treated with various amine-depleting agents by determining the dose of chlordiazepoxide which protected 50% of the mice against the electroshock. The ED_{50} values were estimated and compared for significance of differences by the method of Litchfield and Wilcoxon (7).

Chlordiazepoxide was always the last drug to be administered and was followed in 30 min. by electroshock. Unless otherwise stated, mice were housed in individual plastic cages prior to electroshock. In one experiment the animals were aggregated, in groups of 10, in stainless steel cages.

RESULTS

The effects of the various amine-depleting agents on the ED_{50} value of chlordiazepoxide in the maximal electroshock test are shown in Table I. Both reserpine, at 5.0 mg./Kg., and Ro4-1284, at 100 mg./Kg., antagonized the anticonvulsant effect of chlordiazepoxide as evidenced by a significant increase in the ED_{50} value ($p < 0.05$). Neither α-methyl tyrosine nor α-methyl dopa, at doses of 400 mg./Kg., altered the ED_{50} value of chlordiazepoxide.

TABLE I.—EFFECTS OF AMINE-DEPLETING AGENTS ON THE ANTICONVULSANT POTENCY OF CHLORDIAZEPOXIDE

Treatment	i.p. Dose, mg./Kg.	Pretreatment Time, hr.	ED_{50} of Chlordiazepoxide,[a] mg./Kg.
Saline	...	4	23(19–28)
Reserpine	5.0	24	88(64–121)[b]
Ro4-1284	100	4	66(58–75)[b]
α-Methyl tyrosine	400	4	19(16–22)
α-Methyl dopa	400	4	22(18–26)

[a] 95% confidence limits shown in parenthesis. [b] Significantly different from saline controls ($p < 0.05$).

In order to test the ability of various agents to reverse the effects of reserpine on the anticonvulsant potency of chlordiazepoxide, a series of experiments were performed in which reserpinized mice (5.0 mg./Kg.) received a single dose of the test compound 1 hr. prior to electroshock. This was followed in 30 min. by varying doses of chlordiazepoxide. The ED_{50} values for chlordiazepoxide, in combination with the various compounds, were determined 30 min. later. The results of this experiment are shown in Table II. α-Methyl dopa (400 mg./Kg.), *d*-amphetamine (5.0 mg./Kg.), and 5-hydroxytryptophan (500 mg./Kg.), when administered 30 min. prior to chlordiazepoxide, significantly reduced the ED_{50} value of chlordiazepoxide in reserpinized mice ($p < 0.05$). The 400 mg./Kg. dose of dopa, when administered to reserpinized mice in the isolated situation failed to alter the ED_{50} value of chlordiazepoxide. When this dose of dopa was administered to aggregated mice, however, a significant reduction in the ED_{50} value was produced ($p < 0.05$).

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[1] 2-Hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy 1,2,3,4,6,7-hexahydro-11bH-benzoquinolizine.

TABLE II.—ANTAGONISM OF RESERPINE EFFECT ON THE ANTICONVULSANT POTENCY OF CHLORDIAZEPOXIDE

Treatment	i.p. Dose, mg./Kg.	Pretreatment Time, hr.	ED_{50} of Chlordiazepoxide,[a] mg./Kg.
Reserpine	5.0	24	88(64–121)
Saline	5.0	1	
Reserpine	5.0	24	34(29–40)[b]
α-Methyl dopa	400	1	
Reserpine	5.0	24	52(43–64)[b]
d-Amphetamine	5.0	1	
Reserpine	5.0	24	53(45–63)[b]
5-OH Tryptophan	500	1	
Reserpine	5.0	24	71(56–90)
Dopa	400	1	
Reserpine	5.0	24	57(47–68)[b]
Dopa (aggregated)[c]	400	1	

[a] 95% confidence limits shown in parenthesis. [b] Significantly different from reserpine–saline controls ($p < 0.05$). [c] Housed 10/cage after administration of dopa.

DISCUSSION

The results of this study demonstrate that the effects of amine-depleting agents on the anticonvulsant properties of chlordiazepoxide more closely resemble the effects of similar interactions for diphenylhydantoin than for acetazolamide which have been reported earlier (1, 2). With respect to the effects of reserpine and Ro4-1284, chlordiazepoxide resembles both diphenylhydantoin and acetazolamide in that the anticonvulsant effects of all three of these agents are antagonized by these amine depletors. In the case of interaction with α-methyl dopa, chlordiazepoxide closely resembles diphenylhydantoin in that the anticonvulsant effects of both were unchanged by this amine depletor. The failure of amine depletion by α-methyl dopa to antagonize either diphenylhydantoin or chlordiazepoxide might be explained on the basis of the metabolic fate of α-methyl dopa. α-Methyl dopa is metabolized to α-methyl norepinephrine (8). This metabolite may serve as a false neurotransmittor, an effect which could prevent the antagonism of the anticonvulsants by amine depletion *per se*. This seems unlikely, however, since the authors have found that α-methyl dopa antagonizes the anticonvulsant action of acetazolamide (2). Also, amine depletion by α-methyl tyrosine, which is not metabolized to a catecholamine-like structure (9) is without effect on either diphenylhydantoin (1) or chlordiazepoxide but was found to antagonize acetazolamide (2).

Further similarities between chlordiazepoxide and diphenylhydantoin in these experiments are that the administration of 5-hydroxytryptophan, α-methyl dopa, or *d*-amphetamine to reserpinized mice antagonized the effect of reserpine on the anticonvulsant effects of both chlordiazepoxide and diphenylhydantoin. Since dopa failed to antagonize the reserpine effect in isolated mice the authors studied this interaction in aggregated animals. Dopa has been shown to antagonize the effect of reserpine on the anticonvulsant action of acetazolamide in aggregated but not isolated mice (2). This effect of aggregation may be due to an increased uptake of dopa into the central nervous system. It has been shown by other workers that amphetamine is concentrated in the central nervous system to a greater extent in aggregated than in isolated mice (10).

It appears that the mechanism of the antagonism of the anticonvulsant effect of chlordiazepoxide by reserpine and Ro4-1284 is probably not the result of amine depletion, nor does the anticonvulsant effect of chlordiazepoxide appear to be mediated through brain catecholamines since (*a*) neither of the amine depletors, α-methyl dopa nor α-methyl tyrosine, was effective in antagonizing chlordiazepoxide and (*b*) the reserpine antagonism of chlordiazepoxide was reversed by *d*-amphetamine. On the basis of the results of the present study as well as earlier work from these laboratories (1, 2) the anticonvulsant effect of chlordiazepoxide appears to resemble that of diphenylhydantoin rather than acetazolamide.

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Solubility of Some Steroids in Water

By PETER KABASAKALIAN, ELI BRITT, and MILTON D. YUDIS

The solubility of 21 steroids in water at 25° was determined and compared with previous data whenever available.

SCATTERED data have been reported in the literature for the solubility of a number of steroids (1–8) in water. However, there seems to be no single collection of such information nor is there sufficient data, particularly for the C_1-dehydro corticosteroids, which have gained widespread use in pharmaceutical and medical practice during the past 10 years. A greater need for this kind of information stems from the increased interest in the dependence of drug activity upon solution concentration, which in turn is often related to drug solubility in water. A further application for such data is useful in the design of dissolution rate studies.

It is believed, therefore, that the water solubility of a number of steroids in clinical use should be reported.

EXPERIMENTAL

Finely ground U.S.P. grade or material of equivalent quality was used for all solubility determinations. For each substance excess steroid at two initial solute concentrations, about 1 and 3 mg./ml., and 20 ml. of distilled water were added and sealed in 50-ml. glass ampuls. Each suspension was stirred for 48 hr. at 25 ± 0.1° using a Vibro-Mixer (Chemapec, Inc., Hoboken, N. J.) stirrer, which is known to effect rapid equilibration. A portion of the solution was withdrawn from the equilibrated suspension free of all solid material with a pipet fitted with a washed glass wool filter plug. The filtered solution was diluted appropriately with distilled water depending upon the quantity of substance in solution and was assayed spectrophotometrically at about 240 mμ for the conjugated A-ring ketosteroids and at about 280 mμ for the estrogenic steroids. Reference absorptivity constants were determined in a solution containing 10% methanol–90% water (v/v).

DISCUSSION

The water solubility of 21 steroids has been determined, each at two initial suspension concentrations. In all cases, the solubility for the suspension at the lower concentration was equal to or less than that for the higher concentration. In those cases where a difference was observed, the solubility reported was calculated by extrapolation to zero suspension concentration. The coefficient of variation for replicate measurements made in this study was equal to or less than 10%. The results are summarized in Table I. Available literature data for these substances are also included.

Some generalizations can be drawn from the available data. Steroids possessing the $C_{17,21}$-dihydroxy-C_{20}-keto side chain, such as cortisone, hydrocortisone, prednisone, and prednisolone, show the greatest solubility in water while the solubility of the corresponding C_{21} acetate esters is markedly reduced; the C_{21} primary hydroxyl moiety must function importantly in the solvation process. Nevertheless, the alcoholic function of C_{17} also contributes to solvation as the solubility of the $C_{20}C_{21}$-ketoalcohol, deoxycorticosterone is less than that of the dihydroxyketo analogs. The solubility of the C_{16}-methyl-9-α-fluoroderivatives, dexamethasone and betamethasone, is considerably less than prednisolone and is attributed to reduction of solvation about the side chain due to steric effects of the methyl substituent. The enhancing effect of the C_{11} and C_{17} hydroxyl group is indicated by comparing the solubility of hydrocortisone *versus* cortisone, prednisolone *versus* prednisone, and testosterone *versus* testosterone propionate.

TABLE I.—SOLUBILITY OF STEROIDS IN WATER

Compd.	Solubility in Water, mcg./ml., 25° This Work	Lit.
Estradiol	5.	0.2 (1); 1.3 (2); 1.8 (3)
Ethinyl estradiol	10.	
Estradiol benzoate	0.4	
Testosterone	24.	27 (2); 36(37°)(2); 29 (4); 125(37°) (5); 48(5)
Testosterone propionate	2.	3.7(37°) (6)
Ethisterone	0.4	
Methyltestosterone	32.	36(37°) (1)
Progesterone	9.	6.6 (2)
Deoxycorticosterone	145.	60(37°) (1)
Deoxycorticosterone acetate	4.	
Cortisone	230.	280 (7)
Cortisone acetate	19.	20 (7)
Hydrocortisone	285.	280 (7)
Hydrocortisone acetate	10.	10 (7)
Prednisone	115.	
Prednisone acetate	23.	
Prednisolone	215.	231 (8)
Dexamethasone	84.	
Dexamethasone acetate	13.	
Betamethasone	58.	
Betamethasone acetate	30.	

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Crystal Structure of 2,4-Diselenouracil

By ELI SHEFTER*, MICHAEL N. G. JAMES, and HENRY G. MAUTNER

The crystal structure of 2,4-diselenouracil has been determined to provide some quantitative molecular information about the nature of hydrogen bonding exhibited by this compound in solution. The crystal is monoclinic, with space group P $2_1/c$, with a = 4.41 Å., b = 14.90 Å., c = 10.69 Å., and β = 108.4°. The observed density is 2.42 Gm. cm.$^{-3}$ and that calculated for four molecules in the unit cell is 2.33 Gm. cm.$^{-3}$. The structure was determined by the heavy atom technique and refined by block diagonal least squares to an R value of 0.136. The compound was found to exist primarily in a zwitterionic amidic configuration rather than the keto form. The seleniums formed relatively strong hydrogen bonds of the form N(H)···Se with distances of 3.47 and 3.75 Å.

VARIOUS EXPERIMENTAL observations (1, 2) have indicated that selenium analogs of pyrimidine and purine bases are able to form relatively strong hydrogen bonds in solution. Many of these seleno-bases have also been shown to be pharmacologically active antineoplastic agents (3, 4). It has been implied (5) that this activity may be attributable to the "formation of unusually strong hydrogen bonds when incorporated into deoxynucleic acids." The present X-ray investigation of one of these seleno-derivatives, 2,4-diselenouracil, was primarily undertaken to obtain structural information about the nature of the intermolecular hydrogen bonding.

EXPERIMENTAL

Diselenouracil crystallizes from ethanol–water solutions as yellow dichroic prisms, which exhibited twinning along the (100) plane. Many batches of recrystallized material were examined for the presence of a "single" crystal, but all crystals appeared to be twinned. It was found necessary to use a crystal from which the twin had been dissected for the collection of intensity data. The fragment used showed excellent extinction under the polarizing microscope, but photographs indicated that a small amount of the twin remained; this was estimated to be less than 10% from oscillation photographs.

Oscillation and Weissenberg photographs taken about the a and b axes using Cu radiation (λ = 1.5418 Å.) showed the crystal to be monoclinic, with unit cell dimensions of a = 4.41 Å., b = 14.90 Å., c = 10.69 Å., and β = 108.4° (with estimated standard deviations of 0.5%). The space group, as determined from Weissenberg photographs and confirmed by the determination and refinement of the structure, is P $2_1/c$. The density measured by flotation in a mixture of tetrabromoethane and chloroform was found to be 2.42 Gm./cm.^{3}, and that calculated for a unit cell containing four molecules of diselenouracil was 2.33 Gm./cm.^{3}.

The intensity data were recorded on equi-inclination Weissenberg photographs using multiple film packs. The dissected crystal (dimensions 0.14 mm. along a, 0.04 mm. along b, and 0.02 mm. along c) was first mounted about the a axis for collection of layers h = 0, 1, and 2, and it was then cut in half perpendicular to a and remounted about b to obtain the layers k = 0 and 1. The intensities were estimated visually and corrected for Lorentz polarization effects. The structure factors for the data about the two axes were put on the same scale by comparing common reflections. There were 649 unique reflections that gave measurable values; this represents approximately 55% of all unique reflections in the Cu sphere.

Determination and Refinement of Structure.—The positions of the two selenium atoms were easily located from an unsharpened Patterson synthesis, and the atoms comprising the pyrimidine ring were then found in a Fourier synthesis phased on the seleniums. The atomic identity of the six ring atoms was obtained by electron density calculations at the atomic sites. The refinement of the positional and isotropic thermal parameters of the molecule was carried out by block diagonal least squares, using a program described by Mills and Rollett (6). The weighing scheme used in the refinement was similar to that of Hughes (7). During the final cycles of refinement it was necessary to give zero weight to 36 weak reflections, as they appeared to be significantly influenced by the presence of the twin. The refinement was considered to be complete when the parameter shifts were less than one-third of their estimated standard deviations, which were calculated from the diagonal elements of the normal matrix. The positional and thermal parameters obtained are listed in Table I. The final R value (equals $\Sigma||F_0| - |F_c||/\Sigma|F_0|$) was 0.136 for all 649 reflections. A list of the observed and calculated structure factors can be obtained from the authors. The atom form factors used in the above calculations for carbon and nitrogen were those of

TABLE I.—POSITIONAL AND ISOTROPIC THERMAL PARAMETERS FOR 2,4-DISELENOURACIL

Atom	x/a	y/b	z/c	B_{iso}
N(1)	−0.296	0.088	0.380	3.5
C(2)	−0.248	0.020	0.302	3.1
Se(2)	−0.3976	−0.0951	0.3275	4.1
N(3)	−0.118	0.038	0.199	3.5
C(4)	0.013	0.118	0.173	3.4
Se(4)	0.2002	0.1359	0.0249	3.5
C(5)	−0.056	0.193	0.257	3.9
C(6)	−0.204	0.175	0.361	4.3

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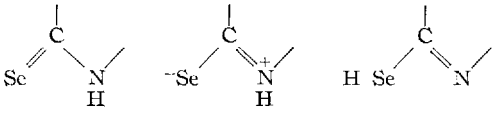
Berghuis *et al.* (8), and for selenium those of Thomas and Umeda (9) were used after being corrected for anomalous scattering. All computations were done on a Ferranti mercury electronic computer, using many of the programs described by Mills and Rollett (6).

RESULTS AND DISCUSSION

From X-ray studies on uracil (10) and various uracil containing compounds (11–14) it has been shown that in the solid state it exists primarily in the diketo form rather than the enol configuration. The C(2)-Se(2) and C(4)-Se(4) bond lengths of 1.89 and 1.99 Å. (E.S.D. 0.05 Å.), respectively, suggests that the electronic structure around the seleniums would be predominantly a zwitterionic amidic configuration (II) rather than I.



I II III

The enol form (III) was eliminated as a possible configuration, as this structure was found to be isomorphous with the crystal structure of dithiouracil (17), for which hydrogen atoms were found attached to the nitrogens. The presence of the amidic configuration rather than the enolic one is also consistent with the dipole moment, ultraviolet spectra, and ionization measurements (2, 18) made on 2-pyridselenone and its *N*-methyl derivative. The sum of the covalent bond radii for Se and C is 1.94 Å. for the single bond and 1.74 Å. for the double bond (15). These results are not surprising in the light of the studies of Mautner (1), which showed that Se has a greater ability than S and O to withdraw electrons from N in pyridine and pyrimidine derivatives.

The E.S.D.'s given above for the Se bonds is twice that calculated from the diagonal elements of the normal matrix, as it is felt that this is a more reasonable approximation to the errors. Relatively large standard deviations for the pyrimidine ring bonds (0.1 Å.) makes it impossible to assess their bond orders and, therefore, precludes any discussion of this part of the molecule.

Though selenium compounds are not usually thought of as hydrogen bond participants, in diselenouracil the seleniums appear to form such interactions. The short Se · · · N distances of 3.47 and 3.75 Å. and the angles around the nitrogen atoms indicate that the seleniums are forming relatively strong hydrogen bonds (Fig. 1). These hydrogen interactions are attributable to the electronic configuration and seem to be a function of the amount of polarization in the C-Se bonds. In the structure of *N*-phenyl-*N'*-benzoylselenourea (16) a Se · · · N hydrogen bond of 3.83 Å. was found, and it could

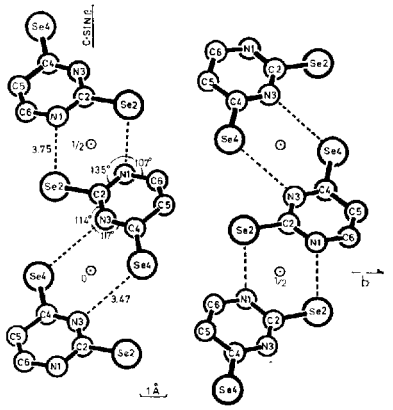


Fig. 1.—Packing diagram of 2,4-diselenouracil as viewed down the *a* axis. Probable hydrogen bonds are indicated by broken lines.

also be attributed to the electron-withdrawing property of the selenium. The importance of such bonding is best realized in connection with the postulate (5) that the antineoplastic activity of selenium derivatives of purine and pyrimidine bases may be related to their ability to form strong hydrogen bonds with their complement base within the helical deoxyribonucleic acid (DNA).

The least squares plane through the eight atoms, indicated they are coplanar; *i.e.*, the deviation of any atom from this plane is less than an E.S.D. of its positional parameter along the normal to the plane. This observation is consistent with the results on uracil and thymine.

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Review Article

Pharmaceutical Sciences—1965

A Literature Review

By ROBERT E. DEMPSKI and GERALD P. POLLI

CONTENTS

Received from the Pharmaceutical Research and Development Department, Merck, Sharp and Dohme Research Laboratories, West Point, Pa.

THIS REVIEW is a continuation of an annual series originated by McKeehan (1). It represents a comprehensive cross-section of the research and development efforts in various disciplines of the pharmaceutical sciences. Numerous periodicals and *Chemical Abstracts*' Pharmaceuticals and Pharmacodynamics sections published during 1965 were searched and selectively abstracted.

Some of the literature related to the pharmaceutical sciences has been reviewed on an annual basis in other publications and is omitted here. For such associated papers in analytical chemistry, antibiotics, bacteriology, biochemistry, biology, cancer, medicine, medicinal chemistry, microbiology, organic chemistry, pharmacology, physical chemistry, physiology, and plant physiology, the reader is referred to reviews in these areas of study. The "Advances in . . . " series,

the "Annual Review of . . . ." series, and the "Progress in . . . " series are particularly pertinent. In order to maintain continuity with previous pharmaceutical science reviews of *J. Pharm. Sci.*, their general format was retained.

GENERAL PHARMACY

The literature continues to abound with articles of interest to investigators involved in all areas of the pharmaceutical sciences. One paper by O'Reilly listed and discussed many of the pharmaceutical reviews published from June 1963 to June 1964 (2). Vice President Hubert Humphrey described the problems in keeping up with drug literature in a survey performed by the National Library of Medicine (3). Two other surveys attempted to outline the problems associated with the collection, distribution, and effective utilization of the vast amount of drug literature (4, 5). The proper procedure for the approval of a new drug or drug cosmetic has been discussed in an outline of current regulation procedures (6). Kass reviewed the type and scope of information an inspector is entitled to receive upon FDA inspection (7). An article written by a group of four physicians explained the practical aspects of drug therapy from the standpoint of the pharmacist, clinician, researcher, and teacher (8).

One review was concerned with how and why generic and trade names are assigned to new drugs (9); Ansel commented on the qualities of a desirable trade name and the intricacies of determining its legal availability, subsequent registration, and protection (10). The utilization of drugs in aerospace medicine was summarized (11). The chemistry and uses of antifungal (12), anthelmintic (13), and antimalarial (14) drugs were presented in three different articles which contained many useful references. The outlook for dimethylsulfoxide, a new drug from lignin, as a therapeutic agent has been described (15). A paper on historical studies of camphor was also published (16). Kuttel recommended a simple and practical design for aseptic compounding in dispensaries (17). Additional surveys considered the preparation and properties of ophthalmic solutions (18, 19) and some aspects of toiletry technology (20).

Preservatives.—The preservation of ophthalmic products was reviewed in a paper with 68 references (21). In another study, many types of preservatives were evaluated for their antibacterial and antifungal properties (22). Evans applied the Ferguson principle to systems of mixed preservatives to ascertain that biological activity is proportional to the degree of saturation of the aqueous phase (23); the same principle was used to determine and correlate the activity of three quaternary ammonium salts against *M. aureus*, *E. coli*, and *C. albicans* with the surface properties of these compounds (24). Propylene glycol exhibited antimicrobial activity when used topically (25). Thoma carried out galenic and analytical studies on the effect of several cellulose derivatives and alginates on the activity of several antiseptics (26). Alkaline glutaraldehyde has been suggested as a general disinfectant for instruments and apparatus that cannot be sterilized by autoclaving (27). Two studies were concerned with the mechanism of action of phenolic disinfectants. One investigated the effects on induction of and accessibility of the substrate to β-galactosidase in *E. coli* (28), and the other explored the effect of 2,4-dichlorophenol on the incorporation of labeled substrates by *E. coli* (29). Another paper assessed the importance of metal ions and the toxic properties of the formyl group in determining the bactericidal activity of various phenols and salicylaldehydes (30). One other report presented data on the hemolysis of erythrocytes by a series of quaternary ammonium salts (31).

Foster published two different articles on the preservation of ophthalmic solutions (32, 33). Test procedures and test organisms suitable for shortening the time required for the selection of an adequate preservative have been disclosed (34). The use of antimicrobial agents in parenteral products was reviewed in a paper containing 22 references (35). Another summary with 38 references on the activity of antibacterials in a two-phase system was presented (36). Krowczynski and co-workers suggested a preservative for aqueous heparin solutions (37). Also, 8-hydroxyquinoline sulfate was satisfactory as a preservative for tuberculin PPD (38). This agent was effective against two yeasts, three molds, *P. aeruginosa*, and *S. aureus*.

Flavor, Aroma, and Color.—A review has been compiled on the use of flavors in the United States, the various methods used for flavor testing, and the difficulties encountered for the taste correlation of drugs (39). Tilgner proposed a flavor dilution profilogram for characterizing the detailed aroma or flavor sensations of a product in dilution steps between the threshold and some standard extract of the undiluted product (40). A combined electrophysiological and sensory test revealed that the gustatory effect of substances which show taste as sour or salty was reinforced by inhibition of cholinesterase on the tongue (41). No changes were observed for bitter or sweet tastes. Taste sensitivities to quinine and 6-*n*-

propylthiouric acid were determined (42). Five new principles for flavoring antitussives have been developed (43). Sorbitol, saccharin, and *N*-cyclohexylsulfamic acid were found to be effective synthetic sweetening agents in pharmaceutical preparations (44). In addition, Edwards commented on the flavor constituents of citrus oils (45).

The philosophy of and methods for the identification of odor and flavor constituents were reviewed by Wick (46). The theory of odor and the relationship between the odor and the chemical properties of flavors have been surveyed in another paper (47). A review of pioneers in aldehydes and ketones was presented (48), and the synthesis, physiochemical properties, and economic factors of lavender were summarized (49). One status report listed all currently approved U. S. certified colors (50); another outlined the current FDA status of all color additives for cosmetic use (51). The effects of grain size and moisture content have been studied with the Pulfrich photometer against 120 color standards (52). A method was described for classifying and describing colors formed when the concentrations of several dyes and carbon black in compressed tablets were evaluated (53). One other paper discussed the physiology and psychology of color sensation (54).

Adjuvants.—The properties and compatibilities of two dextrans with molecular weights of 40,000 and 70,000 were tabulated by Smith (55). Another investigator assessed the effect of heat on aqueous solutions of dextran by measurements of viscosity and reducing power (56). The use of cation exchangers as carriers of drug components for the improvement of their palatability has been described (57). The application of polyethylene glycols in pharmaceuticals was outlined (58). Anomalies in some of the physical properties of spray-dried lactose and granulated magnesium oxide were traced to the presence of fines which could be removed by washing with selected organic solvents (59). A silicone fluid proved useful as a lubricant for artificial eyes (60); it did not adhere to tissue, was insoluble in water, was stable over a wide temperature range, and did not support bacterial growth. The properties of neutrality, inertness, low surface tension, easy viscosity control, miscibility with eye secretions, and a low tendency to support bacterial growth, all contributed to making methylcellulose a valuable agent for preparing an aqueous vehicle for pilocarpine nitrate ophthalmic solutions (61). Polyvinylpyrrolidone has been recommended for use as a binder in tablet making (62). The characteristics of Neen gum, a polyelectrolyte which behaves as a lyophilic colloid, were disclosed (63). Experimental data were also presented on a new gum prepared by fermentation from glucose that could be used for thickening highly concentrated electrolyte solutions (64). Some of the practical aspects of colloids were commented on by Smith (65). The viscosity control of liquids and the forced flow of solids were accomplished by the use of silicas (66). One worker collected data on the common properties of some clays including dispersion, aggregation, and the difference between clays and other mineral colloids with particular reference to exchange capacity (67). Other researchers conducted an infrared study on structuration in bentonite clays (68).

The importance of propylene glycol as a solvent in dermatology was reported by Barr (69). The attributes of hexadecyl alcohol as a new material for cosmetic and topical formulations have been discussed in detail (70, 71). Another paper described the suitability of higher-boiling fractions of ethoxypolysiloxane oil in ointments and suppositories (72). The formulation of creams, lotions, and ointments with silicones was studied in two different publications (73, 74), and the utility of isopropyl myristate (75) and coconut oil derivatives (76) in cosmetics was also demonstrated. Both cholestanol (77) and germ lecithin (78) were effective emulsifiers in making water-in-oil emulsions. King and Sheffield used the triethanolammonium salts of several alkylsulfuric acids in the formulation of dermatologic vehicles (79). The seed husk of *Plantago ovata* has been evaluated for its emulsifying properties (80); the powder produced poor emulsions, whereas the mucilage gave emulsions that compared favorably with those made with acacia. One investigator proposed microcrystalline cellulose as a new ingredient for the formulation of creams, lotions, and ointments (81). Another researcher outlined the pharmaceutical and cosmetic usage of a new colloidal alumina as a topical formulating agent (82). The synthesis, analysis, and physical properties of several allantoin complexes were determined, and their potential dermatological uses were suggested (83). Other investigators evaluated current problems associated with the effects of synthetic detergents on the skin (84).

Stability.—Current formulation stability problems were discussed in two different review articles (85, 86). Papers by Sawatari (87) and Sabalitschka (88) advocated the use of antioxidants as stabilizers. Another report examined the measurement and prevention of oxidative deterioration in cosmetics and pharmaceuticals (89). The thiobarbituric acid–malonaldehyde

reaction was used to measure antioxidant effectiveness in pharmaceutical oils (90). The oxidation of benzaldehyde and methylbenzaldehyde in hydrous solutions of polyoxethylene glycol ethers was measured by a manometric technique (91). Phenolic antioxidants were found to be quite satisfactory for improving the stability of creams containing vegetable oils (92). The rates of autoxidation of linoleic acid in micellar solution were also studied (93). The influence of heat sterilization and the stability of pharmaceutical solutions were surveyed by Speiser (94). The effect of ultrasonic energy was explored in two different studies; it was used to study the hydrolysis of acetylsalicylic acid solutions at various temperatures and pH values (95), and to evaluate its influence on physical and chemical transformations of purine derivatives (96). In another investigation, the effect of X-rays on dihydrouracil in aqueous solution, with and without the presence of oxygen, was revealed (97).

Acetylcholine bromide solutions were shown to be stable at room temperature for 2.5 months and at 5° for 5 months (98). Optimum stability conditions for adrenaline were achieved by dissolving its bitartrate salt in water containing sodium thiosulfate and boric acid (99). In another stability study, solutions of chlorpromazine were found to develop a precipitate if combined with sodium phenobarbital, sodium bromide, or neospasmin (100). Denoel discovered that ephedrine decomposed rapidly in peanut oil, but was extremely stable in mineral oil (101). Three other investigators tested the effect of self-radiation, pH, temperature, and sunlight on the stability of sodium iodohippurate-^{131}I (102). Experiments were also conducted on the stability of isoniazid and its related compounds (103). Sealing morphine hydrochloride solutions under carbon dioxide or nitrogen gave more protection against decomposition than equivalent solutions sealed under air (104). The effect of aging of aqueous pralidoxime solutions on the assay, toxicity, and antidotal activity has been investigated by Lehman and Bloch (105). Two studies were performed on tetracaine hydrochloride solutions. One study evaluated the effect of an ultraviolet lamp on pH and potency (106); the other examined the effect of sterilization and hydrolysis in the pH range of 4–7 (107). The mechanism of thermal rearrangement and decarboxylation of procaine was disclosed (108). Various methods were suggested for the stabilization of procaine hydrochloride solution. Two papers proposed the addition of carbon dioxide and *p*-aminobenzoic acid as stabilizers (109, 110). The addition of EDTA was observed to prevent solutions of procaine from turning yellow, but it failed to inhibit decomposition of the drug (111). Another local anesthetic, procaine, was stabilized with a mixture of sodium thiosulfate and sodium sulfite (112). The stability of retinol acetate (113) and sodium sulfacetamide has also been investigated (114). A compatibility study with certain sulfonamides indicated that insoluble precipitates were formed with salts of several alkaloids, boric acid, and zinc sulfate (115). Other workers conducted a study on the stability of a 10% solution of sodium sulfadiazine in the presence of copper, iron, and hydrogen peroxide (116). In addition, the stability of triiodothyronine (117) and triiodothyronine-^{131}I (118) was carefully determined under different conditions of storage.

Koshy *et al.* described some of the factors involved in the browning of spray-dried lactose (119). In a similar study, the lactose–amine reaction was discovered to be predominately a primary amine–carbonyl reaction and was similar in nature but distinct from the dextrose-, galactose-, and HMF–amine reactions (120). γ-Rays from ^{60}Co were claimed to accelerate the oxidative decomposition of methyl linoleate mixed with lactose (121). Two compatibility studies have been conducted on powder mixtures. The incompatibility of carbinoxamine maleate (122) and calcium phosphorylcholine hydrochloride (123) with 20 and 84 different powder preparations, respectively, was carefully evaluated. Experiments on powders of dehydroacetic acid revealed that the α-form, which is stable at room temperature, is rapidly converted to the β-form at 80° (124). Diffuse reflectance studies were used to observe solid–solid interactions of oxytetracycline, phenothiazine, anthracene, and salicylic acid with various adjuvants (125). Some of the factors influencing the stability of calcium acetylsalicylate and acetylsalicylic acid tablets have been investigated by Kiss, Rozsondai, and Scholz (126). In a study where acetylsalicylic acid was combined with ascorbic acid in tablets, the effect of water vapor pressure on the moisture sorption and the stability of both components was reported (127). No breakdown of digitoxin was found in tablets, injections, or solutions stored in the dark for 5 years (128). Similarly, no decrease in the alkaloid content of ipecac concentrate was observed after 18 months of storage (129). The sedimentation of thyme tincture was prevented by clarification with talc and cooling to 0 to −5° (130). The stability of helveticoside, a glycoside from strophanthidin, was also studied in a long-term investigation (131).

Stability Kinetics.—Garrett published a review with 198 references discussing the prediction of drug stability in pharmaceutical preparations (132). Two other reviews appeared in the literature. Both of these surveys evaluated the use of chemical kinetics for the prediction of drug stability (133, 134). A nomograph chart was devised in one study to facilitate the analysis of stability data obtained in accelerated testing at elevated temperatures (135). A reciprocal heating machine has been found very useful for investigating single-step stability studies under nonisothermal conditions (136). Other workers used model calculations, based on the statistical-mechanical formulation of isotope effects, to predict how analysis of experimentally measured isotope effects may be used to gain information concerning the differences between the reactants and the transition state in a rate process or between the reactants and the product in an equilibrium process (137).

One paper examined the kinetics of solvolysis of various *N*-alkyl-*N*-nitrosoureas in neutral and alkaline solutions (138). The decomposition of *p*-aminosalicylate was found to be first order while sodium *p*-aminosalicylate was zero order in aqueous solution (139). Pseudo first-order rates of spontaneous degradation were ascribed to apomorphine under varying conditions of temperature and pH (140). The hydrolysis of methyl and ethyl esters of benzoic acid, some sterically hindered acids, and benzonitrile by suspensions of sodium hydroxide in DMSO was 10^4 to 10^5 greater than in hydroxylic solvents (141). Activation energies, frequency factors of the Arrhenius equation, and equilibrium constants between chlorothiazide and its intermediate were calculated from the rate constants on the hydrolysis reaction (142). The rate of autolysis of α-chymotrypsin in the pH region of 7 to 11, in the absence of added salt, has been studied through the rate of acid formation in a pH-stat and through the rate of decrease in enzyme activity (143). Garrett and Notari quantified the kinetics of dehydration of cycloheximide to anhydrocycloheximide in the pharmaceutically useful acetate buffer region (144). Another article compared the rate of hydrolysis of thalidomide, *N*-butylphthalidomide, and phthalimide in sodium hydroxide (145). The degradation of hexamine in aqueous solution was considered to be pseudo first order (146). The solvolysis of 5-iodo-2′-deoxyuridine was revealed as first order over the pH range of 3.9 to 12.0 (147). First-order rate constants were disclosed for the decomposition of molten malonic acid from the volume of carbon dioxide evolved (148). In a stability study on mydeton injection solutions, the rate of decomposition was influenced by pH and temperature (149). Two different papers discussed the rate of hydrolysis of procaine under various conditions of temperature and pH (150, 151). The effects of substitution have been correlated with the acid-catalyzed hydrolysis rates of some oxazolidines (152). Other kinetic studies were carried out on tetracaine, parethoxycaine, leucinocaine, procaine, farmocaine, and larocaine (153). Methyl substitution was demonstrated as providing increased ring stability in a study on the hydrolysis of succinamic acid and succinimides (154). Pseudo first-order rate constants were recorded for the base-catalyzed hydrolysis of urea (155).

Antibiotic Stability.—A buffer solution containing boric acid, sodium borate, and polyethylene glycol did not prevent hydrolysis of chloramphenicol (156). The stability of two antibiotics, cranomycin (157) and erythromycin lactobionate (158), has been studied. Tukamoto, Miyake, and Sato reported on the decomposition of dihydrostreptomycin, chloramphenicol, and tetracycline by drug-fast *E. coli* (159). Other investigators have compared the effect of glycerin, paraffin, propylene glycol, white petroleum jelly, polyethylene glycol, lactose, alcohol, sunlight, and darkness on the stability of hamycin preparations (160). Acid degradation studies were performed on kasugamycin (161). A decrease in the optical rotation of lincomycin showed a direct correlation with microbiological assays (162). One study followed the rates of decomposition of methicillin in aqueous solution (163); another determined the effect of sugars on the browning of neomycin (164).

Several papers were presented on the stability of various penicillins. Losses in potency of 2,6-dimethoxyphenyl penicillin were evaluated iodimetrically, by U.V. absorption, and by microbiological assays on *B. subtilis* (165). The stability of aqueous solutions of certain novel penicillins was observed to be reduced by surfactants, preservatives, and thickening agents (166). The decomposition rates of α-phenoxypropylpenicillin, 2,6-dimethoxyphenylpenicillin, and α-aminobenzylpenicillin have been demonstrated to be first order and obeyed Arrhenius' equations (167). In another study, it was concluded that acacia accelerated the inactivation of penicillin, whereas methylcellulose had no effect (168). Other workers, Olszewski and Grabowska, tested the influence of sodium benzoate on the stability of aqueous penicillin solutions (169). In a physical-chemical study on the relationship between potency and hygroscopicity, the effect of humid-

ity on the potency of four semisynthetic penicillins was investigated (170). Schwartz described the effect of ionic interaction on the catalysis of penicillin hydrolysis by certain catecholamines (171). This investigator also concluded that the degradation of penicillin G involved both the catalyzed hydrolysis of the undissociated molecule and a rearrangement of the penicillin ion following proton attack (172). A different study on some penicillin salts correlated the initial product characteristics and the properties after 3 years by statistical means to predict their shelf-life (173). The catalytic effect of buffers on the degradation of penicillin G in aqueous solution has also been examined (174). Other workers considered the cause of the reddening coloration and pigments in colored solutions of streptomycin (175).

Vitamin Stability.—Elevated temperature storage tests and a graphic method of calculation were used by Tardif to determine thermal degradation rates in three polyvitamin tablet formulations (176). In another vitamin stability study, the same worker found no differences in a polyvitamin suspension that had been stored either in the plant or in the pharmacy (177). In other multiple vitamin stability studies, vitamins A, B_{12}, and ascorbic acid were classified as being the least stable (178). The stability of vitamin A, tocopherol, and unsaturated fatty acids in vitaminized vegetable oil exposed to sunlight was also investigated (179). The stability of vitamin A in concentrates and foodstuffs was determined under various conditions of temperature, atmosphere, and time (180). The effect of material quality and the method of preparation upon the stability of aqueous thiamine injections was the subject of one paper (181); another study examined the effect of salts, vehicles, pH, temperature, and vitamins B_2, B_6, and niacinamide upon the stability of thiamine (182). The effect of cocarboxylase on the hydrolysis of thiamine pyrophosphate was reported (183). Kato claimed that powdered mixtures of thiamine tetrahydrofurfuryl disulfide with sodium bicarbonate and acetylsalicylic acid were completely stable (184). Two separate papers were also presented on the stability of isomers of dihydrothiamine (185, 186). In addition, γ-rays from ^{60}Co were found to accelerate the decomposition of thiamine hydrochloride when mixed with calcium carbonate and dibasic calcium phosphate (187). The stability of riboflavin and its phosphate salt in syrup, propylene glycol, glycerol, 70% sorbitol solution, and water has been assessed (188). Macromolecules, *e.g.*, polyvinylpyrrolidone, polysorbate 80, and sodium decyl sulfate, enhanced the rate of aerobic photobleaching of riboflavin by visible light (189).

The mechanism of color formation, the role of furfural, and the decomposition products of ascorbic acid were delineated (190). In a similar study, the rate of formation of furfural by the hydrogen ion-catalyzed anaerobic degradation of undissociated ascorbic acid was depicted as being equal to the rate of disappearance of the ascorbic acid (191). Another anaerobic study considered the formation of carbon dioxide and furfural by decomposition of ascorbic acid at various pH and temperature conditions (192). Finholt *et al.* also studied the anaerobic degradation of ascorbic acid by following the rate of formation of carbon dioxide (193). Otani detected a linear relationship between the color change and the degradation of ascorbic acid in the pH range 1–7 (194). He presented another paper on the relationship between the color change and the oxidation of ascorbic acid in aqueous solution (195). Additional experimental data compared the stability of ascorbic acid and 2-keto-l-gulonic acid in an aqueous medium (196). Two different manuscripts were concerned with the effect of stabilizers on ascorbic acid. Amino acids improved the stability in liquid formulations (197); rutin retarded the oxidation of ascorbic acid in apple juice (198). Another study analyzed the shelf-life of several liquid formulations with ascorbic acid in different vehicles with and without other vitamins (199). The most stable injectible solutions of ascorbic acid were prepared by using a 5% excess of ascorbic acid, purging with carbon dioxide, and sealing under carbon dioxide (200). In tablets, ascorbic acid remained stable longer if made by dry compression or by using a nonaqueous binder and storing in amber containers without moisture (201). Studies in model systems have indicated that ascorbic acid is more stable in aqueous systems and is a more efficient antioxidant than erythorbic acid (202).

The thiazole moiety of thiamine hydrochloride and selected model compounds had no adverse effect on the cyanocobalamin stability (203). A dose of 9.1×10^4 rads of γ radiation from ^{60}Co destroyed 45% of the vitamin B_{12} under test (204). Steric factors were found to have an important influence on hydrolysis of vitamin B_{12} in aqueous hydrochloric acid–dioxane solution at 50° (205). Hydroxocobalamin in liver extract has been stabilized by the addition of ferric and ferrous sulfate ions (206). Janicki and coworkers conducted a study on the stability of vitamin D_2 in irradiated feed yeasts during storage (207). Thiamine, riboflavin, chlorine, ascorbic acid, manganese sulfate, and calcium hypo-

phosphite were all shown to cause decomposition of folic acid in pharmaceutical preparations (208). The discoloration of isonicotinic hydrazide tablets in tropical climates was attributed to the lactose contained therein (209). Other studies compiled data on the incompatibility of some commercial vitamin K_1 injections (210).

PHARMACEUTICAL TECHNOLOGY

Past, present, and future trends in pharmaceutical product development were summarized by Cooper (211). Two progress reports on solid and solid-fluid systems in pharmaceutical engineering have also been published (212, 213). Some experimental work was conducted on the chemical engineering of foam separation (214). Smith advocated that vibration grinding was a faster process than ball milling (215); he also presented several comments on wet *versus* dry grinding (216). A report was made on some powder properties, their mixing performance, and the development of some new theories of mixing (217). The drying characteristics of three commonly employed tablet excipients have been investigated under vacuum in an instrumented rotary double-cone dryer (218). One review discussed the theory of drying (219); another summary compared the methods used for both batchwise and continuous drying (220). Additional comments on some of the principles and applications of spray drying were also published (221).

Two different papers suggested methods and apparatus for the preparation of gold colloids for medicinal use (222, 223). Another investigator summarized nine different methods for measuring particle size (224). A novel method for evaluating dissolution characteristics of capsules was developed by Paikoff and Drumm (225). The simultaneous mixing and segregation occurring in randomly mixed particulate solid systems subjected to agitation have been investigated (226). Similarly, idealized systems of solid particles as represented by steel and glass spheres were studied with reference to their rates of segregation (227). Four separate papers appeared on the lyophilization of pharmaceuticals. These articles included a study on the effect of certain physical-chemical properties on lyophilization (228), a description of a high-sensitivity resistance bridge for low-conductivity measurements at eutectic temperatures (229), a method for programing a mathematical expression for estimating eutectic temperatures from melting point and solubility parameters (230), and determination of the eutectic temperatures of some inorganic salts (231).

Parenterals.—A review on the formulation of parenterals was published by Parrott (232), while Bedaux outlined various aspects for the preparation of infusion solutions (233). Other researchers recommended the use of demineralized water for the preparation of parenteral solutions (234). A thermoelectric vapor phase osmometer was described for measuring osmotic coefficients, isotonicity values, and sodium chloride equivalents of some univalent electrolytes of pharmaceutical interest (235). Isotonic solution values were tabulated for numerous medicinal agents and adjuvants in three separate publications (236–238). The use of polymers in injectables was surveyed in a discussion with 56 references (239). Also, new formulas were suggested for infusion solutions based on acetates and sorbitol (240). The preparation and development of a chloramphenicol intramuscular injection have been described (241). Pinter reported on a method for the preparation and storage of a citric acid solution for the dissolution of lyophilized plasma (242). Nine commercial parenteral preparations containing calcium salts were mixed with 70 commercial preparations containing salts of organic acids and observed for pH changes and precipitates (243). The same investigators compared the compatibility of thiamine tetrahydrofurfuryl disulfide injection with 136 other commercial parenteral preparations (244). In another compatibility study, 270 unique pairs of medication were tested in 5% dextrose solutions. This test resulted in 23 pairs that were physically incompatible (245). An additional 34 drugs intended for intravenous use were cross-matched to test for physical signs of incompatibility (246). A strain of *Pseudomonas* has been isolated from an injection solution containing 10% bivalent mercury diuretic that was resistant to phenyl mercuric borate and sodium ethyl mercuric thiosalicylate (247). A new disposable hypodermic device, called a hypule, has been evaluated by a biological procedure and tested for compatibility with 117 different parenterals (248).

Sterility.—Advances in sterilization techniques were outlined by Ehrlen (249). Four additional reviews discussed the utilization of gaseous ethylene oxide in sterilization (250–253). A method has been developed for the determination of residual ethylene oxide and ethylene glycol in ethylene oxide sterilized pharmaceuticals (254). The utility of cold sterilization in pharmaceutical products was also disclosed (255). Also, emphasis was placed on the cleaning operation and choice of chemicals and detergents used on metal and rubber parts (256). Sterilization

by radiation was also considered (257); while irradiation of tetracyclines and other antibiotics with ^{60}Co did not diminish their antibiotic power (258). An ultraviolet mercury vapor lamp in a quartz tube was employed for sterilizing water received from an ion-exchange method (259). The addition of small amounts of polymyxin B, benzalkonium chloride, chlorobutanol, or various mercury-containing antiseptics to collyriums prevented bacterial contamination (260). The incorporation of 0.3% phenol and autoclaving for 30 min. at 120° provided a new method for the sterilization of oil solutions (261).

Tablets and Capsules.—Some geometrical considerations concerning tablet design to provide a uniform release rate from solution tablets have been investigated (262). It has been ascertained that half-tablets are poor dosage forms, especially when the dosage must be carefully controlled (263). The influence of many different excipients and lubricants on the chemical and physical stability of several medicinals in tablets was demonstrated by Lachman (264). Other researchers described instrumentation for measuring the sign and magnitude of static charges generated by particles flowing through a tablet hopper (265). They also found that tablet lubricants, such as magnesium stearate, polyethylene glycol 4000, sodium lauryl sulfate, and talc, have the ability to lower the accumulation of static charges resulting from the flow of material through a tablet hopper (266). Tableting difficulties due to the nonwetting of lipophilic and aerophilic substances have been overcome by the addition of detergents to tablets (267). A comparative study was made of the absolute water content with relative dampness and dielectric constants on various granules (268). The equilibrium moisture content of several starches, gums, sugars, and hexamine was determined at different relative humidities and temperatures (269).

Two reports were published on the evaluation of several tablet disintegrating agents (270, 271). The latter article indicated that Moriyo starch was as good as or better than cornstarch and Veegum HV as a tablet disintegrant. An *in vitro* disintegration study was performed on 85 different commercial and 14 different hospital preparations according to the "Danish Pharmacopeia" 48 (272). A comparative evaluation was made on the effect of aging for 24 weeks on seven tablet disintegrants (273). Cornstarch was best. The effect of the viscosity of sodium carboxymethylcelluloses used as binding and disintegrating agents on the rate of disintegration of clay tablets was studied (274). In addition, a study was reported on the properties of experimental granulations produced by using aqueous and alcoholic solutions of celluloses, vinyls, acrylamides, pyrrolidones, oxazolidinones, and ethylene oxide condensation products (275). A technique for determining *in vivo* tablet disintegration has also been proposed (276). Some physicochemical properties, including swelling volume, moisture absorption, particle size distribution, surface area, and ion-exchange properties, of the montmorillonites were examined in relation to their application in tablet making (277).

Kirsop reviewed the fundamentals of tablet compression (278). A comprehensive study on compressed tablets evaluated the effect of the method of granulating, excipient, lubricant, disintegrant, particle size, and pressure on the density, disintegration time, and cohesive force (279). It was discovered that the density of compressible systems may be determined without disturbing the system under study by using a densitometer consisting of a sealed source containing ^{170}Tm and a scintillation detector connected to a preamplifier and pulse-height analyzer (280). A "moving-die" apparatus was described for the investigation of die wall friction during compaction (281). This apparatus was used to determine the die reaction during compression of crystalline acetylsalicylic acid, hexamine, sucrose, sodium chloride, and simple granulations of hexamine and sucrose (282). It was also employed to determine the die reaction following compaction of 100 mesh powders of 11 different lubricants (283). Three different papers reported on the physics of tablet compression. One of these articles, by Seitz and Flessland, was concerned with changes in tablet hardness and friability when the operation of a rotary tableting machine was varied (284). Data collected during the measurement of pressure exerted by various substances on the die wall during and after compression of tablets can be related directly to the ease of formation and ejection of these tablets (285). Another comparative study explored the performance of a food grade dextrose and a spray-dried lactose as excipients in the direct compression of tablets (286).

The form or shape of tablets to be coated was studied and an empirical formula was derived from status analysis for tablet form that would express the ease or difficulty of the coating procedure (287). Coatings prepared from 30% gelatin and 2% methylcellulose mixed in a 1:1 ratio with 1:1 potato starch–talc prevented volatilization of peppermint oil from tablets (288). Some spray-dried formulations of sulfaethylthiadiazole were tested for their prolonged-release action (289). Richman, Fox, and Shangraw prepared nonfriable tablets of glyceryl trinitrate by direct compression

employing microcrystalline cellulose as a tablet matrix (290). Gelatinized starch has been used in the preparation of acetylsalicylic acid tablets (291). In an extemporaneous method of preparing enteric-coated capsules, 29 combinations of polyvinyl acetate resins with plasticizers in various solvents were evaluated along with cellulose acetate phthalate (292). The advantages of direct weighing of filled capsules were compared with the indirect weighing of the capsule content to determine weight variations (293).

Suspensions.—Nash discussed the preparation and properties of suspensions intended for oral, parenteral, or topical use (294). Mean diameters and size distribution curves of aqueous barium sulfate suspensions measured by the sedimentation method were nearly identical to results obtained with the Coulter counter (295). A new method of determining the settling rates of suspensions was studied using a specially constructed absorptiometer and a sealed source of β-excited characteristic X-radiation (296). Guar and colloidal substances from linseeds, in low concentrations, induced flocculation of barium sulfate suspensions but in higher concentration hindered interparticle separation (297). The addition of protalbinic acid, lysalbinic acid, or infusion provided various degrees of dispersion for several bismuth compounds (298). The effect of sodium salts of fatty acids on the thermal stability of aqueous dispersions of kaolinite has been investigated (299). Other studies were conducted on the formulation and stabilization of suspensions containing tetracycline base (300), and suspensions containing alkaloids such as noscapine, papaverine, and methyl ephedrine (301). Particles of clay less than 0.5 mm. in size were found to disperse in water more slowly than particles of a larger diameter, 2–10 mm. (302). The influence of the hydrophile–lipophile balance on oily suspensions was also considered (303).

Emulsions.—A review on the stability of oil-in-water emulsions has been published by Garrett (304). In a unique study, the change in the stability of emulsions according to the number of double bonds in the oil molecule was followed by means of the Lederer equation (305). The kinetic theory of droplet coalescence and its applications to emulsion stability has been discussed by Hill and Knight (306). A γ-globulin fraction was discovered to be responsible for the creaming or complete breakage of intravenous fat emulsions *in vitro* (307). Two studies were carried out on deaggregation in oil-in-water emulsions. In one, the rate of deaggregation of an emulsion system containing 2% hexadecane-in-water stabilized with 0.09% dioctyl sodium sulfosuccinate was studied (308). In the other, the influence of *n*-butanol, *n*-hexanol, *n*-octanol, and dioctyl sodium sulfosuccinate on the deaggregation of 2% hexadecane-in-water emulsions was evaluated (309). Two series of mineral oil-in-water emulsions with varying amounts of sodium dodecyl sulfate or polysorbate 80 were also observed for changes in particle size distribution over a 2-year period (310). Another investigator followed the effect of ethoxylated fatty alcohol combinations on the stability of 4% mineral oil emulsions (311).

The preparation, application, and examination of lotions in dermopharmacy was the subject of a review (312). Lin presented an outline for planning laboratory experiments and interpreting results during the process engineering of cosmetic emulsions (313). He also commented on the advantages of cold emulsification over conventional elevated temperature emulsification procedures (314). Several suggestions were made for determining the role of solubility in the formation of emulsions by using the best solvent for the oil to be emulsified (315). Two methods were described outlining the best technique for using the HLB of emulsifiers (316). The interfacial properties of a glycerin and olive oil emulsion were studied (317). The interfacial tension of various natural and synthetic glycerides did not differ greatly, and none of the glycerides had an interfacial tension sufficiently low to emulsify spontaneously but required additional emulsifiers (318). Paraffin oil-in-water emulsions made with a soap and fatty alcohol complex as an emulsifier were examined microscopically for their physical characteristics (319). A fat emulsion concentrate, transparent and stable to autoclaving and containing very low concentrations of nonphosphatide emulsifiers, has been developed (320). Another publication discussed the emulsification and evaluation of a parenteral contrast medium for lymphography (321). In addition, a report was written on the effect of the dispersed phase (water) content on the structural and thixotropic properties of complex emulsions (322). Muys compiled data on the microbiological quality of edible emulsions during manufacture and storage (323).

Ointments and Creams.—A Hoeppler consistometer was used to measure the effect of temperature variations on the heat stability of ointments and creams (324). Another device was developed for determining the oxidation resistance of both oil-in-water and water-in-oil emulsion vehicles (325). The influence of antioxidant mixtures and physiochemical conditions on the stability of 12 different anhydrous and emulsion-type bases has been investigated (326).

Gretskii examined the stability of water–petrolatum emulsions at −10° and −20° by using 5% pentol, sorbitan oleate, or lanolin and 1–24 hr. freezing time (327). This latter investigator and his co-worker also found that emulsion bases composed of mixtures of petrolatum and pentol or sorbitan oleate gave heat stable emulsions (328). Several formulations for barrier creams (329) and clear gels (330) were suggested. In addition, film-forming bases containing aqueous topical adhesives were discussed (331). One author published two different papers on the use of polyorganosiloxane liquids for ointments and liniments (332, 333). Comparative studies were conducted on the solubility and compatibility of polyethylene glycol, polypropylene glycol, and glycerol–polypropylene glycol ether in various topical preparations (334). Dicarboxylic acid esters were claimed to be suitable additives for increasing water-vapor permeability of ointment bases (335). The hardness of topical creams has been increased by increasing the number of hydroxyl groups of partially acetylated mono- and di-glycerides (336). The addition of quaternary compounds increased the water-holding capacity of petrolatum (337). The ointment pendulum of Fueller and Muenzel was employed to evaluate the smearing properties of water-in-oil and oil-in-water emulsion bases (338).

Suppositories.—Anschel and Lieberman published a two part review on suppository bases (339, 340). An easy method has been proposed for the small-scale production of suppositories by pouring the suppository mass into plastic bottles which terminated in points (341). Experiments on changes in the complete deformation time of suppositories during storage showed that with certain fat vehicles the changes were over 100%; there were no changes with polyethylene glycol suppositories (342). Mixtures of polyethylene oxides were characterized for use in suppositories by their melting point, turbidity point, hardness, viscosity, thermal expansion, and rate of dissolution (343). In another study on suppositories the displacement value was found to decrease by reducing the particle size of the insoluble ingredients (344).

Aerosols.—Five different reviews surveyed the pharmaceutical aspects of aerosols (345–349). Another review summarized the function and application of aerosol packaging in pharmacy and medicine (350). Scriba and Hearn presented an outline on current government requirements for packaging and labeling aerosols (351); the hazards of transporting and storing aerosols and their hazards and toxicity during use have also been discussed (352). Some of the operation and maintenance problems associated with processing aerosols with slow-filling valves with a large propellant charge were disclosed (353). Another paper recommended the application of a statistical quality control procedure to a quality assurance program to improve the efficiency of an aerosol filling operation (354).

A review with 144 references considered the topic of monodispersed aerosols (355). Methods were revealed for selecting the best solvent and propellant for achieving a specific desired effect (356). Johnsen and Haase examined the scope of noncondensable gases as aerosol propellants and the data required for the efficient introduction of such gases by the gasser-shaker method into specific products (357, 358). Another discussion was concerned with the effect of transport and storage on aerosol dispensers, propellants, container construction, dispension methods, and protective covers (359). Additional experimental data have indicated a correlation between aerosol product weight loss from leakage through the valve gasket and the choice of propellant, solvent, valve type, and gasket material (360). At 130° F. the reaction of propellant 11 with water was catalyzed by metal (361). The solubility of some lanolin derivatives decreased but at least one increased with storage in pressurized formulations (362). No direct relation was observed to exist between the dispersion of the aerosol and the surface tension, but the dispersion did depend on the salt concentration and the relative humidity of the compressed air used for atomization (363). Also, it was noted that careful control of the formula, the type of actuator, and valve can be used to adjust the chilling effect of aerosol sprays on the skin (364).

Packaging.—Autian reviewed potential problems of packaging cosmetic products with plastic materials (365). A commentary was also presented on mechanical packaging machines available for use in the hospital pharmacy (366). The merits of polyethylene, the plastic most frequently used in cosmetic and pharmaceutical bottles, were examined in detail (367). Other papers were concerned with the application of aluminum containers for packaging pharmaceuticals (368) and suggested methods for the sampling of acceptable metal containers (369). Various manufacturing, packaging, analysis, and control operations for the continuous production of glass vials by a mechanized process have been delineated (370). Coring, permeability, sorption, and leaching were considered the most pressing problems in present closure applications; comments were offered regarding specific areas of improvement in each of these functions (371). Natural

and synthetic rubber stoppers were tested for turbidity, pyrogens, color, metal content, and reducible materials (372). The sorption and diffusion of formic, acetic, propionic, and butyric acids into nylon 66 has been investigated at a number of temperatures and concentrations (373). A similar binding study of sorbic acid with nylon 66 indicated the interaction is primarily one of hydrogen bonding at the amide linkage (374). Sedova *et al.* compared the effect of some rubber stoppers on the quality of injectable solutions during a 12-month period of storage (375). Two brands of disposable syringes selected at random were examined and found to yield two different types of water-soluble extractives (376). Another study indicated that rubber closures were the source of a precipitate found in physiological saline solutions (377). Other investigators recommended a procedure for screening toxicity of plastic materials based on a tissue culture method using monolayers of strain L 929 mouse cells in modified Eagle's medium (378).

A review, citing 49 references, on investigations and standards pertaining to plastic equipment for the collection, storage, transportation, and administration of blood was published with special reference to toxicity problems (379). Two different investigations designed methods for studying the permeability of films and plastic-coated papers (380), and cellulosic or plastic barrier materials, packages, and closures (381). The permeability to water and the stabilities of ascorbic acid and potassium permanganate in several plastic containers were explored by Kimura and co-workers (382). The stability of an enzyme preparation, amylase, was greatly influenced by its packaging material; the activity at 20° and 90% relative humidity decreased in the order of: glass > glassine paper laminated with polyethylene > paper > waxpaper (383). Fluoride solutions in glass containers of aluminum have been stabilized by the addition of EDTA and aluminum chloride (384). Some unique surface tests were utilized for studying the neutrality of glass ampuls for injectable fluids (385). Aluminum tubes provided better protection for anhydrous and hydrated ointment bases containing vegetable oil than orange glass jars (386). Brown tabulated a list of 17 ultraviolet light absorbers used to stabilize plastic packaging materials (387).

EQUIPMENT

Three separate papers discussed automation in the pharmaceutical industry (388–390). Hill published an article on evolutionary operation (EVOP) as a technique for in-plant optimization (391), while Maatman reviewed some of the factors important in the industrial handling and moving of materials (392). Three other investigators evaluated the Sterilab for dispensing sterile products (393). The operation of a simple liquid flow recorder designed to handle a few drops per minute or 50 ml./min. has been revealed (394). The Littleford-Lodige mixer was studied as a method for achieving high-efficiency solid-solid blending; its application as a wet-formulation device was also briefly investigated (395). One other mixer, a stirred flow reactor type, was also tested for its mixing efficiency (396).

A simple constructed device fabricated from materials common to most laboratories was designed for the control of water baths used for nonisothermal studies (397). Another apparatus was developed which permitted rapid determination of thermal diffusivity in foods. A description of its limitations and sources of error was also included (398). Larkins *et al.* described a new automatic recording multigradient capillary viscometer (399). Other workers invented a simple device for measuring the thickness of agar in a Petri dish (400) and illustrated the construction and use of a versatile hot stage microscope for determining phase diagrams of inorganic mixtures (401). During a study on the surface properties of soybean lecithin, a modification of the Wilhelmy or vertical-type film balance was disclosed which is believed to offer certain advantages over other standard film balances (402). One other apparatus has been described for compressing and expanding insoluble monolayers or films present at an oil-water interface (403). A new design was presented for an improved mass-transport cell for the measurement of electrophoretic mobility of concentrated suspensions of particles (404). In addition, a new counter for emulsion photomicrographs was depicted (405). Two papers discussed automated techniques for determining dissolution and reaction rates of antacids. They provided a rapid and accurate profile of the dissolution and reaction rate in addition to the total acid-consuming capacity of the antacid system (406, 407). A new device, called the Heidelberg capsule, has been proposed to telemeter gastric pH (408). The specifications, operation, and production rate of a new encapsulating machine for soft shell products has also been assessed (409).

PHYSICAL PHARMACY

Many pharmaceutical problems were solved through physicochemical means. The theoretical aspects of solid solutions and eutectic mixtures and their application to pharmaceutical systems were discussed by Goldberg, Gibaldi, and Kanig

(410). Other researchers found that the choice of plastic, weight of drug incorporated in the matrix, solubility of the drug used, matrix additives, and the solvent could markedly affect the release rate of drugs from plastic matrices (411). The diffusion coefficients of several physiologically active compounds have been determined in cross-linked thiolated gelatin films (412). It has been shown that stress relaxation effects of gelatin films cross-linked with oxystarch or difluorodinitrobenzene may be represented by the sum of three exponential rates of stress decay (413). Some viscoelastic properties of keratin and collagen fibers immersed in aqueous media have been measured at frequencies between 1 kc./sec. and 20 kc./sec. and at temperatures between 0 and 100° (414). The penetration of chlorpromazine and chlorpromazine sulfoxide into insoluble monomolecular lipid films depended on the surface characteristics of the lipid, pH, and the ionic strength of the underlying solution (415). Additional studies were concerned with the relation between the diffusion coefficients and the electrolytic properties of membranes (416). Measurements of the tensile strength of dry powders of irregular particle shape have been made using a split tilting-plate apparatus based on that described by Thouzeau and Taylor (417). Johnson and co-workers studied the distribution of phenol between water and carbon tetrachloride and the solubility of water in solutions of phenol in carbon tetrachloride (418). Another paper presented a novel means for achieving a superior degree of carbonation with sodium bicarbonate in various latentiated acidifiers (419). The rate of neutralization of hydrochloric acid by dispersed calcium carbonate powder has been examined as a function of pH, temperature, and particle size (420). A tartaric acid buffer has been shown to form a reactive intermediate in aqueous solution capable of rapidly acylating any nucleophilic compound present (421).

Ionization.—The absence of a primary kinetic isotope effect in DMSO was the subject of a paper on ionization rates of weak acids (422). Another article described the mechanisms for the acid dissociation of vitamin B_{12} (423). The acid-base behavior of ephedrine isomers and their oxazolidine derivatives in aqueous and nonaqueous media has been described in detail (424). Also, a new technique was described for measuring the rates of ionization of carbon acids (425).

Solubility.—Wurster and Taylor reviewed the theory of dissolution and methods of study in a paper with 74 references (426). A novel method was developed for determining dissolution rates of multiparticulate systems (427). One other *in vitro* continuous dissolution rate measuring method has been designed and evaluated for determining the dissolution rates of labeled materials from solid dosage forms (428). Results obtained with this method were compared with dissolution rates of similar dosage forms using the U.S.P. disintegration apparatus and the rotating bottle method. An improved holder for rotational disk dissolution studies has been used to determine the relative intrinsic dissolution rates of caffeine monohydrate, aspirin, salicylamide, and acetaminophen (429). The crystal behavior, solubility, and dissolution rates of two anhydrous and one hydrated form of prednisolone in aqueous solution have been investigated (430). The theory for the dissolution rate of polyphase mixtures was probed and applied to several situations involving simultaneous diffusion and rapid equilibria (431). The rate of dissolution of boric acid in aqueous solutions of polysorbates was examined (432); another study explored the effect of complex formation on the dissolution kinetics of *m*-aminobenzoic acid (433). Danckwerts' penetration model was employed to derive equations to explain the theory for the dissolution of solids in a multiparticulate system (434). The dissolution behavior of a weak acid, 1,1-hexamethylene *p*-tolylsulfonylsemicarbazide, and its sodium salt in phosphate buffers has been evaluated by Higuchi *et al.* (435).

One other new method was described for determining the dissolution rate of fine particles of crystalline hydrocortisone acetate (436). A study relating the *in vitro* dissolution rates and solubilities of 45 different compounds representative of various chemical species supported the theory that the initial rate of dissolution of a compound is directly proportional to its solubility (437). In addition, dissolution studies were conducted on both the one-to-one molecular compound and mechanical mixtures of sulfanilamide and sulfathiazole at 15, 25, and 35° (438). As part of a program on the transport, deposition, and dissolution of cholesterol in aqueous medium, the growth, dissolution rates, and nucleation behavior of this compound in saline have been studied by following changes in particle size with the Coulter counter (439). In a similar study, the Coulter counter was used to test the influence of cholate on the precipitation behavior of cholesterol in aqueous media as a function of pH (440).

A new technique has been proposed for determining the solubility product constant (441). Dielectric constants of water–ethanol–sucrose and water–ethanol–sorbitol systems have been experimentally determined and found to be a complex function of composition expressed as weight per

cent (442). The solubilities of acetanilide, *p*-methylacetanilide, *p*-ethoxyacetanilide, aminopyrine, and antipyrine have been measured in dioxane–water mixtures of known dielectric constants (443, 444). Some solubility anomalies were compiled for actinomycin D (445). The water solubilities of five different barbiturates were ascertained by liquid scintillation counting of ^{14}C-tagged compounds using the technique of phase solubility analysis (446). Four different procedures were employed to determine the solubility of cholesterol in water (447). A critical concentration for the solubility of cholesterol chlorobetainate in aqueous solution was detected by specific conductivity, refractive index, and activity coefficient determinations (448). The solubility of iodine has been studied in a series of solvents (449). Nakatani reported on the solubility of compounds related to orotic acid in amine solutions (450). Tagged ^{14}C-pentaerythritol tetranitrate was utilized in determining the solubility of this compound in water and saline (451). Another presentation compared the solubility of polysorbates in simple syrup, glycerol, and propylene glycol (452). The solubilities of several xanthines, including caffeine, theophylline, and theobromine, were examined in dioxane–water mixtures; the solubility curves that were obtained showed a multiplicity of peak solubility values (453). A different type of study provided data on the solubilities of thin films of polyethylene, polypropylene, and polystyrene in many liquid drugs (454). Other investigators studied the solubility of compressed gases in fluorocarbons (455).

Swarbrick published a review article outlining the physicochemical properties of surface-active agents in solution with particular reference to solubilization of materials of pharmaceutical interest (456). One investigation determined the usefulness of test systems consisting of aqueous solutions of anionic surfactants and an excess of ^{14}C-cyclohexane in studying effects related to solubilization (457). The solubilization of several aliphatic and aromatic acids in aqueous solutions of cyclopentamine hydrochloride, ephedrine sulfate, and propoxyphene hydrochloride has been demonstrated (458). A potentiometric method was applied to a study of the solubilization of benzoic acid in systems containing anionic (459) and nonionic (460) surfactants. Another study was concerned with the interaction of urea in varying concentrations with three isomeric monohydroxybenzoic acids (461). The solubilizing capacities for camphor of one anionic, one cationic, and three nonionic surfactants have been investigated and determined by measuring the areas under ultraviolet absorption peaks (462). The use of Z values was proposed as a method for measuring the polarity of the environment in a study on the solubilization of camphor in polysorbate 20 (463). Sorbitan trioleate increased the solubility of hydrous ephedrine alkaloid in liquid petrolatum (464). The effect of various polysorbates on the solubility of sulfanilamide in several different solvent systems was ascertained by Khawam, Tawashi, and Czetsch-Lindenwald (465, 466). Aqueous solutions of urea, 1-acetyl-3-methyl urea, and 1,3-dimethyl urea were discovered to increase the water solubility of testosterone and some other related steroids if the 17-hydroxyl group of the steroid was free (467). The effect of urea on the aqueous solubility of six different dye compounds in water has also been compared (468). In addition, the solubility of theobromine has been increased and stabilized by the addition of various polysorbates in tragacanth mucilage (469).

Complexation.—The formation of copper complexes of sulfur drugs was discussed by Lee (470). Acid dissociation and copper(II) chelate formation constants were compared for glutamic acid and several of its derivatives (471). Data were presented which suggested that coordination complexes may be of major importance in the biological phenomenon of binding with catecholamines (472). Stability constants for complexes of tetracycline with cupric ion have been published (473). An *in vitro* enzyme kinetic study was used to determine the nature of the inhibition of acetylcholinesterase by cupric chelates of glycine and ethylenediamine (474); also, the inhibition of acetylcholinesterase by 1–1 cupric chelates of ethylenediamine and glycine was analyzed and shown to be essentially a noncompetitive type (475). Complexes formed by adrenaline and related compounds with transition metal ions were studied by Jameson and Neillie (476). The inactivation of tetracycline with a cupric–morpholine complex was investigated in another binding study (477). Gel-filtration, ultra-filtration, and ultra-centrifugation were used to study the nature of an iron–dextran complex (478).

Oxytetracycline was shown to form fluorescent complexes with proteins *in vitro* (479); the binding of sulfonamide to serum albumin was also investigated in a similar study (480). Protein binding of radioactive vitamin D_3 added to serum *in vitro* or present in dog serum after intravenous injection has been studied by a variety of methods (481). The interaction of cortisol with bovine

and human serum albumin was observed by an equilibrium dialysis procedure (482). Protein binding of salicylates by rat liver, kidney homogenates, plasma proteins, globulins, and albumin was tested by Stafford (483). Also, the serum albumin binding of several structurally similar xanthine derivatives was evaluated by a spectrophotometric technique (484).

One investigation was conducted to ascertain the interaction of a group of compounds, used in medical and pharmaceutical practice, with several types of insoluble polyamides used in containers or devices for storing or administering drug products (485). The interaction of parachlorometaxylenol with macromolecules was determined by solubility and dialysis procedures (486). An electrophoretic technique was used to illustrate the interaction occurring between sodium carboxymethylcellulose and both methylcellulose and polyacrylamide (487). The interaction between poly-*N*-vinyl-5-methyl-2-oxazolidinone and certain pharmaceuticals in aqueous solution was reported (488). A complex interaction of potato and arrowroot starches with certain drug pharmaceuticals has been noted by Goudah and Guth (489). The binding of certain benzoic acid derivatives by polysorbate 80 and cetomacrogol 1000 was determined by means of an equilibrium dialysis technique (490). An investigation of the binding of polyethylene glycol to carboxymethylhydroxyethylcellulose was also disclosed (491). The hemolytic activity of phenolic preservatives was prevented by their interaction with polyethylene glycols (492).

The reaction of some salts of rare earth metals with group B vitamins has been revealed (493). Another study assessed the interaction of a series of phenyl-substituted carboxylic acids with Schardinger dextrins (494). The equilibrium reactions of caffeine and nicotinamide with lidocaine and saccharin were studied (495); also, flavins and phenols were found to form 1:1 molecular complexes in neutral solution (496). A spectropolarimetric method of determining stability constants of complexes formed through hydrogen bonding has been developed and applied to a camphor–phenol system (497). Gas chromatography was employed during a complexing study of quinine and quinidine with urea, thiourea, and diethylthiourea (498). The binding of chlorpromazine and thioproperazine to rat liver and human leucocytes was reported by Teller (499). The reaction between pyridoxal phosphate and cycloserine has been evaluated (500). Wadke and Guttman presented evidence to indicate that the complexed form of isoalloxazine was resistent to hydrolytic decomposition (501). Complex formation between chlorpromazine and adenosine triphosphate was believed to occur because the surface tension was lowered in the presence of adenosine triphosphate which is not surface active (502). Complex formation involving *cis* hydroxyls in an axial-equatorial position between carbohydrates and dimethylsulfoxide was revealed by proton magnetic resonance data (503).

A study of adsorption complexes formed by the interaction of sodium dodecyl sulfate with gelatin at pH values below the isoelectric point was described (504). Complexation between 1,3,5-trinitrobenzene and several local anesthetic compounds was demonstrated; it was suggested that the amine group was the primary site of reaction, with the tertiary amine taking precedence when present (505). A dark reaction between citrate and iodine was detected by two investigators (506). In addition, Rodgers followed the interaction of hexylresorcinol and amaranth with quaternary ammonium compounds by a conductimetric titration procedure (507). Other studies were concerned with structural determination of complexes by ultrasonic waves (508).

Surface Phenomena.—A review of the basic concepts involved in wetting and adhesion processes was reported by Gray (509). Another review discussed the theory on coagulation of noninteracting particles in Brownian motion (510). Other surveys covered colloidal dispersions, electrokinetic effects, the concept of ζ potential (511), and the coagulation of colloidal powders (512). Higuchi, Rhee, and Flanagan studied the rates of aggregation of polystyrene and polyvinyltoluene particles in aqueous ionic surfactant solutions (513). Solutions to equations for the kinetics of coagulation were analyzed by other workers (514); a mathematical analysis has also been made of two chemically interacting macromolecules settling in a liquid solvent under one-dimensional electrical and cylindrical centrifugal forces that were sufficiently large to overcome diffuse forces (515). Centrifugation current was used to determine the electrokinetic mobility of several electrolytes in the aqueous phase of water-in-heptane emulsions (516). The streaming potential of sulfisoxazole, sulfadimethoxine, and *N'*-acetylsulfisoxazole was measured with Hazel-type cells in sucrose, sodium chloride solution, and sodium chloride in 10% sucrose solution (517).

Two different reviews on the physical chemistry of nonionic detergents have been published (518, 519). Also, a survey on the evaluation of anionic and nonionic emulsifiers according to HLB values was reported (520). The adsorption of three quaternary ammonium salts, having the same

chain length and counterion but differing in the polar group, was measured at the air–water interface (521). The effect of solvent on micellar properties of nonionic surface-active compounds was investigated (522). A conductimetric method was used for determining the critical micelle concentration of sodium decyl and dodecyl sulfates in the presence of sodium chloride (523). Phares predicted reductions in water–air, oil–air, and water–oil interfacial tension produced by nonionic surfactants by using a form of the Langmuir adsorption equation (524). The influence of the sodium chloride concentration on the adsorption of sodium dodecyl, sodium decyl, and sodium octyl sulfate solutions at an air–water interface has been evaluated (525). The Griffin HLB method was utilized to determine the possibility of emulsifying 10 different emulsifiers having HLB values in the range 8–13 (526). The effect of additives on the foaming properties of very dilute surfactant solutions has been reported (527). Some of the physical properties of a series of nonionic detergents with branched hydrocarbon chains of the general formula, $R_2—CH—CH_2O(CH_2CH_2O)_6H$, were described (528).

The adsorption of water on clays was reviewed in a paper with 151 references (529). The relationship between some electrical and thermodynamic properties of adsorbed water on two montmorillonites, a silica gel, and a window glass powder was studied at room temperature (530). The specific surface area of suspensions of montmorillonite and the average thickness of their diffuse double ionic layer were reported (531). The sorption of methylene blue and methylene violet on Fintia bentonite processed by various methods was investigated (532). Bentonite clays have been activated by treatment with 10% hydrochloric acid while heating to improve their adsorptive and catalytic properties (533). Packham examined the coagulation of dispersed clays with hydrolyzing salts while carefully observing the effect of the nature and concentration of clay, pH, and the presence of various ions (534). The adsorption of water on ion-exchange montmorillonite has been discussed (535). It was noted that the heat of wetting of sodium and potassium bentonite was not greatly affected by the addition of small amounts of polyacrylamides (536). Other results were presented which showed clear evidence of discrete charges on clay surfaces (537). The adsorption properties of bentonite clay with regard to vitamin E were considered (538); the adsorption of phenol, *p*-cresol, *p*-nitrophenol, 2,4-dinitrophenol, and puric acid on Fuller's earth was determined (539). The influence of hydrogen ions on the adsorption potential and surface tension of barbital, aminopyrine, and veramon was disclosed in other studies (540). A summary of the effects of the method of preparation on the surface properties of silicas has been presented (541), and the adsorption properties of silica gel precipitated in the presence of some alkaloids has been investigated (542). The reactivities of the surfaces of muscovite, montmorillonite, and chrysotile-asbestos were also explored (543). In addition, Rohdewald evaluated the effect of glycerol, sorbitol, hydrochloric acid, sodium chloride, anionic, cationic, and nonionic surfactants on the properties of talc suspensions (544).

Blaug and Gross reported on the *in vitro* adsorption of nine different anticholinergic drugs by six antacids (545). Steroid adsorption by polyethylene tubing was noted by other workers (546). The critical micel'e concentrations of oxyethylene-oxypropylene polymers were assessed by surface tension and two different spectral absorption methods (547). Additional studies were conducted on the thermodynamic function of the sorption of vitamin B_{12} by the salt forms of sulfo resins (548). The dispersion of inorganic pigments was improved by surface treatment (549). Polypropylene glycols with molecular weights of about 2000 had the highest surface activity and strongest dye dispersing effect (550). It was concluded that with most hygroscopic materials used in pharmacy, the process of water vapor sorption starts to be active in an atmosphere with 50% relative humidity (551). The measurement of interfacial areas of foams and froths has been carried out by a radiographic technique (552). A technique involving reiterated approximations and a high digital computer was employed to solve the problem of surface tension measurement by the Pendant drop technique (553). The hydrophile–lipophile balance of surface-active substances has been determined by a calorimetric technique (554).

Crystallization.—A review of ultrapurification methods based on zone melting appeared in the literature (555). Nickolics, Bidlo, and Nikolics explored the influence of solvents on crystal structure of resorcinol, sulfathiazole, acetylsalicylic acid, phenacetin, barbital, and phenobarbitol (556). The precise crystal and molecular structure of α-D-glucose by neutron-diffraction analysis was ascertained (557); and the crystal and molecular structure of 1,3-dihydro-1-hydroxy-3-oxo-1,2-benziodoxole was determined by X-ray crystallographic methods (558). Polymorphism in potassium sulfate and thallium sulfate has been reported (559). The physical

properties of various anhydrous and hydrous forms of ampicillin were noted (560). A number of compounds were tested for their inhibitory effect on the needle axis growth rate of sodium acid urate crystal (561). Other data revealed the effect of temperature on the linear crystallization rate of salol, betol, salipyrine, antipyrine, and codeine (562).

Rheology.—A new flow equation for pseudoplastic systems was described by Cross (563). An electrical method has been suggested for determining the areas of hysteresis loops (564). A new viscometer was designed for measurements on dilute polymer solutions but could be equally well used for other liquids (565); also, another new glass rotatory viscometer was recommended for certain rheology studies (566). Hiller reported on the effects of temperature and pressure on the rheological behavior of montmorillonite sols (567). The rheology of silica suspensions was investigated as a function of concentration, particle size, polarity of organic solvents, and the pH and ionic strength of the aqueous phase (568). The factors affecting the rheological properties of clay suspensions were observed in another study (569). The thixotropic behavior of "alki" bentonite suspensions (570) and the physicochemical and technological properties of two kaolin varieties were presented (571). The rheology and suspension characteristics of carboxymethylhydroxyethylcellulose–polyethylene glycol systems, evaluated by means of the power law equation, were outlined (572). The concentration dependence of the steady flow viscosity was discussed for aqueous solutions of high viscosity hydroxyethylcellulose (573). The elastic properties of the particle network in gelled solutions of carboxymethylcellulose have been recorded (574). A treatise was published discussing critical gum solution properties that may be determined through analysis (575). Sugar, when used at high levels, offered a means of providing a delay in the development of the viscosity of guar gum formulations (576).

Procedures and instrumentation have been devised for measuring the stress-relaxation modulus of melts employed as soft gelatin encapsulating formulations (577). Other workers explored the viscosity and stability characteristics of the system ascorbic acid–water–polysorbate 20 (578). The flow properties at 25° of concentrated (30–42%) dicalcium phosphate dihydrate paste suspensions and model systems were determined using a concentric cylinder, cone plate, and capillary extrusion viscosimeter (579); in other studies, the thixotropy and dilatancy in complex emulsions and suspensions were investigated (580). Higuchi and Stehle tested the rheometric properties of silica suspensions in dibutyl phthalate, hexadecane, mineral oil, ethylene glycol, and mixtures with and without different surfactants (581). An apparatus, consisting of a sedimentation tube and capillary viscometer, was designed for particle size determination of suspensions and emulsions (582). In one study, the basis and conclusions for rheological characterizations of some pharmaceutical adjuvants were reported (583). The swelling, hygroscopicity, and viscosity properties of bentonite suspensions caused by water absorption were also evaluated (584).

PHARMACOCHEMICAL ASPECTS

This section of the review considers many of the papers on polymers, antibiotics, and radioisotopes which might be of interest to the pharmaceutical scientist. It is not intended to encompass the vast area of pharmaceutical chemistry concerned with synthesis, structure-activity studies, reaction mechanisms, analysis, etc. These related disciplines are reviewed annually in other publications and are, therefore, omitted from this report.

Polymers.—Rodrigues and Barrosa reviewed the current status of polymers of pharmaceutical interest (585). Good agreement was found between theory and experimental data for the stress cracking of high-density polyethylene in octylphenoxy polyethoxyethanol examined from the viewpoint of critical strain (586). An attempt was made to develop a means of assessing the order in an amorphous polymer from density measurements (587). A discussion has been reported on some of the factors responsible for high thermal stability in polymers along with some results of thermal degradation studies on an aromatic polyimide (588). In another investigation, the melting points of polyethylene crystals and the relation between molecular length and chain folding were presented (589). Also analyzed was the effect of changes in structure on chemical reactivity, the effect of structure on physical properties, and the basic principles of a chain reaction mechanism in polymerization (590). Cooper discussed the toxic dangers and disadvantages of plastic materials used in pharmaceutical systems (591).

The effect of the structure of sulfonated polystyrene ion-exchange resins on the sorption of oxytetracycline and chlortetracycline ions has been investigated (592). Measurements of the swelling ratio and exchange capacity of individual ion-exchange resin beads were used to compare resin samples, to study interparticle differences,

and to help characterize resin degradation (593). Lappas and McKeehan employed several esters of poly(ethylene–maleic anhydride) and poly(vinyl methyl ether–maleic anhydride) as coatings to control the release of drugs upon reaching a specific intestinal pH (594). The use of polyvinyl alcohol as a solvent for pilocarpine ophthalmic solutions was reported in publications by different authors (595, 596). A method for preparing an aqueous colloidal dispersion of organic materials such as β-carotene by using water-soluble polymers like polyvinylpyrrolidone has been revealed (597).

Antibiotics.—Reviews on the chemistry and pharmacology of penimepicilina, a new broad-spectrum antibiotic (598), and on the problems and investigative methods of antibiotic binding by blood serum proteins appeared in the literature (599). The properties of some new antibiotics, cephaloridine (600), kasugamycin (601), and copiamycin (602), were disclosed. Also, the preparation and isolation of anhydroerythromycin (603), antibiotic R-12 (604), neohumidin (605), and lemonomycin (606) have been reported. In addition, a new antifungal antibiotic, trichodermin from *Trichoderma viride* was described (607).

The activity in experimental infections, absorption, and elimination of rifamycin B diethylamide in man have been examined (608); also, the *in vitro* activity, absorption, and excretion of cephalothin in normal subjects have been reported (609). Garrett and Miller showed the generation rate constants for viable counts of *E. coli* were linearly dependent on the concentration of tetracycline and chloramphenicol (610). Definitions, standards of identity, strength, quality, purity, tests, and methods of assay were given for dactinomycin (611). A scheme of classification for the antifungal antibiotics of the pentaene group has been presented (612). Gentamicin was tested *in vitro* against 889 strains of pathogenic bacteria (613).

Radioisotopes.—Radioisotopes in pharmaceutical technology were surveyed (614) along with the production of radioactive isotopes for medical use (615). With the exception of the field of chemical kinetics, a brief survey has been presented of the principles of isotope chemistry and their utility in the ever unfolding panorama of scientific research (616). Another brief review was conducted on the preparation and analytical control of radioactive chemicals of the 16th revision of the U.S.P. and the "British Pharmacopoeia" 1958 (617). The preparation of colloidal solutions of zirconyl phosphate with ^{32}P for radiotherapeutic purposes was described (618). The influence of the sample volume on weak β-counting efficiency at 0° in liquid scintillation counting has been reported (619). The factors influencing the loss of ^{14}C from labeled carbonates were published (620); corrections for grain count in autoradiography were also determined (621).

BIOPHARMACEUTICS

The area of biopharmaceutics considers research efforts directed toward studying the influence of pharmaceutical formulations on the biological activity of drugs. Numerous review articles appeared in the literature. Mann cited 144 references while discussing biological aging and its effect on modification of drug activity (622). The evolution of new concepts on the mechanism of drug action was presented (623). Other topics of review included the absorption of pharmaceuticals by diffusion (624) and transport phenomena in artificial membranes (625). A two-part review covering factors influencing drug distribution in the body and pharmacokinetics also appeared in the literature (626, 627). The mechanism of enzyme and hormone actions was the subject of another review (628). In addition, the theoretical aspects of diuresis and the various therapeutic classes of diuretics were surveyed (629).

The kinetics of drug transfer from a buffered aqueous phase through a lipid phase to another aqueous buffered phase were studied (630). The development of a unified statistical mechanical theory of transport across a membrane model in liquid mixtures and electrolytes was discussed (631). A molecular basis for drug activity was considered (632), while the use of quantum chemistry in drug design was explored by Schnaare and Martin (633). On the assumption that the potassium content of the body cell mass remains constant, it has been possible to estimate body cell mass by measuring ^{40}P activity with a whole-body scintillation counter (634).

Milne discussed the potentiation of excretion of drugs (635). Analog computer studies resulted in an equation that can be used to predict the average asymptotic blood levels during a multiple-dose regimen from basic parameters estimated from single-dose studies (636). A theoretical relationship between dose, elimination rate, and duration of pharmacologic effect of drugs has been suggested (637). The importance of the route of administration was demonstrated in the pharmacodynamics of the antitussive action of codeine and ethylcodeine (638). Three unique, new, highly reactive polymeric compounds of magnesium aluminum oxy hydroxide showed 50–60% more antacid activity than presently known aluminum-magnesium antacids (639).

Effects of Physicochemical Properties.—A review, with 21 references, on physicochemical studies of membrane permeation concerned with absorption and excretion of drugs appeared in the literature (640). It was shown that calcium and pH influence the absorption rate of tetracycline and three of its derivatives (641). The water-insoluble salt, lincomycin hexadecylsulfamate, provided greater absorption and activity than the water-soluble salt, lincomycin hydrochloride (642). Poole, Zeigler, and Dugan demonstrated no significant increase in aluminum blood levels after oral ingestion of a water-soluble aluminum complex, potassium glucaldrate, in normal subjects or in those requiring antacid therapy (643). Certain members of some metal-acid complexes of tetracycline and demethylchlortetracycline resulted in higher blood levels (644). Particle size effects were discussed in relation to formula modifications (645); and the effect of griseofulvin particle size reduction was also determined in relation to drug excretion and absorption (646, 647). The *in vivo* distribution of colloidal chromic radiophosphate having different particle sizes has been studied following intravenous, intramuscular, and intracavitary injection into mice (648). The proportion of a sulfisoxazole dose absorbed did not vary with particle size, but the rate at which this proportion was absorbed did change (649). A correlation between the relative percutaneous absorption of topical corticosteroids and the physical properties of solubility and partition coefficient has been revealed (650). The release of steroids from a topical vehicle was stated to be independent of, and uninfluenced by, the presence of a second noninteracting component (651). Wide individual fluctuations were observed in studies on the influence of the rectal route for the administration of sulfonamides for therapeutic purposes (652). A unique relationship has been discovered between the molecular weight and biological effect of dextran (653). When administered to cats, in quantities representing Hatcher doses, there was a proportionality between the absorption rate and the lipid solubility of various lanata glycosides (654).

Effects of Formulation.—Wurster reviewed some of the factors related to the formulation of medicinals for percutaneous absorption (655). Tablet disintegration and physiological availability of drugs were also surveyed in an article containing 58 references (656). The oral absorption of salicylates was found to be a function of the intrinsic rate of dissolution which was affected by formulation parameters such as particle size, disintegrants, and lubricants (657). Incomplete absorption of aspirin was attributed to an excessively low dissolution rate of the dosage form (658). The importance of dissolution rates in producing effective diazoxide blood levels in man was outlined following administration of solutions, capsules, and tablets (659). A method for the evaluation of antimicrobial activity of a bacteriostatic agent in the presence of a surfactant was described (660). Another study disclosed the relationship between the concentration of a surfactant, polysorbate 80, and the rate of absorption of a drug, salicylamide, from the small intestine (661). Modification of *in vivo* promazine absorption by activated attapulgite and activated charcoal in humans using urinary excretion measurements has been demonstrated (662). Three different dosage forms, namely a liquid concentrate, regular sugar-coated tablets, and substained-release preparations, did not influence thioridazine blood levels (663). Only one-fourth to one-third of orally administered tetracycline was absorbed compared to the amount absorbed after intravenous administration (664). A complex model was presented to illustrate the pharmacokinetic parameters related to the absorption, metabolism, and elimination of nalidixic acid in man (665). The absorption and elimination of salicylic acid was investigated after i.m. injection of aluminum aspirin in a neutral oil (666). Serum salicylate levels were also used to evaluate the relative release rate of aspirin from a commercial aspirin tablet and from a hard gelatin capsule (667). Significant differences in blood levels were found for three different theophylline elixirs (668). Additional studies analyzed the effect of viscosity on the gastric absorption of ethanol and salicylic acid (669).

Indomethacin was absorbed satisfactorily from rectal polyethylene glycol suppositories according to blood level studies (670). A preliminary report on drug absorption from the rectum appeared in the literature (671). Suppository administration of theophylline *p*-aminobenzoate of piperazine provided a rate of absorption equal to, or less than, the rate from an equivalent tablet dosage form (672). The pharmacokinetics of rectal application of 4-sulfanilamido-5,6-dimethoxy-pyrimidine has been published (673). The rectal absorption rates of sulfonamides were reduced by water-soluble bases (674). Nonionic surface-active agents were found to decrease the rectal absorption of sulfonamides; this effect was the result of the micelles being too large to pass through the rectal membrane (675). Comparative studies were carried out on the *in vitro* and *in vivo* release of salicylates from fatty suppository bases (676). Anhydrous lanolin increased drug release when cocoa butter was employed as the

fatty suppository base (677). Cod liver oil and polyethylene glycol bases aided in the penetration of chlortetracycline through intact rabbit skin (678). The percutaneous absorption of an ^{35}S-sulfonated polygalacturonic acid from an oil-in-water emulsion base has been determined *in vivo* by following its disappearance after topical application (679). The cutaneous absorption of *S*-dicarbethoxythiamine was studied from various ointment bases (680). A diffusion study of sulfacetamide and sulfathiazole from three different ointment bases through a membrane into water has been reported (681). Dempski and co-workers discussed an *in vitro* study for testing the relative moisture occlusive properties of several topical vehicles and Saran wrap (682). The percutaneous absorption of heparin from commercial ointments was evaluated (683). A new microbiological "agar tube" method has been developed for measuring the release of antibacterial agents from various ointment bases (684). Several unique factors influencing the topical absorption of idoxuridine were demonstrated by Shell (685). One publication presented the pharmacological evaluation of certain ointment bases (686). The diffusion of neomycin from several ointment bases was examined (687); and the absorption of radioactive salicylic acid from 10 ointment bases was also considered (688). The diffusion of iodine and salicylic acid was studied from 29 different ointment bases by impasting the ointment on an agar-gel disk which contained starch solution or ferric chloride (689). In studies concerning the importance of the vehicle for percutaneous absorption, a small uptake of resorcinol and boric acid was noted, but phenol and salicylic acid were readily absorbed, especially from aqueous solutions (690).

Blaug and Canada observed the relationship of viscosity and contact time on the prolongation of action of methylcellulose-containing ophthalmic solutions (691). The intestinal absorption of certain water-soluble acidic dyes and some of their lipoid-soluble complexes has been investigated (692). An investigation of the effect of solubilization of phenobarbital and several other barbituric acid derivatives on *in vitro* and *in vivo* release rates was discussed (693). Succinic acid was found to promote the absorption of iron from the alimentary tract (694); other authors were concerned with the biological availability of riboflavin solubilized with sodium salicylate (695). The onset of action of tubocurarine has been studied in a program evaluating the skin penetrating properties of drugs dissolved in DMSO and other vehicles (696).

Absorption Control.—*In vitro* dissolution tests were developed which correlated quantitatively with dissolution rate-limited drug absorption in man (697). One investigation was noted on the effect of certain tablet formulation factors on *in vitro* drug release and *in vivo* drug absorption (698). Several authors studied the absorption of aspirin from enteric-coated preparations (699–702). Slow-release tablets of ferrous sulphate showed extremely variable absorption when compared to standard ferrous sulphate therapy (703). A radioisotopic technique for determining the efficiency of an enteric-coated tablet *in vitro* was found to be superior to the U.S.P. method (704).

A review of the effectiveness, safety requirements, rational, and clinical pharmacology of prolonged-release drugs was published (705). Frederik and Cass discussed some principles of the clinical evaluation of sustained-release drugs and suggested clinical methods to determine their duration of action (706). Other researchers observed that the action of procaine, isoniazid, sulfamidochrysoidine, and benzocaine was prolonged by combining them with polymers of dextran (707). Sustained-release aspirin formulations have been evaluated by several researchers (708–710). The sustained-release action of polyvinylpyrrolidinone in tablets containing tetracycline hydrochloride or phenoxymethylpenicillin was examined (711). *In vitro* and *in vivo* evaluations were reported for sustained-release amobarbital tablets (712); and controlled-release nitroglycerin tablets used in the treatment of angina pectoris were also tested (713). A novel system was described in which the dissolution rate of theophylline aminoisobutanol in a prolonged-action system was utilized as a parameter of the blood level concentrations (714). The release rates of sustained-release tablets were followed automatically by employing a photometer (715); another simple apparatus was described that was based on continuous elution for the *in vitro* control of sustained-release preparations (716). By using the half-change method of artificial intestinal and stomach juices, the elution ratio of medically active substances from Bellergot tablets with prolonged action has been investigated (717).

Absorption Mechanism.—Reviews appeared covering the mechanism of absorption of weak acids and alkalies through the lipid barrier (718), and the importance of specific binding, nonspecific binding, and the role of chemical configuration of the effective substance and its receptor (719). Five different models for the transport of methionine and sodium butyrate by intracellular plasma have been described and discussed (720). A discussion of the influence of a drug on its own

absorption was outlined (721). Of particular interest was an article which presented a theoretical study of the potential effect of drug binding by plasma proteins on drug distribution (722). The mechanism of absorption of sulfonamides has been studied by measuring the rectal absorption rate in anesthetized rats (723). The absorption of methyl ethyl ketone under normal, dehydrated, and hydrated skin conditions was investigated by Wurster and Munies (724, 725).

The intestinal absorption of vitamin B_{12} was evaluated by several authors (726–729). Deferrioxamine had no significant influence on the intestinal absorption of hemoglobin iron (730); however, in other studies, desferrioxamine B reduced the intestinal absorption of iron (731). The effect of neostigmine on the intestinal absorption of sulfisoxazole (732) and the effect of acetazolamide on the excretion of sulfisomezole into human parotid saliva were explored (733). Beckett and Rowland examined the urinary excretion kinetics of amphetamine in man and showed that the excretion of the unchanged drug was dependent upon urinary pH (734). The effect of cetrimide and phloridzin on the intestinal absorption of glucose in man was disclosed (735). A significant increase in the excretion rate of sodium salicylate has been observed upon the addition of an equal amount of glucosamine hydrochloride to an orally administered dosage form (736). The modification of protein binding and urinary excretion by the simultaneous use of two drugs was reported (737). The effects of acetazolamide, sodium perchlorate, ouabain, and iodide loading on the processes controlling ^{131}I and inulin-^{14}C distribution in the brain and cerebrospinal fluid were compared in nephrectomized rats (738). Silver proteinate was found to inhibit the absorption of iodine in the rat intestine (739). Benzoyl thiamine monophosphate produced thiamine levels in humans 4 times greater than those produced by thiamine hydrochloride (740). Also, it was noted that chymotrypsin did not augment the absorption or increase the activity of an oral dose of tetracycline (741). In addition, intestinal absorption of glycine was inhibited by phlorizin but not by rutin (742), while fat absorption in rats was delayed by Triton (743).

Kinetic Studies.—The kinetic factors of the digestive absorption of drugs were reviewed in a paper citing 181 references (744). Another article commented on the theoretical aspects of the elimination kinetics for a number of model drugs which have a high affinity for plasma protein (745). Radiophosphorus, ^{32}P, was employed to determine the absorption rate of dibasic sodium phosphate from fatty suppository bases in rabbits (746). In studying the percutaneous absorption of dinitroisopropylphenol in rats, absorption increased with time to reach a maximum after 7 hr. (747). An analog computer program suitable for using blood-level *versus* time data as an input and producing *in vivo* dosage for availability *versus* time pattern as its output was developed and tested by Stelmach, Robinson, and Eriksen (748).

The determination and significance of the biological half-life of pharmaceuticals were surveyed in a review with 63 references (749). In addition, the biological half-life of several drugs appeared in the literature, namely, 140 min. for psicofuranine (750), 9 min. for noscapine (751), and 11.3 hr. for meprobamate (752). Several investigators studied the pharmacokinetics of aspirin metabolism and distribution (753–755). The utility of goldfish as test animals for evaluating biological membrane permeation led to a detailed analysis of the drug transfer kinetics for 4-aminoantipyrine (756). Data were compiled on the excretion, distribution, and metabolism of doxapram after intravenous injection into dogs (757). A new method was presented for calculating per cent absorbed *versus* time plots from metabolite blood level data (758). Also, a relation between the rate of elimination of tubocurarine and the rate of decline of its pharmacological activity was demonstrated by Levy (759).

Drug Absorption.—The influence of certain factors on the absorption and excretion of drugs was reviewed by two different authors (760, 761). Schlagel discussed the comparative efficacy of topical anti-inflammatory corticosteroids in a review article containing 219 references (762). In other studies, the excretion of ^{3}H-labeled dihydromorphine was presented (763) and whole-body liquid scintillation counting was found suitable for testing the kaliuretic properties of diuretic agents (764).

Blood levels from chloramphenicol palmitate compared well with those from chloramphenicol *per se* at equivalent dosage levels (765). After a single dose of chloramphenicol, both the biologically active form and inactive metabolites have been found in human milk (766). The absorption and excretion of erythromycin and monomycin were measured by Lagert (767), while Fischer and Riegelman evaluated the absorption and distribution characteristics of griseofulvin from other blood level data (768). The absorption, diffusion, and excretion of lincomycin has been studied following oral, i.m., and i.v. administration (769). Plasma appearance times and values of ^{47}Ca did not cor-

relate with the absorption of radiocalcium from the intestine (770). A double isotope method for the measurement of intestinal absorption of calcium in man was presented in another publication (771). The absorption, distribution, and utilization of iron in a fat-soluble form was evaluated against similar data for ferrous sulfate and ferrocene (772). In addition, the absorption of ^{59}Fe-hemoglobin has been investigated in iron deficient and iron supplemented rats and compared with the absorption of ^{59}Fe as ferrous sulfate (773).

The absorption of steroid hormones was assessed by measuring the disappearance rate from the human small intestine during steady-state perfusion of aqueous solutions through a transintestinal tube (774). It was reported that when aloxiprin and aspirin were administered orally, they had similar mean rates of excretion, but aspirin provided higher blood levels (775). Cotty *et al.* evaluated aspirin blood levels following the ingestion of commercial aspirin-containing tablets by humans (776). The absorption, distribution, and elimination of 6,7-dimethyl-4-hydroxyquinoline hydrochloride, administered orally and intravenously to animals has been studied (777), and the absorption and distribution of oxafuradene in the dog were also determined (778). By means of urinary excretion studies in man, the absorption and excretion of ^{14}C-bethanidine were measured (779). It has been suggested that the acetylated phenolic laxatives, bisacodyl and diphesatin, became absorbable from the intestines and active, pharmacologically, after deacetylation (780). Data were presented on the absorption, distribution, and elimination of *N,N'*-dimethyl-*N,N'*-bis[3-(3,4,5-trimethoxybenzoyloxy) propyl] ethylenediamine dihydrochloride (781). Another publication ascertained the absorption, excretion, and metabolic fate of ethambutol in man after oral and intravenous administration (782); the absorption, distribution, and excretion of morphocycline in rabbits were also discussed (783). Other data were compiled showing that only a limited amount of riboflavin can be absorbed from the intestinal tract (784). Another investigator carried out research on the gastrointestinal absorption and pharmacokinetics of selected compounds in man (785). The absorption of isoniazid and some of its derivatives has been noted (786). The absorption, metabolism, and elimination of thiabendazole in farm animals, along with a method for its estimation in biological materials, were presented by Tocco *et al.* (787). Peak plasma levels for sulfamethoxydiazine were found in 6–8 hr. after oral administration (788); also, a similar study by the same authors comparing the antibacterial activity and chemical levels of the same chemical in human serum and plasma was published (789). Amundson, Johnson, and Manthey determined the urinary excretion of *d*-propoxyphene hydrochloride in man (790). The absorption and elimination profile of isoproterenol in anesthetized and unanesthetized dogs has been announced (791, 792). The rate of excretion of a new antiviral agent, amantadine hydrochloride, was discovered to be first order (793). The absorption of terephthalic acid through the digestive tract and its excretion in urine of rats were examined (794). The urinary excretion of three oral cholecystographic agents, iopanoate, tyropanoate, and bunamiodyl, has been studied (795). Another investigation was concerned with the absorption of *d*,1-1-(3-hydroxyphenyl)-1-hydroxy-2-ethyl aminoeltane in the gastrointestinal tract (796).

PHARMACOGNOSY[1]

One review was published on the investigation of *Lupinus* alkaloids (797); another surveyed the physical and chemical properties of aromatic acids from crude drugs of the *Umbelliferae* family (798). Other reviews were concerned with aurones (799), a tabulation of 43 alkaloids used in modern medicine (800), recent advances in the chemistry of *Rutaceae* alkaloids (801), the present status of *Cannabis* research (802), and the application and relationship of plant tissue culture to medicinal plant study (803).

The morphine content of opium from poppy plants under cultivation has been examined by two investigators (804). In a study on the stability of erichroside from *Erysimum cheiranthoides*, it was shown that lower pH's caused hydrolysis, whereas higher pH's caused isomerization (805). Sciuchetti and Born reported on the effect of dimethylsulfoxide alone and in combination with *N*-dimethylamino succinamic acid or 2-chlorethyl trimethylammonium chloride on the growth and alkaloid biosynthesis of *Datura tatula* (806). In a study on morphine losses in poppy capsules (*Papaver somniferum*), it has been revealed that the maximum loss of morphine in freshly crushed poppy capsules occurred in the first 8 days of storage at 20° (807). A description of various rhubarb roots used in the drug trade was listed (808). An interesting article on Mexican witchcraft drugs, *Ipomoea violocea* and *Salvia divinorum*, outlining their active principles, appeared in the literature (809). The production and consumption of opium and coca derivatives

[1] The writers thank Dr. G. H. Svoboda for his suggestions concerning the preparation of this section.

TABLE I.—PHARMACOGNOSTIC INVESTIGATIONS

Plant	Ref.
A	
Abies amabilis	(817)
Acacia angustissima	(818)
Acacia confusa	(819)
Aconitum japonicum	(820)
Actinidia polygama	(821)
Aesculus hippocastanum	(822)
Afzelia xylocarpa	(823)
Alangium lamarckii	(824, 825)
Amanita muscaria	(826, 827)
Amaranthus caudatus	(828)
Amaryllidaceen species	(829, 830)
Ammi majus	(831)
Anabasis aphylla	(832)
Angelica hirsutiflora	(833)
Angelica japonica	(834, 835)
Angelica pubescens	(836)
Aniscocyclea grandidieri	(837)
Anthocleista procera	(838)
Apocynum cannabinum	(839)
Araucaria imbricata	(840)
Arctium lappa	(841)
Ardisia macrocarpa	(842)
Argyreia nervosa	(843)
Arisaema ringens	(844)
Artemisia absinthium	(845)
Artemisia taurica	(846)
Artocarpus heterophyllus	(847)
Asarum europaeum	(848)
Asclepias tuberosa	(849)
Asimira triloba	(850)
Aspidosperma dasycarpon	(851)
Aspidosperma species	(852)
Atropa belladonna	(853)
B	
Baccharis rosmarinifolia	(854)
Berberis hauniensis	(855)
Bixa orellana	(856, 857)
Boletaceae species	(858)
Bryophyllum daigremontianum	(859)
Buxus microphylla	(860)
Buxus sempervirens	(861, 862)
C	
Cassia occidentalis	(863)
Catharanthus lanceus	(864)
Catharanthus roseus	(865–867)
Ceanothus americanus	(868)
Cedrus deodara	(869)
Centaurea species	(870)
Chelidonium majus	(871)
Chionographis japonica	(872)
Chondria armata	(873, 874)
Cinnamomum species	(875, 876)
Cissampelos pareira	(837, 877)
Clausena anisata	(878)
Clerodendron infortunatum	(879)
Cochlospermum gossypiune	(880)
Convallaria keiskei	(881)
Convallaria majalis	(882)
Coptis chinensis	(883, 884)
Corydalis species	(885)
Crotalaria species	(886)
Croton cumingii	(887)
Cyclea madagascariensi	(837)
Cymbopogon mortini	(888)
Cymbopogon nardus	(889)
Cynara scolymus	(890)
D	
Dalbergia lanceolaria	(891)
Daphne genkwa	(892)
Daphne papyracea	(893)
Datura callus	(894)
Datura innoxia	(895, 896)
Datura meteloides	(897)
Datura sanguinea	(898)
Datura tatula	(899)
Daucus carota	(900)
Delphinium species	(901)
Desmodium caudatum	(902)
Digitalis ciliata	(903)
Digitalis davisiana	(904)
Digitalis lanata	(905, 906)
Digitalis purpurea	(908–913)
Digitalis species	(907)
Digitalis thapsi	(914)
Doryphora sassafras	(915)
E	
Eledone moschata	(916)
Embelia ribes	(917)
Enantia species	(918)
Erysimum canescens	(919)
Erythroxylon monogynum	(920)
Eschscholtzia californica	(921)
Eucalyptus staigeriana	(922)
Eupatorium semiserratum	(923)
F, G	
Fumaria species	(924)
Gaillardia pulchella	(925)
Genista lusitanica	(926)
Gentiana bellidifolia	(927)
Glycosmis arborea	(928)
Glycyrrhiza glabra	(929)
Gualteria psilopus	(930, 931)
Gutierrezia sarothrae	(932)
Gymnema sylvestre	(933)
H	
Heimia salicifolia	(934, 935)
Helenium thurberi	(936)
Heracleum candicans	(937)
Heracleum mantegazzianum	(938)
Hibiscus abelmoschus	(939)
Hippophae rhamnoides	(940)
Hydnellum diabolus	(941, 942)
Hyoscyamus albus	(943)
Hypericum perforatum	(944)
I, J	
Indigofera endecaphylla	(945)
Ipomoea digitata	(946)
Ipomoea operculata	(947)
Ipomoea species	(948)
Juniperus virginiana	(949)
L	
Lecidea tenebrosa	(950)
Lepidium sativum	(951, 952)
Libanotis intermedia	(953)
Limnophila rugosa	(954)
Litsea cubeba	(955)
Lophophora williamsii	(956)
Luffa operculata	(957)
Lycium halimifolium	(958)
Lycopodium species	(959)
M	
Machilus species	(960)
Mammea americana	(961)
Mangifera indica	(962)
Mentha piperita	(963)
Mentha pulegium	(964)
Mentha species	(965)
Metaplexis japonica	(966)

(*Continued on next page.*)

TABLE I.—(*Continued*)

Plant	Ref.
M	
Minthostachys verticillata	(967)
Mitragyna speciosa	(968)
Monodora myristica	(969)
N	
Neolitsea acuminatissima	(970)
Neolitsea pulchella	(971)
Nepeta ciliaris	(972)
Nicotiana glutinosa	(973)
Nuphar japonicum	(974)
Nuphar luteum	(975)
O	
Ochrosia sandwicensis	(976)
Ocimum canum	(977)
Ocimum species	(978)
P	
Papaver caucasicum	(979)
Papaver somniferum	(980–982)
Papaver species	(983, 984)
Parmelia cryptochlorophaea	(985)
Peganum harmala	(986)
Pelea christophersenii	(987)
Petasites japonicus	(988)
Peucedanum ruthenicum	(989)
Peumus boldus	(990)
Physalis alkekengi	(991)
Penellia ternate	(844)
Piper methysticum	(992)
Pirus serotina	(993)
Piscidia erythriva	(994)
Plantago asiatica	(995)
Plantago species	(996)
Podophyllum peltatum	(997)
Prunus mahaleb	(998)
Psoralea species	(999, 1000)
Pycnanthemum albescens	(1001)
R	
Rauwolfia mannii	(1002)
Rauwolfia vomitoria	(1003)
Rhamnus frangula	(1004–1006)
Rhizoma zingiberis	(1007)
Rhododendron dauricum	(1008)
Rubus idaeus	(1009)
Rudbeckia species	(870)
Rumex hymenosepalus	(1010)
Ruta graveolens	(1011, 1012)
S	
Salicornia herbacea	(1013)
Salvia officinalis	(1014)
Samadera indica	(1015)
Sapium sebiferum	(1016)
Saracococa pruniformis	(1017, 1018)
Scopolia parviflora	(853)
Securinega suffruticosa	(1019)
Shorea talura	(1020)
Similax sieboldi	(1021)
Solanum atropurpureum	(1022)
Solanum khasianum	(1023)
Sophora flavescens	(1024)
Sterculia candata	(1025)
Sterculia setigera	(1026)
Sterculia urens	(1027)
Strychnos henningsii	(1028)
Strychnos nux-vomica	(1029)
T	
Tabernaemontana laurifolia	(1030)
Talictrum simplex	(1031)
Tanacetum vulgare	(1032)
Taxus baccata	(1033)
Thalictrum minus	(1034)
Thalictrum species	(1035–1037)
Thymus serpyllum	(1038)
Thymus vulgaris	(1039, 1040)
Tinomiscium philippinense	(1041)
Torulopsis utilis	(1042)
Tribulus terrestris	(1043)
Trifolium arvense	(1044)
V	
Vaccinium bracteatum	(1045)
Valeriana procurrens	(1046)
Valeriana species	(1047)
Veratrum album	(1048)
Viburnam opulus	(1049)
Viburnum prunifolium	(1050)
Vinca major	(1051)
Vinca rosea	(1052)
Voacanga bracteata	(1053)
Voacanga globosa	(1054)
W, Y, Z	
Withania somnifera	(1055, 1056)
Yucca glauca	(1057)
Zanthoxylum hamitonianum	(1058)

in France since 1950 were summarized (810). Another report stated the growth of *Atropa belladonna* was strongly inhibited by a heavy water concentration greater than 50% (811). An alfalfa trypsin inhibitor was demonstrated to be thermally stable and also stable in the pH range 2–12 (812). Other studies have been conducted on two sulfur-containing alkaloids from Congo trees (813). The presence of aloin was discovered in *Aloe* species from the section *Anguialoe* Reynolds (814). The effect of dimethylsulfoxide and tributyl 2,4-dichlorobenzylphosphonium chloride on growth and alkaloid synthesis in *Datura ferox* has been described (815). Alkaloid artifacts were difficult to distinguish from active plant alkaloids formed in plant extracts by ammonium hydroxide and acetone (816).

Pharmacognostic Investigations.—This section of the review is primarily concerned with those references pertaining to isolation and identification of plant constituents. Table I lists alphabetically each plant studied, followed by appropriate references to the bibliography.

Methodology.—The pH of the medium and the nature of the organic solvent were the main factors affecting the extraction of alkaloids by organic solvents (1059). A comparison was carried out on turbo-, vibro-extraction, and maceration methods for the preparation of various tinctures in the "Yugoslav Pharmacopeia" II (1060). The extraction of drugs from plants

has been improved by the use of surface-active agents (1061). Laboratory experiments demonstrated the effectiveness of a domestic vibrator for obtaining extracts from various types of plant materials (1062).

Ovadia and Skauen reported that ultrasonic energy had an accelerating effect on the extraction of alkaloids (1063). The kinetics of extraction of alkaloids and other extractables from the rhizome of *Scopolia carniolica* by pressing was studied (1064). Another procedure was described for preparing rye fluid extracts containing 0.05% alkaloids (1065). The properties of clove oils produced under different conditions of distillation from the buds and stems were investigated (1066). Optimum conditions for glycoalkaloid diffusion in the *Solanum laciniatum*–sulfuric acid system were reported (1067), as well as optimum conditions for the extraction of alginic acid from seaweeds on the Saurashtra coast (1068). Various methods for obtaining deterpenated oils were reviewed in a paper with 22 references (1069). In addition, thin-layer chromatographic patterns were noted for Umbelliferous drugs and their adulterants (1070).

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[2] *Index Medicus* abbreviations were used for journals not listed in *Chemical Abstracts*.

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Research Articles

In Vivo Pharmacodynamic Evaluation of Oral Dosage Forms by Whole Body Liquid Scintillometry

By GERALD HECHT*, JOHN E. CHRISTIAN, and GILBERT S. BANKER

A large volume liquid scintillation detector was used to determine rates of excretion, absorption, and intercompartmental clearance in ambulatory dogs. A γ-emitting test substance was administered intravenously, orally in aqueous solution, and orally in sustained and delayed-release dosage forms. Unanesthetized female dogs were partially restricted, catheterized, and fasted prior to dosing. Where applicable, plots of log per cent whole body retention as a function of time were resolved into linear components following apparent first-order kinetics. The rate constants of these components were calculated and compared. Sustained-release forms were prepared which exhibited zero-order release characteristics *in vitro* and *in vivo*, and the characteristics of an enteric coated dosage form were compared after *in vitro* and *in vivo* testing. The procedure provided such parameters as absorption, excretion, release, and the effect of formulation techniques and formula variation on the biological availability of the test substance employed without the necessity of excreta and blood sampling and analysis.

THE PROBLEM of evaluating the biological availability and pharmacodynamic properties of any drug, new or old, in a new dosage form, is of increasing importance due to the greater potency and specificity of drugs, the greater complexity of dosage forms, and the increasing demands of the drug laws and new drug application requirements.

Several authors (1–9) have attempted to standardize the *in vitro* testing of various oral dosage forms, not necessarily as a means of simulating the characteristics which would be expected *in vivo*, but rather as a means of insuring control and correlation with data collected *in vivo*.

Many methods for the *in vivo* evaluation of oral dosage forms have been devised, but undoubtedly, those supplying the most pertinent information are concerned with the evaluation of absorption,

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Research Articles

In Vivo Pharmacodynamic Evaluation of Oral Dosage Forms by Whole Body Liquid Scintillometry

By GERALD HECHT*, JOHN E. CHRISTIAN, and GILBERT S. BANKER

A large volume liquid scintillation detector was used to determine rates of excretion, absorption, and intercompartmental clearance in ambulatory dogs. A γ-emitting test substance was administered intravenously, orally in aqueous solution, and orally in sustained and delayed-release dosage forms. Unanesthetized female dogs were partially restricted, catheterized, and fasted prior to dosing. Where applicable, plots of log per cent whole body retention as a function of time were resolved into linear components following apparent first-order kinetics. The rate constants of these components were calculated and compared. Sustained-release forms were prepared which exhibited zero-order release characteristics *in vitro* and *in vivo*, and the characteristics of an enteric coated dosage form were compared after *in vitro* and *in vivo* testing. The procedure provided such parameters as absorption, excretion, release, and the effect of formulation techniques and formula variation on the biological availability of the test substance employed without the necessity of excreta and blood sampling and analysis.

THE PROBLEM of evaluating the biological availability and pharmacodynamic properties of any drug, new or old, in a new dosage form, is of increasing importance due to the greater potency and specificity of drugs, the greater complexity of dosage forms, and the increasing demands of the drug laws and new drug application requirements.

Several authors (1–9) have attempted to standardize the *in vitro* testing of various oral dosage forms, not necessarily as a means of simulating the characteristics which would be expected *in vivo*, but rather as a means of insuring control and correlation with data collected *in vivo*.

Many methods for the *in vivo* evaluation of oral dosage forms have been devised, but undoubtedly, those supplying the most pertinent information are concerned with the evaluation of absorption,

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blood levels, blood clearance, and excretion rates. Heimlich *et al.* (10, 11) used urinary excretion for the calculation of the half-lives and excretion constants for phenylpropanolamine hydrochloride and trimeprazine in sustained-release dosage forms, and Wagner and Nelson (12–14) used per cent absorbed *versus* time plots to elucidate kinetic models for drug absorption.

Large volume liquid scintillation counters have been employed to measure the parameters of whole body retention and excretion of selected γ-emitting radionuclides in humans (15–20). These procedures involve administering a dose of a γ-emitting test substance and determining the whole body radioactivity at zero time and at succeeding time intervals thereafter. Data of this type have been heretofore primarily expressed as whole body retention and have not been specifically correlated to urinary excretion or rate changes in blood levels for the purpose of describing the pharmacodynamics of a drug or a dosage form. For the system studied, whole body activity appears to be an effective method of picturing total compartmentalization and absorption-distribution-excretion kinetics.

In this study, a model system was devised wherein a γ-emitting radionuclide (sodium ^{131}I-orthoiodohippurate)[1] was administered intravenously and orally in various dosage forms, for the purpose of studying by whole body liquid scintillometry the parameters of absorption, excretion, and intercompartmental clearance of the radionuclide and the effect of variations in formula composition on these parameters.

The method described in this study would be applicable to the investigation of (*a*) any drug or test substance which could be labeled with a γ-emitting radionuclide to illustrate drug pharmacodynamics, (*b*) the factors affecting dosage form release mechanisms and properties *in vivo* according to dosage form design and the physicochemical properties of the radionuclide compound, (*c*) drugs which alter the sodium and potassium content of the body.

EXPERIMENTAL

General Considerations.—Since the evaluation of oral dosage forms was of primary interest in this study, the length of time over which whole body radioactivity determinations are normally made in such an investigation (12 hr. or longer) provides certain requirements of the γ-emitting test substance. These requirements may be listed as follows. (*a*) The test substance must not be concentrated or bound in specific tissues of the body such as the thyroid gland or liver. (*b*) The test substance should be completely eliminated by the kidneys. (*c*) The excretion rate must be rapid enough to clear a major portion of the material from the body in 3 or 4 hr. or less. (*d*) The material should not be degraded in the body to release a radioactive metabolite which would confuse interpretation of the data.

Instrumental Methods.—Whole body activity was determined in the 4 π large volume liquid scintillation counter at Purdue University (21).[2] The instrument was calibrated to differentially count the γ-emissions of ^{131}-iodine having an energy of 0.364 mev. Dosages were adjusted to provide count rates of 150,000 counts per minute or less in order to avoid having to make data corrections for coincidence loss which were found to become significant at about 250,000 counts per minute.

The *in vitro* evaluation of the release of the test material from sustained and delayed-release oral dosage forms, and the evaluation of whole blood disappearance of the test substance, was conducted in a 2-in. Harshaw[3] NaI(Tl) well crystal and appropriate electronics, calibrated to differentially count the γ-emissions of ^{131}-iodine having an energy of 0.364 mev.

In vitro release tests were conducted on a rotating bottle apparatus, as described by Souder and Ellenbogen (9), at 36–38°, and at a speed of 40–45 r.p.m.

Animal Methodology.—Seven adult dogs were utilized in this study. The dogs averaged from 8–10 Kg. in weight, and from 2 to an estimated 8 years of age. The dogs were from two groups of animals, the first group consisting of three mixed breed female animals, ranging in estimated age from 2–8 years of age, and the second group consisting of four female AKC registered beagles ranging from 2–2.5 years of age. All animals were immunized for rabies, distemper, and hepatitis, and were wormed. A maximum of four animals were used in any single experiment. On the day before an experiment was conducted, each participating animal was removed from its run and was placed in a metabolism cage. All food and water were withheld for a period of 12 hr. prior to initiation of each experimental procedure.

Catheterization of the female animals to facilitate repeated bladder emptying immediately prior to each whole body radioactivity determination was accomplished using a No. 14 French scale Bardex[4] indwelling catheter, which was fixed in place immediately prior to dosing and removed immediately after the experimental period. To prevent removal of the catheter by the animal, partial restriction was necessary. To accomplish this restriction, each animal was fitted with a "girdle" formed from pieces of polyethylene sheet 1/8 in. thick, 7–8 in. wide, and as long as necessary to completely surround the thoracic and abdominal areas. The girdle was secured by a leather strip which passed in front of the forelimbs and across the chest.

The animals were dosed immediately after bladder drainage. Immediately prior to the determination of whole body activity at each subsequent interval, the bladder was drained, and rinsed with 50 ml.

[1] Hippuran-^{131}I, Volk Radiochemical Co., Chicago, Ill.

[2] The Sinco-P was converted from 2 to 4 π geometry prior to the initiation of this experimentation.

[3] Harshaw Chemical Co., Cleveland, Ohio.

[4] Davol Rubber Co., Providence, R. I.

of sterile sodium chloride injection U.S.P. The urine so collected, the rinse, and washings from the collection graduate were placed in 1-L. polyethylene bottles,[5] brought to 1-L. volume with water, and placed in the geometrical center of the whole body scintillation counter for determination of cumulative excretion values.

So that the animals would not move about unduly to produce geometry errors while in the whole body counter, they were placed in a cylindrical fiber drum[6] of 8-gal. capacity. All counts collected on a single animal at each time interval were averaged and corrected for background, decay, and variation of counter efficiency. Each urine sample was similarly treated, with the exception that decay corrections were unnecessary since the activity of a urine phantom containing an amount of test substance identical to that administered the animals was determined each time a urine sample was counted.

A time span of not less than 4 days was allowed between subsequent uses of an individual animal.

Mass Absorption Effects.—In the initial experimentation it was necessary to determine the effects of the variability of whole body mass absorption, caused by variations in geometry and body mass surrounding the gastrointestinal tract of the animal on the reproducibility of whole body count rates as measured in the Sinco-P. An insoluble sealed source of ^{131}-iodine was prepared by imbedding an ^{131}I Radiocap[7] in an epoxy resin polymer.[8] Each sealed source was tested *in vitro* in simulated gastric and intestinal juices for absence of leach-out prior to animal dosing. Periodic determinations of whole body radioactivity were made over a 24 hr. period in the whole body counter for the animals administered the sealed sources. In all cases the sealed ^{131}I source was recovered intact from the fecal material, and the urine and feces were monitored and found to be free of radioactivity.

Dosage Regimen.—The animals used were fasted, restricted, and catheterized as described earlier. After bladder drainage and rinsing, the following dosage regimen was followed. (*a*) A dose of 1.0 ml. of a sterile aqueous solution of sodium ^{131}I-*o*-iodohippurate containing 0.14 μc./ml. was injected into each of four animals *via* the cephalic vein. (*b*) A solution containing 10 mg. of nonlabeled sodium-*o*-iodohippurate[9] and approximately 0.1 μc. of sodium ^{131}I-*o*-iodohippurate was administered orally to each of four animals. In regimens 1 and 2 whole body radioactivity was determined at zero time and at scheduled intervals thereafter. Values for whole body retention and cumulative per cent excretion were plotted as a function of time. (*c*) A solution containing 10 mg. of nonlabeled sodium-*o*-iodohippurate, and from 15–22 μc. sodium ^{131}I-*o*-iodohippurate was administered orally to four animals. At scheduled intervals following this oral administration, 1 ml. of blood was removed from the cephalic vein and analyzed for per cent of administered dose per milliliter in the Harshaw NaI(Tl) well crystal described earlier. (*d*) Ten milligrams of nonlabeled sodium *o*-iodohippurate

[5] Plax Corp., Hartford, Conn.
[6] Continental Can Co., Inc., New York, N. Y.
[7] Abbott Laboratories, Oak Ridge, Tenn.
[8] Armstrong Products Co., Inc., Warsaw, Ind.
[9] Mallinckrodt Chemical Works, St. Louis, Mo.

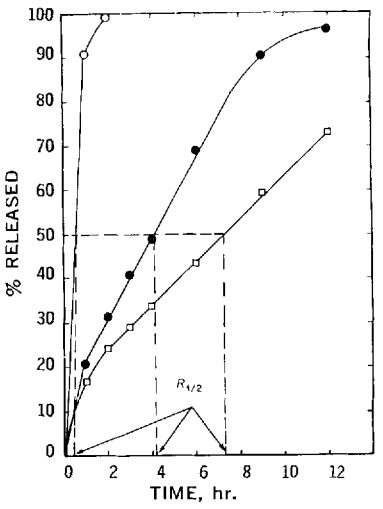


Fig. 1.—*In vitro* release, release half-times, and rate constants of sodium ^{131}I-*o*-iodohippurate contained in sustained-release oral dosage forms. Key: □, 50% polymer, $R_{1/2}$ 7.3 hr., k_0 0.082%/min.; ●, 30% polymer, $R_{1/2}$ 4.2 hr., k_0 0.168%/min.; ○, 10% polymer.

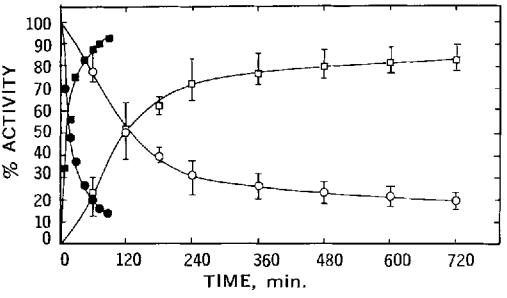


Fig. 2.—Comparison of per cent whole body retention and cumulative per cent excretion for sodium ^{131}I-*o*-iodohippurate administered orally and intravenously in aqueous solution. Key: ○, oral whole body retention; □, oral cumulative excretion; ●, intravenous whole body retention; ■, intravenous cumulative excretion.

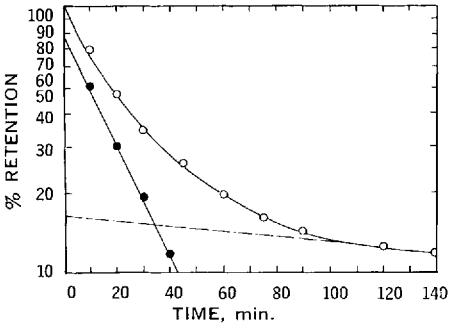


Fig. 3.—Whole body retention of sodium ^{131}I-*o*-iodohippurate administered intravenously in aqueous solution. Key: ○, whole body retention; ●, fast component; — —, slow component.

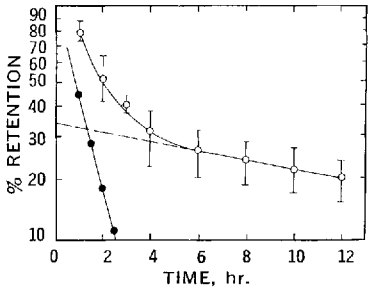


Fig. 4.—Whole body retention of sodium ^{131}I-*o*-iodohippurate administered orally in aqueous solution. Key: O, whole body retention; ●, fast component; — —, slow component.

and approximately 0.1 μc. sodium ^{131}I-*o*-iodohippurate were administered to four animals in each of two sustained-release dosage forms which exhibited zero-order release kinetics *in vitro*. (*e*) Ten milligrams of nonlabeled sodium *o*-iodohippurate and approximately 0.1 μc. of sodium ^{131}I-*o*-iodohippurate was administered to four animals in an enteric coated delayed-release dosage form.

Preparation of the Solid Dosage Form.—Sustained-release oral dosage forms of sodium ^{131}I-*o*-iodohippurate containing 0.1 μc. of ^{131}I per tablet were prepared. Three groups in all, each weighing 400 mg., were prepared containing 10, 30, and 50%, respectively, of carboxypolymethylene polymer.[10]

Care was taken to compress the tablets to the same thickness and hardness.

The *in vitro* release characteristics of these three formulas are shown in Fig. 1, with the release half times ($R_{1/2}$) and zero-order rate constants (k_0) for the 30 and the 50% formulas.

Four-hundred-milligram tablets, each containing 10 mg. of nonlabeled sodium *o*-iodohippurate and approximately 0.1 μc. of sodium ^{131}I-*o*-iodohippurate were prepared and coated with a mixture of cellulose acetate phthalate,[11] diethylphthalate,[11] and acetone. These tablets were designed to release their active principle in a delayed manner in the weakly acid or alkaline reaction of the proximal small intestine, but not in the strongly acid stomach.

When tested *in vitro* on the rotating bottle apparatus at 36–38°, the tablets prepared in this manner were seen to release less than 5% of the radioactive principle after 2.5-hr. exposure to simulated gastric juice U.S.P. without pepsin, and 100% of the radioactive principle within 15 min. after exposure to simulated intestinal fluid U.S.P. without pancreatin.

[10] Carbopol 934, B. F. Goodrich Co., Cleveland, Ohio.
[11] Eastman Organic Chemicals, Rochester, N. Y.

RESULTS AND ANALYSIS

Statistical evaluation of the data accumulated on corrected whole body activity determined intermittently during 24 hr. following administration of a sealed source of iodine-^{131}I showed the individual determinations to be within two standard deviations of the mean. These limits represent an average of plus or minus 5.45% of the mean, indicating minimal influence of mass absorption.

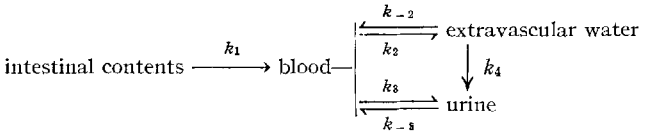
The average results of whole body retention, and cumulative excretion, of ^{131}I-*o*-iodohippurate after intravenous and oral administration in aqueous solution, are compared in Fig. 2 and plots of log per cent whole body retention as a function of time are presented and resolved into apparent first-order processes by the method of Sapirstein *et al.* (22) and Tauxe *et al.* (26) as shown in Figs. 3 and 4.

A simplified compartmental analysis of sodium ^{131}I-*o*-iodohippurate after oral administration is shown in Scheme I.

In Fig. 3, the plot for intravenous administration, the fast component A (Table I), is considered to be k_3, and the slow component B, k', a combination of k^{-2} and k_4 (the constant of tubular secretion). Figure 4 for oral administration is analyzed in the same way, designating the fast component A, k_3, and the slow component B, K' a combination of k'_{-2} and k_4.

Table I compares the apparent first-order rate constants and biological half-lives (T_B) derived from the linear components in Figs. 3 and 4.

Figure 5 shows the correlation of observed and theoretical whole body retention of sodium ^{131}I-*o*-



Scheme I

iodohippurate administered intravenously and orally in aqueous solution. The theoretical whole body retention is derived by subtracting the cumulative per cent excreted from 100%. If the theoretical per cent whole body retention perfectly matched the observed per cent whole body retention, the straight line shown in Fig. 5 would connect such points in complete agreement. The extent of departure of the points in Fig. 5 is a reflection of the departure of the observed data from the theoretical data. The data for intravenous administration can be seen to depart somewhat from complete agreement, while the data of oral administration exhibit excellent correlation.

Figure 6 illustrates the cumulative excretion, theoretical whole body retention, and whole blood disappearance following oral administration of sodium ^{131}I-*o*-iodohippurate in aqueous solution. The data can be seen to be extrapolated to the 12-hr. value for use in Fig. 7. Figure 7 is a plot of the log per cent whole body retention, and log of the per cent of the dose in the blood as a function of time. The rate constants derived from the linear components of this figure are shown in Table II. The fast component of the theoretical whole body retention curve is represented by k_3 and the slow

TABLE I.—APPARENT FIRST-ORDER RATE CONSTANTS AND BIOLOGICAL HALF-LIVES OF THE LINEAR COMPONENTS OF FIGS. 3 AND 4

Parameter	Intravenous Components A	Intravenous Components B	Oral Components A	Oral Components B
k_3	0.048 min.$^{-1}$	...	0.014 min.$^{-1}$	...
K'^a	...	0.0026 min.$^{-1}$	...	0.0011 min.$^{-1}$
T_B	14.4 min.	4.43 hr.	49.5 min.	10.5 hr.

[a] $K' = k_{-2} + k_4$.

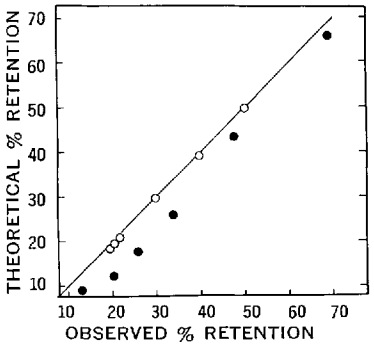


Fig. 5.—Correlation of observed and theoretical whole body retention of sodium ^{131}I-*o*-iodohippurate administered orally and intravenously in aqueous solution. Key: ○, oral; ●, intravenous.

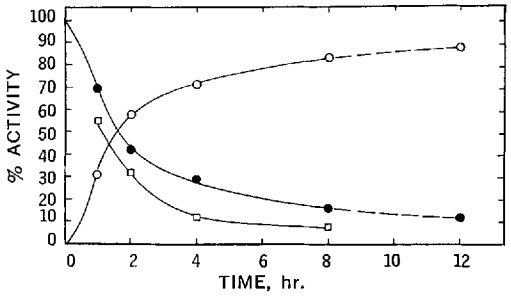


Fig. 6.—Cumulative excretion, theoretical whole body retention, and whole blood disappearance following oral administration of sodium ^{131}I-*o*-iodohippurate in aqueous solution. Key: ○, cumulative excretion; ●, theoretical retention; □, amount of dose in blood (%/ml. × 10^4).

component, $K'(k_{-2} + k_4)$. The fast component of the whole blood disappearance curve is felt to represent a macroscopic rate expressed by the constants $k_2 + k_3$. The slow component is represented by the constant, k_{-2}.

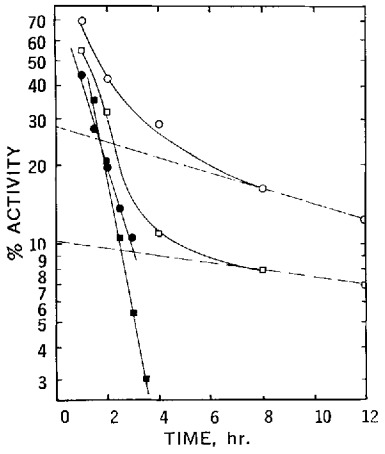


Fig. 7.—Theoretical whole body retention and whole blood disappearance of sodium ^{131}I-*o*-iodohippurate following oral administration in aqueous solution. Key: ○, whole body retention; ●, fast component; — —, slow component; □, amount of dose in blood (%/ml. × 10^4); ■, fast component; ——, slow component.

Figure 8 shows the correlation of whole body retention and whole blood disappearance. The deviation from perfect correlation noted in this figure is felt to be a reflection of the fact that whole blood disappearance is faster than excretion.

Figure 9 is a comparison of the whole body retention *versus* time plots from sodium ^{131}I-*o*-iodohippurate contained in the two sustained-release tablet formulations which exhibited two different zero-order release rates *in vitro* and the curve for sodium ^{131}I-*o*-iodohippurate in aqueous solution, and Table III presents the rate constants and half times for these systems, compared to those derived from Fig. 1. Figure 10 shows the comparison of the whole body retention *versus* time plots for sodium ^{131}I-*o*-iodohippurate contained in an enteric coated delayed-release tablet.

TABLE II.—COMPARISON OF APPARENT FIRST-ORDER RATE CONSTANTS OF WHOLE BLOOD DISAPPEARANCE AND WHOLE BODY RETENTION OF SODIUM ^{131}I-*o*-IODOHIPPURATE ADMINISTERED ORALLY IN AQUEOUS SOLUTION

	k_3	k_{-2}	K'^a	$k_2 + k_3$
Whole blood disappearance	...	0.0072 min.$^{-1}$		0.0204 min.$^{-1}$
Theoretical whole body retention	0.0120 min.$^{-1}$		0.00112 min.$^{-1}$	...
Biological half-life	58 min.	96 min.	12.5 hr.	30 min.

[a] $K' = k_{-2} + k_4$.

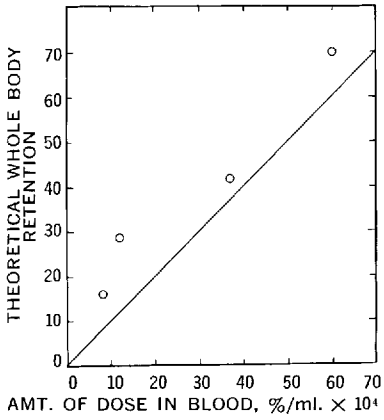


Fig. 8.—Correlation of theoretical whole body retention with the per cent of dose per milliliter of blood for sodium ^{131}I-*o*-iodohippurate administered orally in aqueous solution.

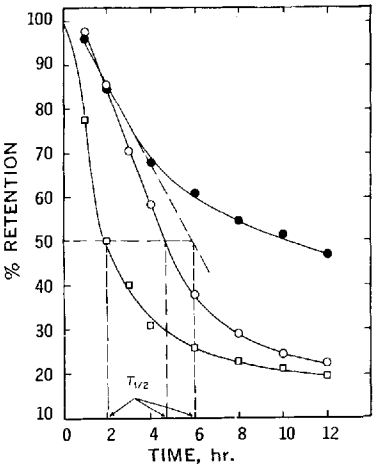


Fig. 9.—*In vivo* comparison of sodium ^{131}I-*o*-iodohippurate administered orally in aqueous and in two sustained-release dosage forms. Key: ○, 30% carboxypolymethylene polymer; ●, 50% carboxypolymethylene polymer; □, aqueous solution.

DISCUSSION

Sodium ^{131}I-*o*-iodohippurate was found to satisfactorily meet most of the requirements for a γ-emitting test substance outlined earlier. It does not concentrate in the thyroid gland or liver. It is at least 90% excreted by the kidneys.

Mathematical treatments of the excretion of substances excreted both by glomerular filtration and tubular secretion are given by Sapirstein *et al.* (22) for creatinine and by Conn *et al.* for sodium-*o*-iodohippurate.

Benzoic acid is detoxified in man by conjugation with glycine to form hippuric acid and by conjugation with glucuronic acid to form benzoyl glucuronide. Analogously, ^{131}I-orthoiodobenzoic acid forms the similar corresponding metabolites, one of which is ^{131}I-*o*-iodohippuric acid (23). Thus, it was felt that if a test substance were supplied to the body as a metabolite, degradation of the test substance would be reduced and interpretation of accumulated data would be simplified.

TABLE III.—COMPARISON OF THE *In Vivo* AND *In Vitro* ZERO-ORDER RATE CONSTANTS, RELEASE HALF TIME, AND BIOLOGICAL HALF-LIVES OF SODIUM ^{131}I-*o*-IODOHIPPURATE IN SUSTAINED-RELEASE DOSAGE FORMS

	30% Carboxypolymethylene Polymer	50% Carboxypolymethylene Polymer
In vitro k_0	0.168%/min.	0.082%/min.
In vivo k_0	0.227%/min.	0.147%/min.
Apparent $T_{1/2}$[a]	4.65 hr.	6.0 hr.
T_B[b]	3.60 hr.	5.7 hr.
$R_{1/2}$[c]	4.20 hr.	7.3 hr.

[a] Determined from 50% whole body retention values in Fig. 9. [b] Calculated from the expression for the half life of a zero-order process. $T_{1/2} = 1/2\ Co/k_0$. [c] Determined from 50% released values in Fig. 1.

It is possible that when administered intravenously, a certain amount of sodium ^{131}I-*o*-iodobenzoylglycine may be converted to the glucuronide, and further it is possible that on oral administration the iodobenzoic acid moiety may be liberated from its glycine conjugate by the effect of intestinal enzymes, then absorbed and detoxified by conjugation with glucuronic acid or glycine.

Regardless of the metabolic pathway followed by sodium ^{131}I-*o*-iodohippurate on oral administration, it has been observed that reproducible values for the fast and slow components of a plot of log per cent whole body retention as a function of time are readily obtainable.

It will be noted from Table II that the rate constant (k_3) for the fast component upon intravenous administration of sodium ^{131}I-*o*-iodohippurate was 0.048 min.$^{-1}$, while the k_3 value for sodium ^{131}I-*o*-iodohippurate administered orally in aqueous solution was 0.0143 min.$^{-1}$. If the k_3 obtained after intravenous administration is considered to be the renal excretion rate constant, then the k_3 obtained after oral administration, which is approximately one-third of the value of the intravenous k_3, must be the rate constant for some rate-limiting process in the passage of sodium ^{131}I-*o*-iodohippurate from the gastrointestinal tract to the urine. This rate-limiting step is hypothesized to be the intestinal absorption rate of sodium ^{131}I-orthoiodohippurate. This hypothesis is supported, but not confirmed, by the fact that the whole body clearance becomes a zero-order process when sodium ^{131}I-*o*-iodohippurate is administered orally in a sustained-release dosage form which exhibits zero-order release kinetics on *in vitro* testing.

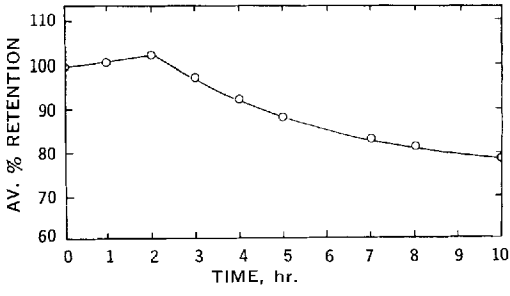


Fig. 10.—*In vivo* whole body retention of sodium ^{131}I-*o*-iodohippurate administered orally in a delayed-release dosage form.

It will be noted from Fig. 9 that contrary to the characteristics observed for the sustained-action dosage forms *in vitro* in Fig. 1, the zero-order elimination begins to deviate from linearity at 4–5 hr. after dosage. This deviation may be due to decreased biological availability of sodium ^{131}I-*o*-iodohippurate. This may occur due to enzymatic degradation of the compound to form one which is not readily absorbed, due to liberation of free ^{131}-iodine which would concentrate in the thyroid gland, or more likely may occur due to the pH of the environment being such that the compound is ionized and thus not readily absorbable. This phenomenon may also be observed from Fig. 10 where only about 22% of the administered dose was excreted over a 10-hr. period.

It has been demonstrated that the property of biological availability of a test substance, a parameter most important in dosage design and formulation, can be readily evaluated by the whole body monitoring of the γ-emissions of the test substance. An insight into *in vivo* release rates from sustained and delayed-release oral dosage forms is gained along with the effect of these dosage forms on the biological availability of the test substance in the body. Furthermore, specific information can be gained on the effect of the dosage form on absorption, excretion, and distribution of the test substance in the body. In addition, in the test substance under study, whole body retention of the radionuclide could be correlated very well to the excretion rate of the drug orally administered and fairly well to drug intravenously administered. The whole blood disappearance rate of the radionuclide could also be correlated to whole body retention as determined by the whole body scintillometry method described.

The added advantage of simplicity of animal and sample handling is also evident. Since urine samples in the larger animals must be accumulated on the short term basis by catheterization, the excretion of the test substance can be evaluated after urine drainage in as little as 3 min. without the necessity for further urinalysis. This is accomplished by determining the per cent whole body retention in a large volume liquid scintillation detector. Sinco-P, the whole body scintillation counter at Purdue University, was used in this study, but smaller and less expensive units are available which will readily accommodate a dog the size of a 20-lb. beagle (24, 25).

SUMMARY AND CONCLUSIONS

The use of the large volume liquid scintillation counter at Purdue University was investigated for its applicability to the evaluation of the pharmacodynamic properties of selected drugs and oral dosage forms. Use of data accumulated on the whole body retention of ^{131}I-orthoiodohippurate sodium was illustrated. Semilog plots of per cent whole body retention as a function of time were constructed and analyzed on the basis of first-order kinetics. Data obtained from *in vivo* analysis were compared with *in vitro* data for sodium ^{131}I-*o*-iodohippurate in sustained and delayed-release oral dosage forms. These studies indicated the following.

1. A large volume liquid scintillation counter is useful in obtaining information on the pharmacodynamic properties of a test substance in oral dosage form.

2. Whole body retention data may be readily correlated to cumulative excretion rates and blood disappearance rates.

3. Meaningful rate constants are obtainable from semilogarithmic plots of whole body retention as a function of time.

4. The biological availability of a test substance may be readily determined from whole body retention data.

5. Rates of release of a test substance in sustained or delayed-release dosage forms may be obtained *in vivo* without the necessity of blood and urine analysis.

6. Sodium ^{131}I-*o*-iodohippurate was found to be a suitable γ-emitting radioactive test substance.

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Hemolysis of Erythrocytes by Antibacterial Preservatives III

Influence of Dimethyl Sulfoxide on the Hemolytic Activity of Phenol

By HOWARD C. ANSEL and WILLIAM F. LEAKE*

The influence of dimethyl sulfoxide (DMSO) on the hemolytic activity of phenol has been investigated *in vitro*. Although DMSO freely penetrates the erythrocytic membrane and, in high concentrations, is destructive to red cells, it did not enhance but greatly reduced phenol-induced hemolysis of both washed and unwashed erythrocytes. This interference may be manifest through chemical interaction with phenol or through a direct cellular effect.

DIMETHYL sulfoxide (DMSO) is currently the subject of numerous reports and investigations directed toward determining its efficacy as a pharmaceutical adjunct and therapeutic agent. In a review of the medicinal and pharmaceutical aspects of DMSO, Block (1) has collated preliminary reports that indicate the varied potential of DMSO as a primary pharmacologic agent in producing analgesia, tranquility, diuresis, and reduced inflammation.

DMSO has been the subject of conflicting reports concerning its ability to act as a penetrant carrier of drugs through biologic membranes. Some investigators (2–4) attribute greatly increased drug penetration to the presence of DMSO whereas others (5, 6) report effects much the same as those produced by more common vehicles and experimental controls.

DMSO has been employed as a solvent in certain biochemical procedures (3, 7) and as an endocellular cryophylactic agent for the protection of cells, including erythrocytes, and tissues against freeze damage during low-temperature preservation (3, 7, 8–14).

The present investigation represents a continuation of studies (15–17) to determine the hemolytic activity of antibacterial preservatives alone and in the presence of pharmaceutical adjuncts. The controversial reputation of DMSO as a penetrant carrier of drugs prompted the question of whether its presence would affect the hemolytic activity of an antibacterial preservative. The hemolytic activity of certain antibacterial preservatives including phenol has been shown to be indicative of their antimicrobial activity (15). An alteration in the hemolytic response of erythrocytes to phenol in the presence of DMSO would be suggestive of a like alteration in antimicrobial activity. This information would be useful should future pharmaceuticals containing DMSO as a solvent, penetrant carrier, or primary pharmacologic agent require the presence of an antibacterial preservative. Phenol was selected for the present study since its hemolytic activity has recently been characterized in this laboratory (15, 16).

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EXPERIMENTAL

Materials.—Dimethyl sulfoxide was experimental drug grade (Crown Zellerbach Corp., Camas, Wash.). Phenol and sodium chloride were reagent grade.

Blood Samples.—Rabbit blood, obtained by heart puncture in the manner described by Grosicki and Husa (18), was used throughout this study. Each blood sample was collected just prior to its use and was verified for osmotic normalcy during the course of each experiment (15).

Quantitative Determination of Per Cent Hemolysis.—The colorimetric method employed for the determination of the degree of hemolysis occurring in each test solution has recently been described (15). In brief, it involved the addition of 0.05 ml. of defibrinated blood to duplicate pairs of colorimeter tubes each containing 5 ml. of test solution. The test mixtures were incubated in a water bath for 45 min. at 37° after which the unhemolyzed cells were settled by centrifugation and the absorbance readings of the hemolysate determined with a Klett-Summerson photoelectric colorimeter. Each absorbance reading was compared with a total hemolysis reading obtained by laking red cells in distilled water. The degree of hemolysis occurring in each test solution was calculated as a per cent of total hemolysis. The data reported represent the average of a minimum of two like experiments.

During and after the 45-min. incubation period, each test mixture was macroscopically observed for color changes, precipitation, and other signs

of denaturation. Throughout the investigation signs of blood denaturation were observed in solutions of high DMSO concentration. The following experiment was conducted to determine which test solutions affected hemoglobin such that colorimetric determinations no longer remained an accurate measure of degree of hemolysis. Washed blood cells were laked in distilled water, and like amounts of the resulting hemoglobin solution added to 5-ml. volumes of water, to individual solutions of phenol, sodium chloride, and DMSO, and to combinations of these ingredients as employed in the hemolysis experiments. The absorbance of each test solution was determined prior to and after the addition of hemoglobin solution and the final readings adjusted by subtracting the blank readings. Variations in the final readings were indicative of alterations in the hemoglobin. Each solution was also macroscopically examined for discoloration and precipitation.

Experimental Solutions.—Aqueous solutions were employed throughout the investigation. Concentrations of DMSO are expressed as per cent v/v and phenol and sodium chloride as per cent w/v.

Solutions of DMSO ranging in concentration from 0.5 to 99.5% were prepared and their absorbance readings determined. These readings served as blank readings that were subtracted from the colorimetric readings of subsequent hemolysis experiments.

Solutions containing 0.5 to 90% DMSO in 0.6% sodium chloride were similarly handled. The sodium chloride was added to provide protection to the erythrocytes against osmotic hemolysis. Hemolysis which occurred could be attributed to the presence of DMSO.

The primary objective of this investigation was to determine the influence of DMSO on the hemolytic activity of phenol. Previous studies (16) showed that phenol in the presence of 0.6% sodium chloride induced trace hemolysis of unwashed rabbit erythrocytes at approximately 0.41% phenol concentration and caused total hemolysis when present at 0.47% concentration. In the present study 0.44% phenol was employed. At this concentration 50 to 90% hemolysis usually occurs and the effect of added DMSO, whether it be increased or decreased hemolysis, could be readily detected. Hemolysis studies were conducted on test solutions containing 0.6% sodium chloride, 0.44% phenol, and concentrations of DMSO varying from 0.5 to 80%. Solutions containing sodium chloride and phenol but not DMSO served as the controls.

Similar experiments utilizing washed red blood cells were conducted to reveal the influence of serum on the DMSO–phenol activity. Erythrocytes separated from defibrinated blood by centrifugation were washed approximately 5 times with 0.6% sodium chloride until the washings tested free of protein to 0.5 *N* mercuric chloride T.S. After the final washing, the cells were resuspended in 0.6% sodium chloride to the approximate volume of the original blood sample. The cell suspension was employed in the same manner as the unwashed blood samples.

Kinetic Studies.—The experimental design of this investigation was such that the hemolytic effect of the various test solutions was determined after a constant 45-min. incubation period. Test solutions that were fundamental to the experiments or in which the erythrocytes responded diversely were selected for a time study. The hemolytic activity of these solutions was determined at intervals of from 5 to 15 min. during a period of 60 min.

RESULTS AND DISCUSSION

DMSO Solutions.—Erythrocytes incubated in aqueous DMSO solution ranging in concentration from 0.5 to 25% completely hemolyzed (Fig. 1). The color of the hemolysate appeared normal and there were no macroscopic signs of blood denaturation. Thirty and 40% DMSO solutions also induced total hemolysis; however, the resulting hemoglobin solutions were slightly turbid. At 50 and 60% DMSO concentrations, the hemolysate was no longer red but amber. At 80% DMSO concentration the test mixtures contained bulky brown-colored sediments with no sign of intact red cells or hemoglobin.

DMSO is not capable of maintaining the integrity of erythrocytes, for it permeates the red cell membrane (19) and allows the influx of water and the hemolytic consequence. To prevent the osmotic hemolysis of erythrocytes, the presence of an extracellular ingredient to which the red-cell membrane is impermeable is required. It should be noted that in the successful preservation of blood against freeze damage, the optimal concentration of DMSO is 15 to 20% (20) and an agent that contributes to the tonicity of the solution is added (10, 19).

The deleterious effect of high concentrations of DMSO on blood was noticed throughout this investigation. The discoloration of blood, the flocculation of its components, and the precipitation of hemoglobin were observed consistently in test solutions containing more than 40% DMSO.

In a toxicologic study of DMSO, conducted to determine the feasibility of employing the material as the vehicle during the intravenous administration of water-insoluble antitumor agents, Willson *et al.* (21) attributed perivascular inflammatory reactions and intravascular thrombi in dogs to the use of undiluted DMSO injections. They found hemolytic anemia to occur in both rats and dogs subjected to repeated intravenous injections of DMSO. Both hematuria and hemoglobinuria were also noted. The latter claimed to be the result of intravascular hemolysis.

Similar observations were reported by DiStefano and Klahn (22) who studied the hematologic effects of DMSO on cat blood *in vivo* and *in vitro*. Their work indicated that DMSO is a potent hemolytic agent through direct action on the blood cells and that the degree of dilution of DMSO is an important determinant of its hemolytic activity.

DMSO–NaCl Solutions.—The presence of 0.6% sodium chloride protected erythrocytes against osmotic hemolysis in solutions containing less than 20% DMSO (Fig. 1). Trace hemolysis was detected in saline solutions containing 25% DMSO, and 25% hemolysis occurred when 40% DMSO was present. In sodium chloride solutions containing 50% DMSO and greater there was no evidence of intact cells but rather a brown flocculant sediment and a green amber-colored supernatant liquid. The results show that although sodium chloride is

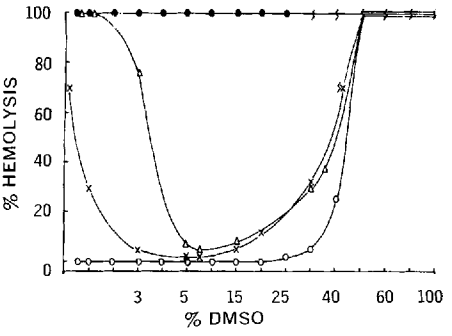

Fig. 1.—Hemolytic activity of solutions containing DMSO. Key: ●, aqueous DMSO; △, DMSO plus 0.6% NaCl and 0.44% phenol using washed erythrocytes; ×, DMSO plus 0.6% NaCl and 0.44% phenol using defibrinated blood; ○, DMSO plus 0.6% NaCl; ǂ, denaturation as color changes and/or flocculation.

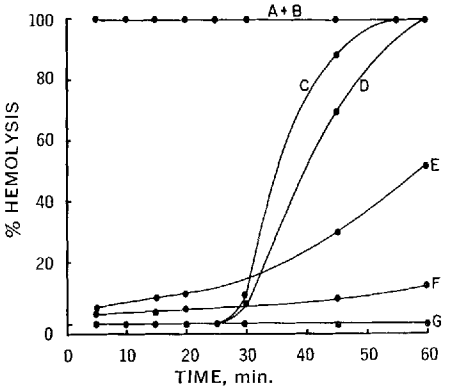

Fig. 2.—Kinetic study of the hemolytic activity of various test solutions. Key: A, distilled water; B, 30% DMSO; C, 0.6% NaCl plus 0.44% phenol; D, 0.6% NaCl plus 0.44% phenol and 0.5% DMSO; E, 0.6% NaCl plus 0.44% phenol and 30% DMSO; F, 0.6% NaCl plus 30% DMSO; G, 0.6% NaCl.

capable of preventing osmotic hemolysis in solutions of less than 20% DMSO strength, it is incapable of preventing the cytotoxic effects of higher concentrations of DMSO.

DMSO-NaCl-Phenol Solutions.—Figure 1 shows the influence of varying concentrations of DMSO on the hemolytic activity of 0.44% phenol in 0.6% sodium chloride. It should be noted that in the absence of DMSO, the phenol–salt solution induced 70% hemolysis of the erythrocytes. With the phenol and sodium chloride concentrations held constant, the addition of DMSO altered the hemolytic response. As the DMSO concentration was increased to 7%, phenol-induced hemolysis was virtually eliminated. With greater concentrations of DMSO, hemolysis increased steadily until denaturation ensued at 40% DMSO concentration and prevented further quantitation. At that concentration a red flocculant sediment was observed, indicating that a portion of the hemolysate was no longer in solution. At concentrations of DMSO exceeding 50%, the sediment was the often-seen brown flocculant material underlying a green-amber supernatant liquid.

Hemoglobin solution, prepared by lysing washed erythrocytes in distilled water, was increasingly discolored and precipitated by all test solutions containing DMSO in concentrations of 40% and greater; therefore, in experiments employing DMSO in high concentrations the colorimetric readings were no longer an accurate indication of the degree of hemolysis, for the hemoglobin released by laked cells was at least partially precipitated and colorimetrically undetectable.

Washed Erythrocytes.—Under the conditions of this study, erythrocytes were found to be more easily hemolyzed by phenol in the absence of blood serum. A standard test solution of 0.44% phenol in 0.6% sodium chloride consistently induced complete hemolysis of washed erythrocytes after 45 min., whereas an average of 70% of the unwashed cells lysed in one series of experiments (Fig. 1) and 90% in another (Fig. 2). The greater degree of lysis with washed cells could be attributed to the absence of the protective action of the serum proteins and to the increased fragility of the erythrocytes caused by the repeated washings. The influence of DMSO on phenol-induced hemolysis, however, followed a similar pattern for both the washed and unwashed red blood cells (Fig. 1). As the DMSO concentration in the phenol–salt test solution was increased to 7%, hemolysis decreased from complete hemolysis to about 5% hemolysis. As the DMSO concentration was further increased, hemolysis increased until denaturation occurred at 40% DMSO concentration.

Interpretation of Results.—The data seem to suggest the biologic inactivation of phenol as part of a chemical complex with DMSO or by virtue of increased cellular resistance to its hemolytic capabilities.

The observation that antimicrobial agents are inactivated by various chemical agents resulting in a loss of antimicrobial and hemolytic activities is not new (16, 23–28). Although phenol and DMSO have been shown (14) to react under certain laboratory conditions (dissimilar to those of the present study) the addition of small amounts of DMSO to solutions of a number of other organic chemicals has produced evidence of complex formation (3).

In a separate investigation (29) involving the hemolytic activity of the antimicrobial agent chlorhexidine diacetate, DMSO had an influence on hemolysis similar to that shown with phenol. Chlorhexidine diacetate was employed in hemolytic concentrations, as phenol had been, in the presence of 0.6% sodium chloride and varying amounts of DMSO. Curiously, the hemolytic activity of chlorhexidine diacetate was decreased with increasing amounts of DMSO with minimal hemolysis occurring in the presence of 9% DMSO; greater concentrations of DMSO resulted in increased hemolysis.

The possibility of a direct influence of DMSO on the erythrocyte rendering the phenol ineffective in its hemolytic activities was explored. In pursuing the premise that DMSO was directly affecting the red cell membrane, experiments were designed in which washed erythrocytes were incubated in saline and varying amounts of DMSO with the intention of later removing the DMSO and subjecting the cells

to phenol to determine whether the cells were permanently altered by the DMSO pretreatment. Erythrocytes were incubated with 1, 7, 20, and 30% concentrations of DMSO. Attempts to remove the DMSO by washing the cells with 0.6% sodium chloride were unsuccessful. The red cells pretreated with 20 and 30% DMSO solutions completely hemolyzed on the first attempted washing. Appreciable hemolysis occurred in the 7% DMSO pretreated cell sample and trace hemolysis in the 1% DMSO pretreated cells as washing was attempted. The same washing fluid was efficient in the concurrent washing of the control cells subjected to the same conditions except for the exposure to DMSO. In blood preservation work, the use of DMSO as an endocellular cryophylactic agent has stimulated investigators to seek an effective method of removing DMSO from within the cells prior to transfusion (20). As in the present work, efforts to wash the cells free of DMSO have resulted in hemolysis (20). It has been found possible to dialyze erythrocytes free of DMSO, but the required dialysis time has been found prohibitive to practical application (20).

Kinetic Studies.—As can be seen in Fig. 2, both distilled water and 30% DMSO induced total hemolysis within the first 5 min. of the experiment. Hemolysis occurring in the 30% DMSO test solutions can be largely attributed to osmotic hemolysis resulting from the penetration of this hygroscopic material (3, 14) through the erythrocytic membrane (19). It is not unlikely, however, that the high concentration of DMSO also exerted damaging changes to the cell structure.

The addition of 0.6% sodium chloride to 30% DMSO reduced the hemolytic effect of the latter such that at the conclusion of the experiment, only 12% hemolysis had occurred. Since 0.6% sodium chloride is sufficient to prevent osmotic hemolysis, the hemolysis that did occur can be attributed to the cytotoxic activity of DMSO.

Phenol at a concentration of 0.44% in the presence of 0.6% sodium chloride induced trace hemolysis after 25 min. of incubation and total hemolysis after 55 min. The inclusion of 0.5% DMSO to the phenol–sodium chloride solution slightly decreased the rate of hemolysis. The inclusion of 30% DMSO to the phenol–sodium chloride solution prompted an initial hemolytic response that progressed steadily throughout the duration of the experiment. At the conclusion of the study, however, only about half of the erythrocytes were lysed in contrast to total hemolysis which occurred in the other solutions containing phenol.

In summary, DMSO has been shown to interfere with the hemolytic activity of phenol *in vitro*. This interference may be manifest through chemical interaction or through a direct cellular effect. It would be interesting to investigate the antimicrobial activity of phenol and other preservatives in the presence of DMSO. Such a study would further elucidate the analytical value of erythrocytes in predicting microbial responses to preservatives. It would also provide useful information relative to the preservation of pharmaceuticals containing DMSO.

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Use of a Precision Coagulation Timer in the Biological Assay of Thrombin

By H. PATRICK FLETCHER, RICHARD J. BURNHAM, ROBERT J. COLE, and NORRIS W. DUNHAM

A new *in vitro* assay for the determination of thrombin potency has been studied using a precision coagulation timer. This instrument showed a more objective and quantitative clot-time determination of the human and bovine plasmas. The 2 × 2 and 3 × 3 assay designs were employed utilizing the dose-response relationship established between thrombin and the plasma. The differences between the present and proposed methods and between plasma of the two species are discussed.

THE PRESENT method used for the assay of thrombin is essentially the same as that published by the Federal Security Agency, National Institutes of Health, Bethesda, Md., 1946. In this method, two dilutions of unknown bovine thrombin are chosen so that one has a slightly higher and the other a slightly lower clotting time than a dilution of N.I.H. standard thrombin. The potencies (units/ml.) of the unknown thrombin dilutions, which are based on their assumed potencies, are plotted with the clotting times and these points are connected by a straight line. A point on this line which corresponds to clotting time of the standard thrombin is determined. A ratio is set up by comparing the potency corresponding to this point with the assigned potency of the standard. The potency of the unknown thrombin is obtained by multiplying the assumed potency by the ratio, as follows:

$$\frac{\text{assigned potency (of standard) units/ml.}}{\text{obtained potency (of standard) units/ml.}} \times \text{assumed potency (of unknown)} = \text{potency of unknown}$$

The clotting times in this method are visually determined by observing the clot in a small test tube after the plasma has been added to the thrombin and the clotting time is recorded by using a stop watch. During the observation time, the test tube is tilted once every second until coagulation occurs.

Due to its simplicity, this assay can be done rapidly. However, the dependence on subjective judgment for the end point determination and a complete lack of any statistical validity tests appear as serious disadvantages of this method. Thus, it was the purpose of this study to attempt the automation of the end point determination and to design a new assay in which the procedure will have to pass at least one validity test.

In this study we used a precision coagulation timer.[1] This instrument is being used by many clinical laboratories for the determination of prothrombin times.

For the determination of the clotting times, a plastic cup[2] containing 0.2 ml. of plasma is placed in the reaction well and the timer is automatically started when 0.1 ml. of thrombin is delivered from a pipet. After 1.5 sec. delay, the probe arm inserts the two electrodes into the plasma–thrombin mixture. The electrodes consist of a stationary electrode and a moving electrode which moves in and out of the plasma–thrombin mixture. When the moving electrode has risen above the reaction mixture, it reaches a contact point which would complete the circuit except for the gap between the two electrodes. Thus, when thrombin has converted the fibrinogen to fibrin, the latter is partially pulled out of the reaction mixture by the moving electrode and completes the electrical circuit between the electrodes. This electrical short stops the timer and the clotting time is recorded. The clotting times are obtained at 37° since the instrument is equipped with heating blocks which maintain this constant temperature.

The moving electrode completes a cycle (in and out of the reaction mixture) every 0.5 sec. Since the circuit can only be completed when the moving electrode has moved out of the reaction mixture, the clotting time end point can occur in an interval which is one-half of a cycle. Thus, in 1 sec. there are two intervals of approximately 0.25 sec. duration in which the end point can be detected. This means that the exact clotting time is not always given to the tenth of a second as the timer indicates. However, since this is a constant error, and it is the relative clotting times which are important in the biological assay, the clotting times to the tenth of a second as shown on the timer are used for the assay calculations.

Although there is some chance for error in the end point determination, this instrument has the advantage of a more objectively determined clot

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[1] Fibrometer, Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.
[2] Supplied with instrument.

time than the method now being used. It is anticipated that this increased objectivity will reduce the variation in obtained potencies between operators.

EXPERIMENTAL

Assay.—For the 2 × 2 assay, thrombin dilutions of 5.64 and 8.46 units/ml. (a log 1.5 interval) were prepared for both standard and production material. The dilutions were made up in 10-ml. volumetric flasks with normal saline. The individual dilutions were numbered so that a random order could be used for the collecton of data. For the 3 × 3 assay design, 5.64, 7.05, and 8.81 units/ml. dilutions of thrombin were used (a log 1.25 interval). The dilutions for unknown thrombin were based on their assumed potencies. The 50% plasma solutions were prepared by adding 5 ml. of distilled water and 5 ml. of normal saline to a vial containing 5 ml. of lyophilized human plasma.[3]

Two tenths of a milliliter of plasma solution was delivered to the plastic cup by using the autopipet. One rack of cups containing plasma was placed in the heating block. At the same time, the appropriately numbered cups containing the thrombin dilutions were also placed in the heating block. At the end of the 3-min. warm-up period, one of the cups containing plasma was placed in the well directly beneath the electrodes. One-tenth milliliter (0.1) of the thrombin solution was delivered to the plasma *via* the autopipet. The autopipet activates the timer when the plunger is depressed and the switch is in the "on" position. When coagulation occurs, the timer automatically stops, and the time of the end point is recorded.

The data for this assay were gathered as quickly as possible due to the effect of the 37° temperature on the thrombin and plasma. According to the manufacturer, plasma begins to decompose after 15 min. at this temperature. In this study it was found that effects on the plasma are insignificant if the plasma was used within 6 to 7 min. when at 37°.

The data were arranged under the appropriate dilutions (six determinations per dilution) and the clotting times in seconds were converted to logs. The logs are treated as numbers rather than as exponents in the computations of the statistics of the assay. In this laboratory the computations were carried out by an IBM 1620 computer. The computations for this 2 × 2 assay were described by Bliss (1).

Dose-Response Curve.—In order to design a new assay, the dose-response relationship was determined. Since there are many unknown variables in plasma coagulation, several dose-response curves were determined using both human and bovine plasmas and also for N.I.H. standard and production thrombins.

Although the curves shifted slightly from day to day, the shapes of the curves were the same even for the plasmas of the two different species. The dose-response relationship is a curved line. Examination of these lines led to the selection of the middle part of the curve for the assay work. The dose-response relationships in this area were somewhat linear and the sensitivity of the response in this area was more suitable for assay work. The linearity of this part of the dose-response curve was confirmed when orthogonal analysis of curvature was not significant in this area. However, the variance due to curvature was significant for the entire dose-response curve, and curvature still remained a problem in many of the assays. However, this problem was partly solved by plotting log dose *versus* log response as will be discussed later.

From the dose-response curve study it was determined that the dilutions of thrombin which resulted in clotting times between 10–22 sec. were useful dilutions for assay purposes. This useful area of the curve was determined for the different types of plasma used, *e.g.*, bovine, human plasma[3] half-strength, human plasma full-strength.

Statistical Design of Assay.—At the outset of this study it was felt that a statistical design was needed which would allow the operator to establish limits for the assay. At the beginning of this study the statistical design used for the three-dose balanced assay of corticotropin injection U.S.P. XVII (referred to as the 3 × 3 assay) was chosen since it contained four different validity tests. The present assay method contains no validity tests. Although the validity tests in the 3 × 3 assay design are far from perfect, they do serve as a source of confidence for the operator. The passing of these validity tests depends upon the "F" values obtained by dividing the mean squares resulting from deviation from parallelism, curvature, differences in curvature, and the combination of these three by the error variance. The weakness in these validity tests is that a high error variance can increase the probability that the validity test will be passed. Thus, it is up to the operator and the laboratory to limit the error variance which will be accepted. This may also be accomplished by placing limits on the "L" value. All of the computations for the statistical designs used in the study were programmed for the IBM 1620 computor.

RESULTS AND DISCUSSION

It became apparent in the early part of this study that temperature had an effect on the clotting times when the thrombin solutions remained at 37°. Thus, it was important that each dilution of thrombin be kept standing at 37° for the same length of time prior to clotting time determination. In order to insure this, tubes containing the dilutions of thrombin should be placed in the heating block at the same time. After the 3-min. warm-up period, the clotting times for the different thrombin solutions are determined using a random order design. In this design for the collection of data, the effect of standing at 37° will be distributed over the different dilutions.

For the 3 × 3 assay, the responses (clot times) are statistically compared to the log dose for the standard and unknown thrombin dilutions. In this assay, we were using a statistical design which was intended for a linear dose-response relationship for data which possessed a curved dose-response relationship. Therefore, several of the assays failed the curvature validity test. A log × log transformation of the data was attempted in order to obtain a straight line.

The portion of the dose-response curve in which we were working became more linear when log dose was plotted against log response (Fig. 1.) Another

[3] Normal Plasma Control (5-ml. size), Baltimore Biological Laboratory, Division of B-D Laboratories, Inc., Baltimore, Md.

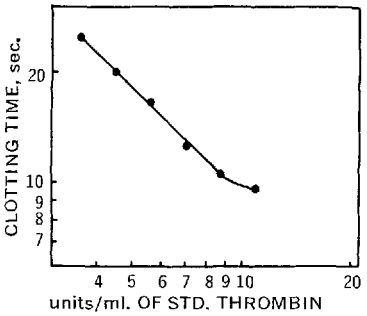

Fig. 1.—Log × log dose-response curve for N.I.H. standard thrombin and human plasma. Each point represents mean of six clotting times.

advantage of using the log × log transformation is that it tends to decrease the differences in the response variation which were often noted between the long and short clotting times.

A study to determine whether the log × log transformation reduced the sensitivity of the validity tests was conducted by intentionally making increased "errors" in one of the test dilutions in consecutive assays. The effect of these increased "errors" on the mean squares resulting from deviation from parallelism, curvature, and difference in curvature was observed and a comparison was made between semilog and log × log designs.

The results of this comparison indicated that the log × log transformation did not make the assay less sensitive than the semilog assay. The assays failed and passed in the same instances. The mean squares resulting from deviation from parallelism were increased in the assays where intentional errors were made in dilutions.

A 2 × 2 (log × log) assay (two standard and two unknown dilutions) was also used in this study and is now being used in place of the 3 × 3 design. This 2 × 2 design is thought to be more practical for routine work since it requires less time and reduces the amount of time the thrombin is kept standing at 37°.

The 2 × 2 assay has only one validity test to pass and this is deviation from parallelism. This is one of the more important validity tests since the log × log transformation reduced the problem of curvature. It was also observed that the potencies of 2 × 2 assays differed very little (0.5 to 1%) from the potencies obtained with the 3 × 3 design.

Results of many assays have shown that bovine plasma at 37° gave a high error variance. Analysis of the row effect, which represented the effect of time at 37°, revealed that the variance resulting from time was significant. It is probable that after a short time, the bovine plasma was breaking down at 37°. This temperature effect was not present when human plasma was used in the same time interval and at the same temperature. It was also revealed that in assays using human plasma, the thrombin was more efficient when the 0.2 ml. of plasma solution consisted of 50% plasma rather than 100% plasma. More thrombin was required by the 100% plasma to obtain the same clotting time as the 50% plasma. Apparently the optimal substrate concentration is closer to the 50% plasma solution than to the 100% solution.

The statistical data indicated that there was no

TABLE I.—COMPARISON OF POTENCIES OBTAINED WITH PRESENT AND FIBROMETER METHODS

Lots	Potencies, units/Vial — Present Method[a] Bovine Plasma	Present Method[a] Human Plasma	Proposed Method Bovine Plasma	Proposed Method Human Plasma
A	1130	1053	1436	1280
B	1293	1210	1612	1335
C	1115	1088	1378	1190
D	5113	4825	5665	5350
E	5538	5225	6302	6005

[a] 3 × 3 semilog assay design used.

TABLE II.—COMPARISON OF RESULTS FROM THE DIFFERENT METHODS FOR THE CALCULATION OF POTENCY

Lots	Potencies, units/Vial — Proposed Method[a]	Point Assay Proposed Method	Point Assay Present Method[b]
A	1271	1227	1130
B	1333	1263	1293
C	1191	1271	1115
D	5440	5230	5113
E	6000	5665	5538

[a] 2 × 2 (log × log) assay design used with human plasma.
[b] Bovine plasma used.

advantage in using 100% plasma solution rather than 50% solutions. The error variance remained about the same in both instances.

Samples from five production lots of thrombin, three 1000-units/vial and two 5000-units/vial, were assayed with bovine and human plasma solutions. These results were compared with the results obtained using the present N.I.H. method. The result of this study appears in Table I. Examination of these data reveals that the thrombin was more potent when assayed with bovine plasma than with human plasma. It was observed that the precision coagulation timer method employing human plasma at 37° results in a higher potency for thrombin than does the present method employing bovine plasma at 28°. This increase in assayed potency was about 50–150 units for the 1000 units/vial material and about 250–400 units/vial for the 5000 units/vial material. Part of this increase is probably due to the increased temperature from 28° to 37°. However, the results in Table II indicate that part of this difference in potency between the present and proposed precision coagulation timer methods is due to the method employed to calculate the potency. When the single point assay which is used to calculate the potency in the present method was applied to the new method, the potency values are closer together.

SUMMARY AND CONCLUSIONS

It is felt that this new assay offers advantages over the assay presently being used. Human plasma which is used at 37° is closer to the conditions which exist when thrombin is used clinically.

The automated end point eliminates the reliance on subjective observation on the part of the analyst. The statistical design which contains a validity test can be a source of increased confidence in the results.

REFERENCE

(1) Bliss, C. I., *Drug. Std.*, **24**, 41(1956).

Blood Level Distribution Patterns of Diazepam and Its Major Metabolite in Man

By J. ARTHUR F. DE SILVA, B. A. KOECHLIN, and G. BADER

The effect of different diazepam dosage regimens on the blood level patterns of diazepam (I) and of its metabolite *N*-desmethyl diazepam (II) in man was studied, using specific GLC and TLC techniques, and an ultraviolet assay measuring both components. Single oral doses of diazepam (10 mg.) produced low (0.18–0.21 mcg./ml.) and rapidly declining diazepam (I) blood levels. Repeated daily doses (30 mg.) caused a progressive increase of diazepam (I) levels. The metabolite (II) appeared 24–36 hr. after the first dose, and, thereafter, the levels increased rapidly, approaching those of I. Upon discontinuing the drug after repeated dosing, components I and II disappeared from blood very slowly, II persisting longer than I. The patterns indicated a redistribution into blood of I and II previously stored by tissues. Following chronic massive doses (150–200 mg./day), levels of I averaged 1.60 mcg./ml. (after 1 week of continuous dosage), while II continued to increase until an apparent equilibrium was reached at a ratio of I–II of 1:2.5. Only traces of the urinary metabolites, oxazepam and 3 OH-diazepam, were identified in blood.

DIAZEPAM,[1] 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one (Ro-S-2807) is a psychotherapeutic agent, synthesized by Sternbach and Reeder (1), which among the other members of the benzodiazepine class of compounds is distinguished by tranquilizing and antitension activity combined with marked muscle tone regulating properties (2). The correlations between pharmacological activity and chemical structure of the 1,4-benzodiazepine derivatives have been reviewed by Childress and Gluckmann (3).

Distribution and fate of diazepam labeled with tritium in the C_5-phenyl ring have been studied by Schwartz *et al.* (4). Following the oral administration of 10 mg. of diazepam, blood level maxima of about 0.10 mcg./ml. were found after 1–2 hr. Blood level fall-off patterns indicated a rapid and extensive uptake by tissues. Although the radioactivity in the blood appeared to represent mainly the intact drug, diazepam was shown to be excreted exclusively in the form of its metabolites. The major metabolic pathways were shown to consist of demethylation at the nitrogen in position 1, addition of a hydroxyl group at carbon 3, and conjugation of the respective derivatives (Scheme II). Oxazepam glucuronide was the predominant metabolite in urine. *N*-Demethylated diazepam was also found in urine, and in addition could be detected in the blood several hours after a 10-mg. single diazepam dose. The excretion rates of the radioactivity indicated an over-all drug half-life of 2–3 days.

Blood level studies on larger groups of patients under different diazepam dosage conditions employing a U.V. spectrophotometric analytical procedure yielded inconclusive results on account of the limited sensitivity and specificity of that method. Subsequently, a gas liquid chromatographic (GLC) assay was developed capable of measuring nanogram quantities (10^{-9} Gm.) of diazepam with a high degree of precision and selectivity (5). This procedure established its usefulness in investigations of the placental transfer of diazepam (6), and also permitted the determination of the major metabolite in blood from diazepam treated patients in a single assay.

The objective of the present study was to establish blood level distribution patterns of diazepam and its metabolite produced under a variety of diazepam dosage conditions, by means of carefully designed and controlled clinical studies, and by very sensitive and specific analytical methodology. Blood level curves are reported for single therapeutic doses, administered by oral, intravenous, and intramuscular routes, for repeated oral therapeutic doses and for chronic treatment with massive doses. Three independent analytical procedures and improved modifications thereof are described and their scopes are compared, including (*a*) a quantitative spectrophotometric assay of more limited sensitivity and specificity but of usefulness in toxicology; (*b*) a modified quantitative gas liquid chromatographic procedure differentiating between the intact drug and its *N*-demethylated metabolite; and (*c*) a qualitative thin-layer chromatographic (TLC) technique capable of definite identification of the individual benzodia-

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[1] Marketed as Valium by Hoffman-La Roche, Nutley, N. J.

zepin derivatives in extracts of biological materials.

ANALYTICAL PROCEDURE

U.V. Assay.—The U.V. method measures both intact diazepam and its major metabolite, *N*-demethylated diazepam (Ro 5-2180). Both of these compounds are extractable into diethyl ether from blood at pH 7.0 and exhibit similar U.V. absorption spectra with maxima at 240 and 285 mμ, respectively (Fig. 1), the absorbance at 240 mμ being twice that at 285 mμ.

Two-milliliter aliquots of oxalated specimens of control blood taken from the patient prior to medication and a 2-ml. specimen of control blood containing 5.0 mcg. of standard diazepam from a stock solution were run along with the unknowns. The samples were extracted twice with ether by a procedure described previously (5), and the extracts were combined. The combined ether extracts were washed twice with 5 ml. of 0.1 *N* NaOH, centrifuged, and the NaOH layer removed with a capillary pipet. The ether was extracted with 2 ml. of 2 *N* HCl which was then washed twice with 10-ml. portions of ether, centrifuged, the ether removed by aspiration. The ether washed HCl extract was transferred into a micro quartz cell (0.6-ml. capacity) with a clean glass capillary pipet. Using a 1-mm. pinhole slit in a Beckman DU spectrophotometer in conjunction with the micro quartz cell, the U.V. absorption spectrum of control, internal standard, and sample blood extracts were scanned by measuring their respective absorbances at 230, 240, 250, 265, 275, 285, 295, and 320 mμ to establish the presence of the characteristic diazepam spectrum with maxima at 240 and 285 mμ.

The absorbances (A) of the internal standards and the unknowns at 240 and 285 mμ were corrected for the corresponding control absorbance. The concentration of the unknowns was calculated from the corrected absorbance values at 240 mμ of the internal standard. The recovery of 5.0-mcg. amounts of diazepam and Ro 5-2180 added to blood was 90 ± 5.0% and was determined from the absorbance at 240 mμ in a 1-cm. cell of standard solutions of diazepam and Ro 5-2180, respectively. The A_{240} for 1 mcg./ml. solutions was 0.091 and 0.101, respectively. The method has a sensitivity limit of 0.3–0.5 mcg./ml. of blood, and a range of linearity up to 10 mcg./ml. of final solution (Fig. 2) and is sufficiently sensitive to measure blood levels resulting from doses greater than 50 mg.

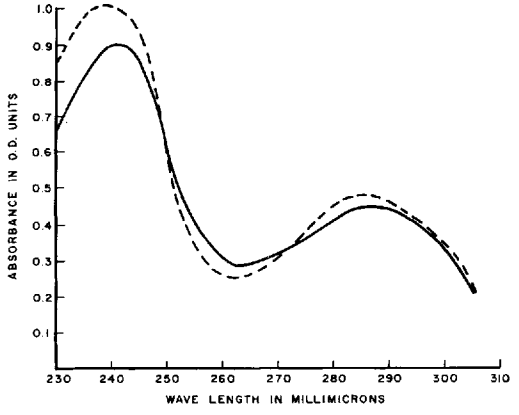


Fig. 1.—U. V. absorption spectra of diazepam and its metabolite (Ro 5-2180) in 2 *N* HCl (10 mcg./ml.). Key: ———, diazepam A_{240} = 0.091/mcg.; ·····, metabolite A_{240} = 0.101/mcg.

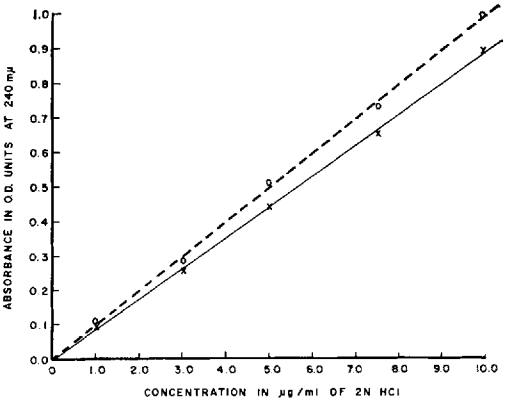


Fig. 2.—Standard curves of diazepam and its metabolite (Ro 5-2180) at 240 mμ. Key: ×, diazepam; O, Ro 5-2180.

Separation of Diazepam and Its Metabolites by Thin-Layer Chromatography.—Thin-layer chromatography was employed for the qualitative identification of the presence of diazepam and its metabolites extracted from blood and assayed cumulatively in the 2 *N* HCl acid extract by the U.V. method. Following spectrophotometric measurement, the acid extract was neutralized with 2 *N* NaOH to a blue end point, using bromothymol blue indicator, and extracted into diethyl ether which quantitatively removes diazepam and the metabolites. The residue of this extract was dissolved in 0.2 ml. of *n*-hexane (Fisher spectrograde) and quantitatively transferred onto a thin-layer plate of Silica Gel G (Stahl) containing a fluorescent indicator. Pure standards of diazepam and other possible metabolites were run alongside the sample extracts for identification of the compounds.

The plates were developed for about 2 hr. in chloroform–acetone, 90:10, and then viewed under ultraviolet shortwave light to identify the compounds on the plate. A typical chromatogram is shown in Fig. 3. The results were further verified by two-dimensional thin-layer chromatography using either chloroform–heptane–ethanol (10:10:1) followed by chloroform–heptane–acetic acid–ethanol (5:5:1:0.3) or chloroform–acetone (90:10) followed by chloroform–heptane–ethanol (10:10:1) as the developing solvents. A typical chromatogram is shown in Fig. 4.

Determination of Diazepam and Its Metabolites by Gas-Liquid Chromatography.—The more sensitive and specific gas chromatographic method is capable of quantitatively resolving diazepam and its major metabolite (Ro 5-2180) in blood, after they are hydrolyzed to their respective benzophenones (5) (Scheme I). By this method the amount of diazepam and Ro 5-2180 can be determined in the same sample of blood in a single assay.

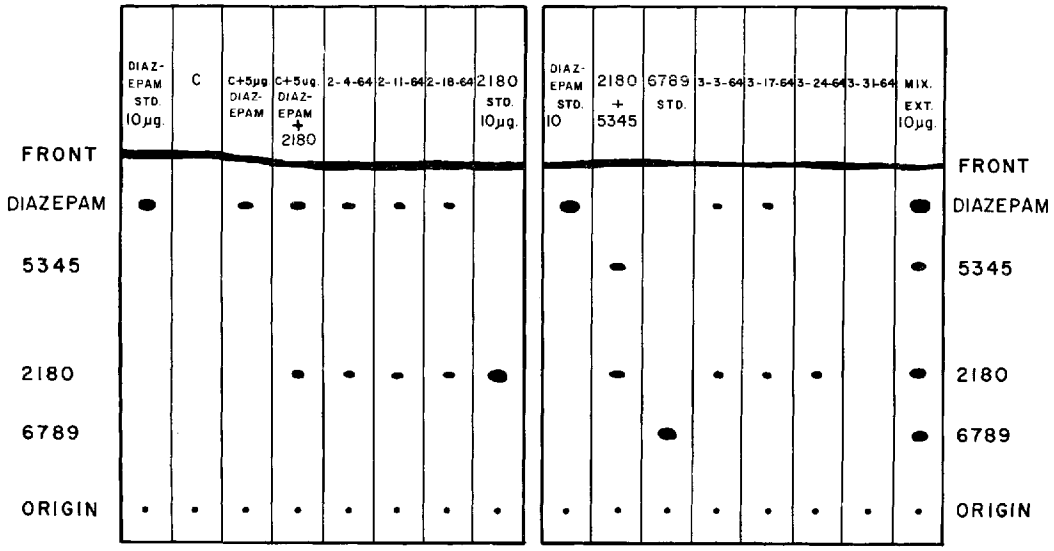


Fig. 3.—Thin-layer chromatograms of blood extracts showing the presence of intact diazepam and its *N*-demethylated metabolite, Ro 5-2180, in a patient maintained on high doses of diazepam for an extended period of time. Silica Gel G (Stahl) with fluorescent indicator. Solvent: chloroform–acetone, 90:10 v/v, 2 hr.

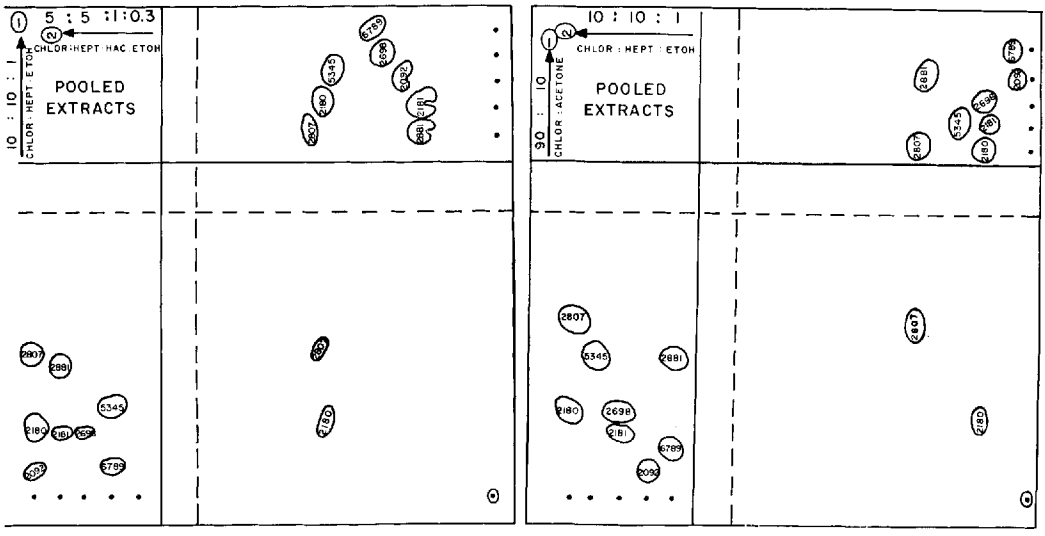
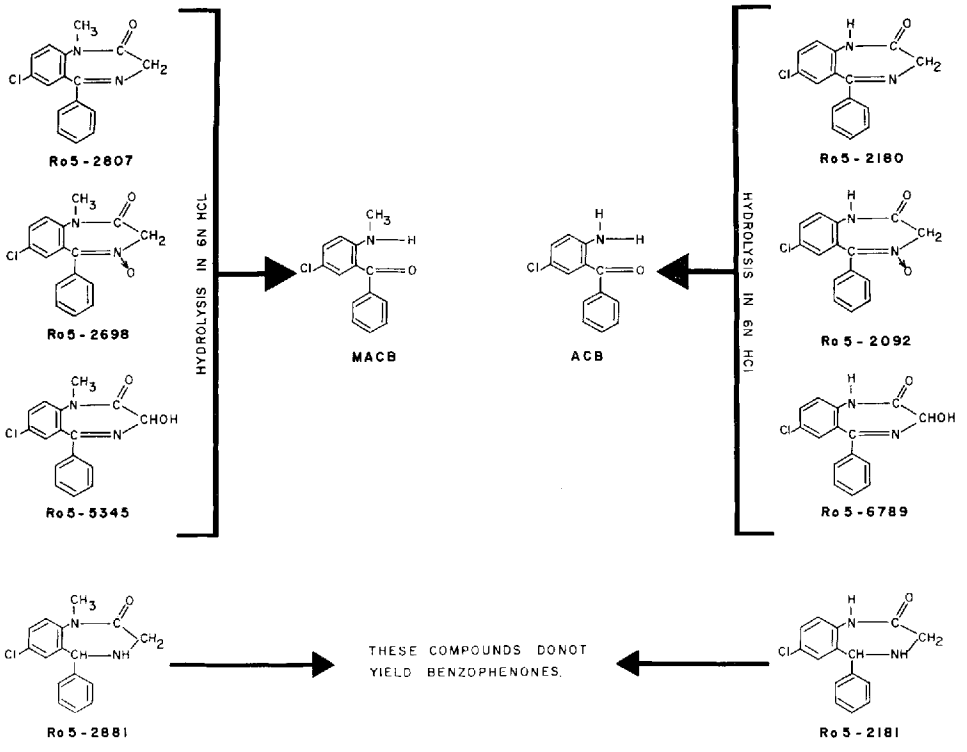


Fig. 4.—Two-dimensional thin-layer chromatograms of pooled blood extracts showing the presence of intact diazepam (Ro 5-2807) and its major metabolite (Ro 5-2180).

An improved procedure is described employing a liquid phase of 2% Carbowax 20M–terephthalic acid (CBW-20M-TPA), a synthetic polyester (7), which was found to be superior to Carbowax 20M used in the original method. It is a more polar phase with greater temperature stability, bleed resistance, column life, uniform coating characteristics, and produces very sharp well-resolved symmetrical (Gaussian shaped) peaks (Fig. 5). Its physical characteristics make it very selective for a number of benzophenones. The sample preparation for gas chromatography was carried out exactly as published (5).

Gas chromatographic parameters were obtained on a Jarrell-Ash instrument model Universal 26-700 equipped with an electron capture detector (No. 26-755) containing a 100 mc. titanium tritide β ionization source. Column: a 2-ft. column of 2% Carbowax 20M–terephthalic acid polyester phase on silanized Gas-Chrom P 100/120 mesh contained in $\frac{1}{4}$ in. stainless steel tubing was used. Carrier gas: nitrogen (oil pumped and dry) passed through a molecular sieve before entering the column was adjusted to a flow rate of 150–170 ml./min. measured at room temperature. The column head pressure was 17–20 psig on the second stage of the gas regulator. Condition of column head pressure and flow rate may be varied to obtain a retention time



Chemical structures and reactions of diazepam (Ro 5-2807) and some of its analogs. MACB = 2-methylamino-5-chloro-benzophenone. ACB = 2-amino-5-chloro-benzophenone.

Scheme I

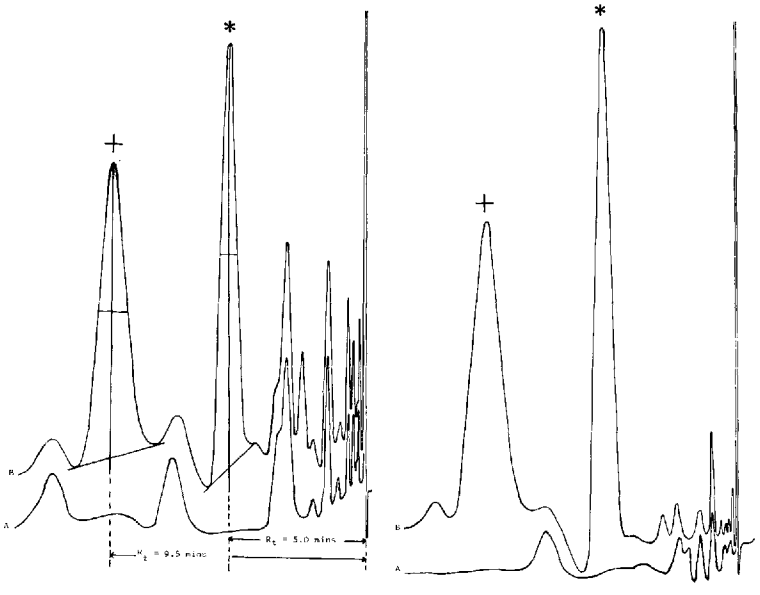


Fig. 5.—Chromatogram of diazepam and its *N*-demethylated metabolite determined by GLC as the MACB and ACB derivatives from blood extracts of patient L. R.; 30 mg. of diazepam per day for 10 days. Key: *, MACB; +, ACB; A, control blood from patient; B, patient blood after medication. Left, GLC assay of blood ether extract (10/100 μl.). Right, two-dimensional TLC of pooled blood ether extract and GLC assay of the two components (2/300 μl.).

(R_t) of 5–6 min. for MACB (diazepam) and 9–10 min. for ACB (Ro 5-2180) for effective resolution from adjacent peaks.

Temperatures: injection port, 250° ± 2.0; detector, 210° ± 2.0; oven, 215° ± 2.0 (isothermal); amplifier range, 10×10^{-9} amp. full-scale deflection (fsd); recorder, Bristol, output, 10 mv.; time constant, 1 sec. (fsd); chart speed, 1.25 cm./min. = 30 in./hr.; detector voltage, 20–30 v. d.c. Optimal detector voltage has to be determined for each compound assayed and should be checked frequently to compensate for any changes in detector response due to variation in standing current. Minimum detectable amounts of MACB = 5.0×10^{-9} Gm. (5 nanograms) and ACB = 10×10^{-9} Gm. (10 nanograms).

Preparation of Column Substrate.—The inert support Gas-Chrom P 100/120 mesh (Applied Sci-

ence Laboratories, State College, Pa.) was silanized according to the method described by Horning *et al.* (1959) to inactivate any adsorbent sites present. Two grams of Carbowax 20M–terephthalic acid (TPA) polyester phase (Wilkens Instrument and Research, Inc., Walnut Creek, Calif.) dissolved in 500-ml. of hot methylene chloride and 98 Gm. of silanized Gas-Chrom P 100/120 mesh were shaken for 30 min. on a reciprocating shaker with intermittent release of pressure. The material was transferred into a flat dish and the solvent evaporated off on a hot plate with continuous stirring. The coated substrate was then dried overnight in an oven at 200°, cooled to room temperature, and stored in an airtight container. A 2-ft. piece of stainless steel or aluminum tubing was packed with the prepared substrate and conditioned for 48–72 hr. at 230–240°. The column was then ready for use, and had a useful life span of at least 6 months of continuous use.

Preparation of Standard Curves of MACB and ACB.—2 - Methylamino - 5 - chlorobenzophenone (MACB) and 2-amino-5-chlorobenzophenone (ACB) synthesized by Sternbach *et al.* (8), of at least 99% purity, were dissolved in *n*-hexane (Fisher spectrograde) to yield separate stock solutions of 1 mg./ml. From this solution suitable dilutions were made in *n*-hexane to yield final solutions covering the range of 5 ng./10 µl. to 30 ng./10 µl. Three 10-µl. aliquots of each of the final solutions were injected into the gas chromatograph and from their average peak area a standard curve of peak area (cm.^{2}) *versus* nanograms of MACB or ACB was drawn. A standard curve should be determined for each day of analysis because column performance and detector response to these compounds changes with time.

Preparation of Standard Solutions of Diazepam and Ro 5-2180.—Diazepam and Ro 5-2180, synthesized by Sternbach and Reeder (1), of pharmaceutical grade purity (>99%), was used in the preparation of pure standard solutions.

Ten milligrams of each pure compound was weighed into separate 100-ml. volumetric flasks, and dissolved in 10–15 ml. absolute ethanol, warming the flask if necessary to effect solution. The solutions were made to volume with absolute ethanol, and should be water white in color.

The stock solution (A) contains 100 mcg./ml. One milliliter of solution A was transferred into another 100-ml. volumetric and diluted to volume with distilled water. The standard solution (B) contains 1 mcg./ml.

Suitable aliquots of solution B were used for obtaining recovery data from blood. Solutions A and B should be made fresh daily.

RESULTS AND DISCUSSION

The blood level distribution patterns of diazepam and its *N*-demethylated analog (Ro 5-2180) in man were determined by a gas-chromatographic procedure (5), which has an over-all recovery of 90% ± 6.0 and a sensitivity limit of 0.02–0.03 mcg. of diazepam/ml. of blood. The recovery of Ro 5-2180 is of the order of 70% ±7.0 with a sensitivity limit of 0.05–0.10 mcg./ml. (Table I.)

Diazepam Blood Levels Following Single Doses.—Blood level fall-off curves following single 10-mg. oral doses (Fig. 6), indicated that peak blood diazepam levels ranging from 0.18–0.22 mcg. were obtained 1 hr. after dosing, after which these levels declined rapidly within 6 hr. (half-life = 2–3 hr.) to a plateau level of 0.04–0.05 mcg./ml. This level was maintained for 12–24 hr. after which a gradual decline was seen (half-life = 27–28 hr.). These data appear to confirm the fall-off pattern using ^{3}H-labeled diazepam where the ether extractable radioactivity was measured by liquid scintillation counting.

In another study using a parenteral formulation (F-126) a group of three patients received a single 10-mg. intravenous dose of diazepam, while another group of three patients received the same formulation as a single 10-mg. intramuscular dose. The blood level curves (Fig. 7) indicate that the blood level maxima obtained with the parenteral formulation were of the same order of magnitude as those obtained with the oral doses. As before, the maxima were followed by a decline in the blood level

TABLE I.—RECOVERY OF DIAZEPAM AND RO 5-2180 ADDED TO 1 ml. OF BLOOD, DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

Diazepam Added, ng.	Total MACB Recovered, ng.	Diazepam Recovered, ng.[a]	% Recovery
Recovery of Diazepam			
100.0	78.0	90.5	91.0
100.0	75.0	87.0	87.0
200.0	147.0	171.0	86.0
200.0	152.0	176.0	88.0
200.0	144.0	167.0	84.0
200.0	148.0	172.0	86.0
200.0	164.0	190.0	95.0
200.0	172.0	199.0	99.8
200.0	162.0	188.0	94.0
200.0	160.0	186.0	93.0
200.0	143.0	165.0	83.0
200.0	143.0	165.0	83.0
200.0	155.0	179.0	85.0
200.0	159.0	184.0	92.0
200.0	164.0	190.0	95.0
200.0	163.0	189.0	94.0
200.0	162.0	188.0	94.0
200.0	167.0	194.0	97.0
200.0	170.0	198.0	99.0
200.0	166.0	193.0	97.0
300.0	225.0	261.0	87.0
300.0	218.0	253.0	84.0
300.0	210.0	244.0	81.0
300.0	227.0	263.0	88.0
300.0	205.0	238.0	80.0
		Over-all av.:	90.0% ± 6.0
Recovery of Ro 5-2180			
Ro 5-2180 Added, ng.	Total ACB Recovered, ng.	Ro 5-2180[b] Recovered, ng.	% Recovery
400.0	265.0	307.0	77.0
400.0	260.0	302.0	76.0
400.0	225.0	261.0	65.0
400.0	228.0	265.0	66.0
600.0	436.0	506.0	84.0
600.0	434.0	503.0	84.0
600.0	355.0	412.0	69.0
600.0	338.0	392.0	65.0
600.0	320.0	371.0	62.0
600.0	344.0	399.0	67.0
600.0	356.0	413.0	69.0
600.0	360.0	418.0	70.0
600.0	344.0	399.0	67.0
600.0	338.0	392.0	65.0
		Over-all av.:	70.5% ± 7.0

[a] ng. MACB × 1.16 = ng. diazepam. [b] ng. ACB × 1.16 = ng. Ro 5-2180.

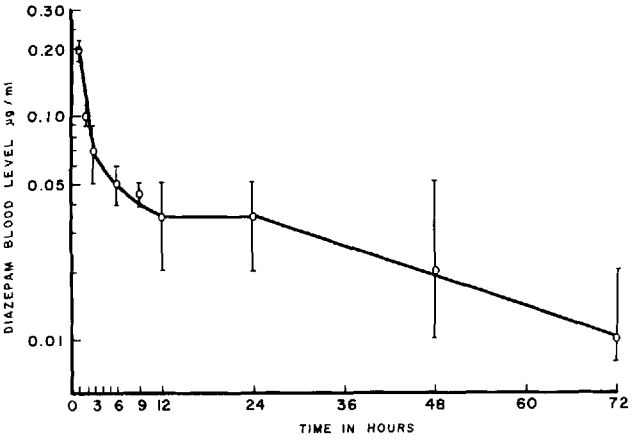


Fig. 6.—Blood level fall-off curve in man following a single oral 10-mg. dose of diazepam.

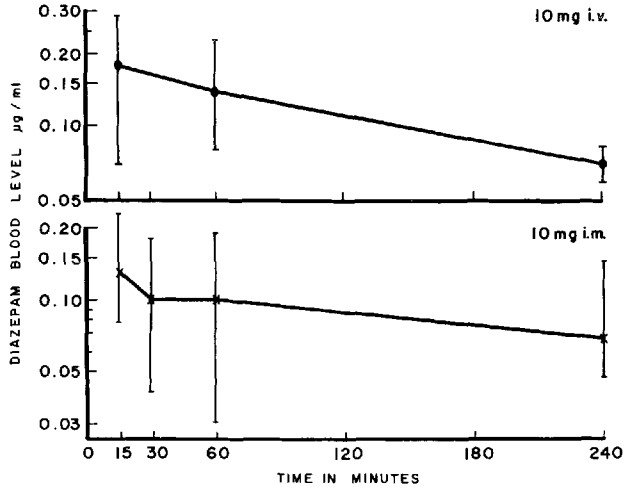


Fig. 7.—Blood level fall-off curves in man following a single 10-mg. dose of diazepam in a parenteral formulation by i. v. and i. m. routes.

within 4 hr. (half-life = 2–3 hr., indicating removal into tissue storage depots. No measurable amounts of the *N*-demethylated metabolite of diazepam were seen after these 10-mg. single doses.

Blood Level Distribution Pattern of Diazepam and Its Metabolite During Administration of Repeated Doses.—In this series of experiments the blood level patterns of diazepam and its *N*-demethylated metabolite were studied in two patients during periods of repeated daily dosage and immediately following the discontinuation of drug treatment.

Total daily oral doses of 30 mg. of diazepam (lying within the normal therapeutic range) were administered in divided doses either in the form of regular 10-mg. tablets (t.i.d.) or in the form of 15-mg. (7.5 + 7.5 mg.) repeat action tablets (b.i.d.). Blood specimens were drawn for analysis every 12 hr. (8 a.m. and 8 p.m.) during the entire experimental period; and, in addition at 1, 4, and 6 hr. after the first dose.

One patient (L. R.) received the drug for 10 consecutive days which represented a cumulative dose of 300 mg. He was taken off medication for 7 days, after which diazepam treatment was resumed for 10 more days, giving a second cumulative dose of 300 mg. The other subject (H. W.) was treated for 5 days with a cumulative dose of 150 mg. After a drug free period of 7 days, he received diazepam treatment for another 5 days or a second 150-mg. cumulative dose. The two dosage forms were alternated and their sequence was reversed in a crossover fashion in the two subjects. This was of no consequence to the particular purpose of this study.

All the specimens were assayed for intact diazepam and its *N*-demethylated metabolite by GLC. The blood levels of diazepam and of Ro 5-2180 are plotted against time in Figs. 8 and 9, respectively. In both patients the curves for diazepam and the metabolite are seen to follow basically very similar patterns. Diazepam blood levels after the expected initial drop from the maxima after the first dose, increased with each day of continued medication. In the first subject (L. R.) the diazepam levels rose from a 24 hr. value of 0.18 mcg./ml. to a final value of 0.8–1.0 mcg./ml. during the 10-day period of medication. In the second subject (H. W.) there was a corresponding rise from an initial 24 hr. level of 0.12 mcg./ml. to a maximum of 0.50 mcg., within 4 days. The metabolite Ro 5-2180 could not be detected initially in either subject up to 36 hr. after the first dose, but from then on its blood levels showed a steady increase, gradually approaching the concentrations of the parent compound. This pattern was more systematic in patient L. R., especially during the first 10-day period of medication.

In both experiments discontinuation of the drug did not show an immediate change in the blood level

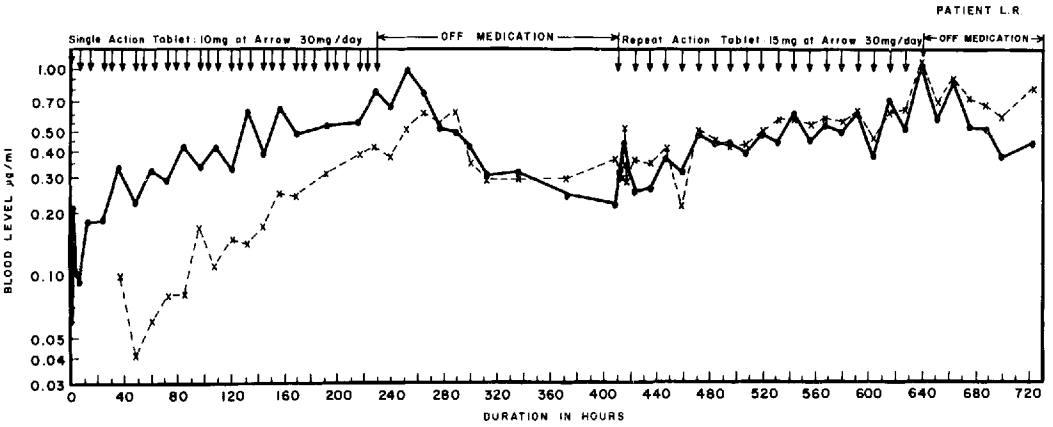


Fig. 8.—Blood level distribution pattern of diazepam and Ro 5-2180 in man following administration of single action tablets *vs.* repeat action tablets. Key: ●, diazepam Ro 5-2807; ×, metabolite Ro 5-2180.

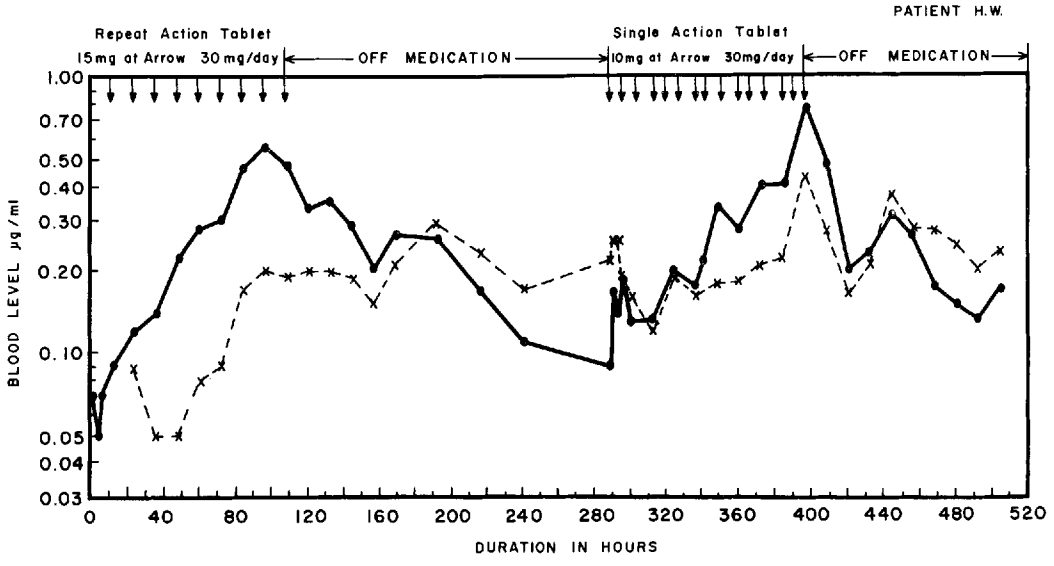


Fig. 9.—Blood level distribution pattern of diazepam and Ro 5-2180 in man following administration of repeat action tablets *vs.* single action tablets. Key: ●, diazepam Ro 5-2807; ×, metabolite Ro 5-2180.

of either component. The diazepam levels showed an erratic but definite decline with time but were still measurable at the end of the 7-day fall-off period. The metabolite levels fluctuated without a distinct downward trend. As a result, the ratio of the two components changed gradually in favor of the metabolite. By the end of the drug-free period the metabolite levels consistently exceeded those of intact diazepam.

Uptake of the drug during the second period of medication produced an increase in the cumulative blood levels over and above the residual threshold levels after the first fall-off period. Owing to the high residual metabolite levels, the ratio of the metabolite to diazepam was maintained close to unity, especially in patient L. R. where the earlier cumulative dose had been higher.

These blood level curves bring out the important fact that the concentration of diazepam, the ratio of diazepam to its metabolite, and also the rate of elimination of these components from blood depend not merely on the dose administered at the time of sampling but also depend greatly on the preceding dosing history and its ultimate duration. These levels are governed specifically by the cumulative amount of drug administered continuously and by the duration of time of continuous medication. The blood level distribution pattern is influenced by all these parameters, and it is also indicative of drug accumulation in some tissue storage depots.[2] Release from such depots back into the blood stream is manifested by the slow and erratic saw-toothed fall-

[2] Tissue storage depots implies those compartments of the body which are associated in drug accumulation, metabolism, and excretion. Such depots are defined in the terminology of biopharmaceutics and pharmacokinetics as "shallow" compartments of soft extra vascular tissues, *e.g.*, liver, kidney, intestinal tract, and other associated organs and "deep" compartments of tissues such as bone, marrow, muscle, fat, all of which are accessible and interconnected through the blood circulatory system. Consequently, chemical compounds carried in the blood can be stored and/or metabolized in these depots, and later released back into circulation prior to excretion. [See Wagner, J. G., *J. Pharm. Sci.*, **50**, 359(1961); Doluisio, J. T., and Swintosky, J. V., *Am. J. Pharm.*, **137**, 144(1965); **137**, 175(1965).]

off pattern seen after the discontinuation of dosing. The faster buildup and the much slower disappearance of the metabolite levels indicate a relatively slower metabolism and excretion of this product.

Blood Level Distribution Pattern of Diazepam and Its Metabolite During Chronic Administration of Massive Doses.—An opportunity to study the blood level distribution pattern of diazepam and its metabolites under more extreme conditions of chronic dosing was offered by a unique therapeutic experiment. It involved a male patient (L), age 53, with a history of chronic alcoholism and related psychic disorders who was able to tolerate daily doses of diazepam as high as 200 mg. without major side effects (9).

Blood specimens were taken at weekly intervals. They were originally analyzed by the U.V. spectrophotometric assay for "total" diazepam, and then reexamined by the differential GLC procedure (Table II).

TABLE II.—BLOOD LEVELS OF DIAZEPAM AND ITS METABOLITE IN A HUMAN[a] GIVEN 150–200 mg./DAY FOR 8 WEEKS

Medication, Wk.	Date Blood Drawn	Dose	Blood Level, mcg./ml. 1 Diazepam	GLC 2180	Method Ratio Met./Diaz.	2 Diaz. Eq. of 2180	Total 1 + 2	Total Diazepam U.V.
0 (Control)	1/21/64	Diazepam Tablets	...	...	...	...	...	N.M.[b]
	1/23/64	150 mg./Day began						
1	1/28/64		1.51	1.39	0.92	1.47	2.98	3.70
2	2/4/64		...	...	...	...	...	4.10
3	2/11/64		1.42	2.58	1.83	2.74	4.16	3.90
4	2/18/64		2.00	2.90	1.45	3.07	5.07	5.00
5	2/25/64		1.42	2.38	1.68	2.52	3.94	4.10
	2/26/64	200 mg./day began						
6	3/3/64		1.46	3.19	2.18	3.38	4.84	4.70
7	3/10/64		1.88	3.25	1.73	3.45	5.33	4.40
8	3/17/64		1.57	4.18	2.66	4.43	6.00	4.22
	3/18/64	Withdrawal began						
9	3/24/64	90 mg./day	0.65	2.49	3.83	2.64	3.29	3.98
10	3/31/64	20 mg./day	0.30	0.16	0.53	0.17	0.47	3.70
11	4/3/64	Off diazepam, on placebo	0.09	N.M.	...	...	0.09	N.M.
12	4/7/64	On placebo	N.M.	N.M.	...	...	...	N.M.
13	4/10/64	On placebo	N.M.	N.M.	...	...	...	N.M.
14	4/16/64	On placebo	N.M.	N.M.	...	...	...	N.M.

[a] The patient was under the care of Dr. K. S. Ditman, Alcoholism Research Clinic, Department of Psychiatry, Center for Health Sciences, University of California at Los Angeles. [b] N.M. = not measurable.

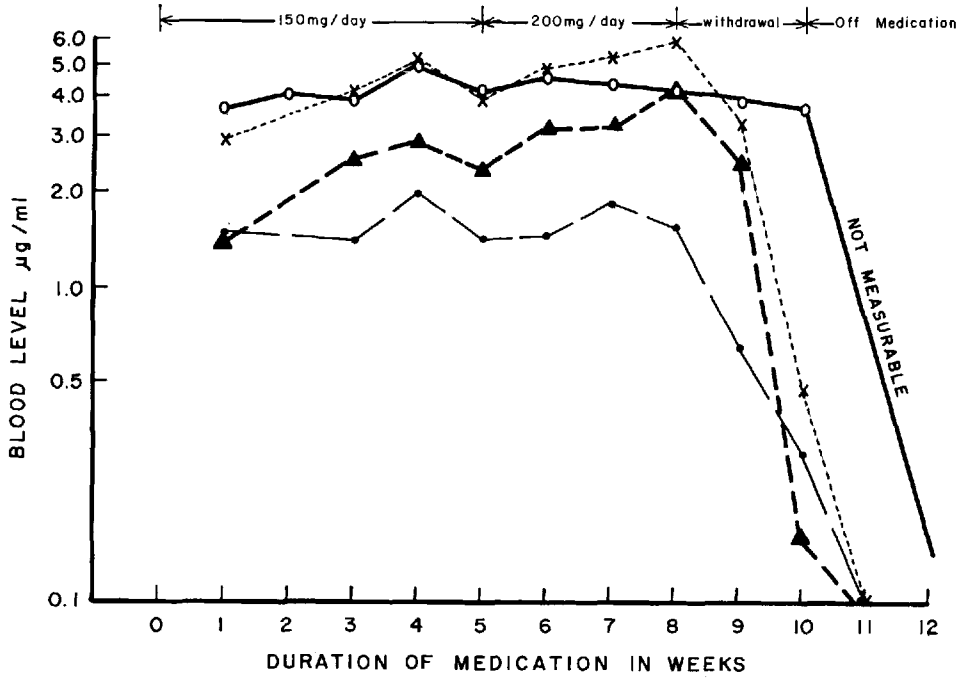


Fig. 10.—Blood levels of diazepam and its metabolite (Ro 5-2180) from a human given 150–200 mg./day orally for 8 weeks followed by gradual withdrawal of medication. Key: ●, diazepam (GLC); ▲, metabolite (2180) (GLC); ×, total diazepam equivalent GLC (calcd.); ○, total diazepam + metabolite (U. V.).

The blood level distribution pattern of the two individual components, of the calculated sum of the two, and of the total spectrophotometrically measured material are shown in Fig. 10. After a cumulative dose of 750 mg. within the first week the blood level of intact diazepam was 1.51 mcg./ml. but then tended to stabilize itself rapidly with continued medication at 1.60–1.64 mcg./ml. After 4 weeks of continuous medication at a dosage of 150 mg./day, followed by 3 weeks at an increased dosage of 200 mg./day, the blood level of intact diazepam did not show a significant increase over the first week level of this component in blood. This seemed to indicate than an equilibrium was established between drug uptake and its disposition by metabolism, excretion, and/or tissue distribution. The levels of Ro 5-2180 equaled those of the parent component by the end of the first week, but continued to rise to about twice this value during 2 more weeks of treatment. Thereafter, with fluctuation of the absolute blood levels of the two components, the ratio of the metabolite to intact diazepam maintained a fairly constant value of about 1.8, with the exception of the last week of treatment at the highest daily dose when a further increase of this ratio was noted. A more pronounced rise of this ratio during the final phase of gradual drug withdrawal was the result of a relatively faster decline of the intact diazepam blood level over that of the metabolite.

The curves for the total drug levels as measured by the two procedures indicated reasonable agreement, except for the discrepancies during the withdrawal period, which remain unexplained.

The clinical observation of an abnormally high drug tolerance suggested the possibility of either a congenital or of a drug-induced increased metabolic rate in this subject. An increased rate of diazepam metabolism due to congenital factors would manifest itself by a slower buildup of the blood levels, by a lower stabilization level, and by a more rapid fall-off after withdrawal. A drug-induced stimulation of a metabolizing enzyme system, *e.g.*, the liver microsomal enzyme system, would be reflected in a gradual lowering of the stabilization level during continued dosing at a constant rate. The blood level curves obtained provided no conclusive evidence of such factors in operation in this patient. The diazepam blood level of 1.51 mcg./ml. produced by a cumulative dose of about 750 mg. during the initial phase appears to be in keeping with a corresponding value of 0.8–1.0 mcg./ml. produced by a 300-mg. total dose, and of a value of 0.50 mcg./ml. following a 150-mg. total dose in the earlier low dose experiments. This, therefore, indicated similar rates of drug accumulation in all three patients. The stabilization levels of both diazepam and Ro 5-2180 maintained a fairly constant value throughout the entire period of dosing at the high level. The blood level fall-off rates during the withdrawal period were difficult to evaluate in terms of possible changes in metabolic rates due to the stepwise reduction of drug discontinuation and to the insufficient number of samples measured during this period.

Since the GLC method is capable of resolving diazepam from its metabolite Ro 5-2180, the specificity of the method depends on the absence of other analogs of diazepam, which if present in blood, and/or are ether extractable, would be hydrolyzed to MACB and ACB, respectively, and give erroneous values for diazepam and Ro 5-2180 (Scheme I). Blood specimens from the high dose patient were pooled and analyzed first by one-dimensional and then by two-dimensional thin-layer chromatography to observe the presence, if any, of other possible metabolic analogs which might interfere with the GLC assay.

The chromatoplates (Figs. 3 and 4) showed only the presence of intact diazepam and its major metabolite Ro 5-2180. This was further verified in

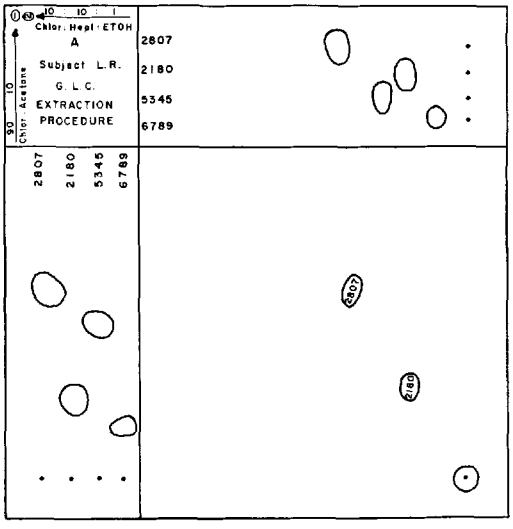


Fig. 11.—Two-dimensional thin-layer chromatogram of pooled blood extracts of a patient (L. R.) who had received 30 mg. of diazepam per day for 10 days, using the extraction procedure for GLC assay, showing the presence of only diazepam and its *N*-demethylated metabolite, Ro 5-2180.

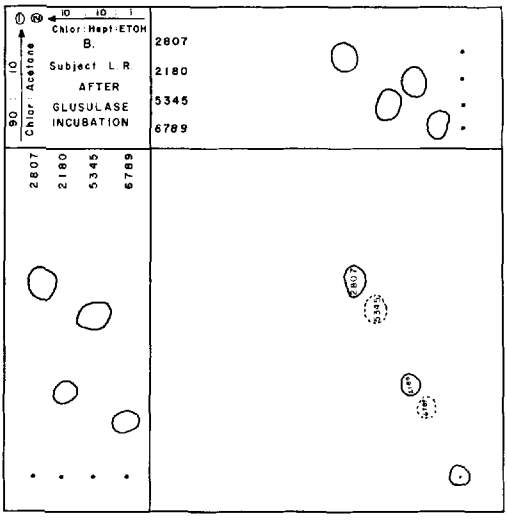
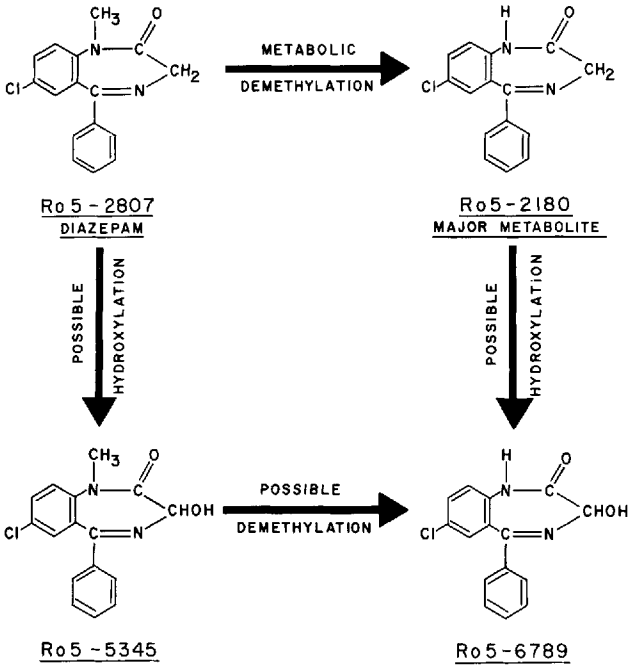


Fig. 12.—Two-dimensional thin-layer chromatogram of pooled blood extracts of a patient (L. R.) who had received 30 mg. of diazepam per day for 10 days. Using the same extraction procedure as in Fig. 11, but after incubating the blood with Glusulase enzyme, shows the presence of diazepam, Ro 5-2180, Ro 5-6789, and Ro 5-5345.



Possible metabolic pathways of diazepam in humans.

Scheme II

pooled blood ether extracts from the patients (L. R. and H. W.) of the low dose experiments (Fig. 11). The areas on the plate corresponding to diazepam and the metabolite were scraped off, extracted with ether, and then taken through the GLC procedure. The chromatograms (Fig. 5) establish the formation of MACB and ACB (benzophenones) from the hydrolysis of their respective parent compounds found in the blood, thus establishing the specificity of the GLC method for diazepam and Ro 5-2180. By two analytical procedures (TLC and GLC) diazepam and its *N*-demethylated analog Ro 5-2180 have been shown to be the major ether extractable components present in the blood of patients treated with the pharmaceutical formulations of diazepam.

Since the 3-hydroxy compounds, Ro 5-5345 and Ro 5-6789 (oxazepam) (10), have been shown to be significant metabolites of diazepam in human urine in the form of conjugated glucuronides (4), their presence in the blood as such was also investigated. Aliquots of blood were pooled (32 ml.), acidified with diluted HCl to pH 5.5, and incubated in a 37° water bath with 0.5 ml. of Glusulase enzyme (Endo Labs, Inc., Richmond Hill, N. Y.; activity = 100,000 units of glucuronidase, 50,000 units of sulfatase/ml.) for 2 hr., swirling every 15 min. The samples were then buffered to pH 7.0, extracted with ether, and analyzed by two-dimensional TLC using the same solvent systems as before.

The chromatoplates (Fig. 12) showed the presence of significant amounts of diazepam and Ro 5-2180 and also trace amounts of Ro 5-5345 and Ro 5-6789. After acid hydrolysis and GLC analysis, the identity of diazepam and Ro 5-2180 recovered from the chromatoplates was again established with significant amounts of their respective benzophenones, whereas Ro 5-5345 and Ro 5-6789 present in trace amounts gave much less significant but measurable amounts of MACB and ACB, respectively. It was estimated that the pooled blood contained approximately 0.19 mcg./ml. of diazepam and 0.13 mcg./ml. of Ro 5-2180, whereas Ro 5-5345 (0.008 mcg./ml.) and Ro 5-6789 (0.004 mcg./ml.) were present and/or extractable in trace amounts as the free hydroxy compounds only after incubation with Glusulase. Of these four compounds only diazepam and Ro 5-2180 were directly extracted and quantized by the GLC procedure, the other two being present as nonextractable conjugated glucuronides in the blood, which were only released as the free hydroxy compounds after incubation with Glusulase enzyme.

Studies on the metabolism of diazepam in the dog (11) have indicated a metabolic pathway in this species which is similar to man. However, when rabbits were fed chronic doses of diazepam (600–800 mg./Kg.), Jommi *et al.* (12) detected the presence of two other possible metabolites in addition to those found in man and in the dog. These were identified after strong acid hydrolysis as 2 methyl amino-5-chloro-4′-hydroxybenzophenone and 2 amino-5-chloro-4′-hydroxybenzophenone, being derived from their respective 1,4-benzodiazepines. Metabolites corresponding to these two compounds have not yet been reported in man or dog.

The findings of Schwartz *et al.* (4), on the metabolism of diazepam in man using tritiated diazepam labeled in the C_5 phenyl ring, were verified in man using TLC and GLC as analytical techniques with diazepam in pharmaceutical formulations used as the vehicle of administration. The metabolic pathways of diazepam in man are summarized in Scheme II and the data obtained indicate that the principal pathway is through the *N*-demethylation of diazepam to Ro 5 2180, which is then hydroxylated at position C-3 to Ro 5-6789. There is also evidence for a hydroxylation at C-3 of diazepam to yield Ro 5-5345 which could then be *N*-demethylated to give Ro 5-6789. The 3-hydroxy compounds are excreted as conjugated glucuronides, whereas the *N*-demethylated

metabolite Ro 5-2180 is excreted as the intact compound. No measurable amounts of intact diazepam have yet been found to be excreted in human urine, and the 3-hydroxy compound Ro 5-6789 (oxazepam) (13, 14) is a major urinary metabolite of diazepam in man.

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Polarography of Various *N*-Alkyl-*N*-nitrosoureas

By EDWARD R. GARRETT and ANTHONY G. CUSIMANO

The irreversible polarographic reductions of various *N*-alkyl-*N*-nitrosoureas were studied from a pH of 1.1–6.7. Below pH 3.2, the limiting current, $i_{lim.}$, of *N*-methyl-*N*-nitrosourea (NNMU) is controlled by diffusion. Above this pH, adsorption or chemical reaction is the electrode-controlling process and buffer effects are observed. Similar results were obtained for *N*-ethyl-*N*-nitrosourea (NNEU) and 1,3-dimethyl-1-nitrosourea (SRI-1384), except that the buffer effects were greatly diminished for the latter compound. The acetate buffer effects on $i_{lim.}$ have been quantified and have been attributed either to adsorption of the nitrosourea (NNMU) and the buffer components onto the microelectrode, or to a reaction between these species. The polarographic method offers a sensitive (1×10^{-6} *M*) and reliable (0.91 per cent standard error among days) assay procedure for the nitrosoureas. All of the compounds studied in this series were more easily reduced than the parent methyl compound. A correlation between the change in the half-wave potentials and the Taft substituent constants (σ^*) was noted for simple alkyl groups such as methyl, ethyl, butyl, etc. A correlation between the half-wave potentials and the apparent first-order rate constants (k, sec.$^{-1}$) for hydroxyl ion catalyzed solvolysis was noted for several of the compounds studied.

THE ANTILEUKEMIC and antiviral activities of various *N*-nitrosoureas have been reported (1–4). They have a high degree of lipoid solubility, are essentially nonionized, and are not bound to any extent by plasma proteins.

Degradation studies (5) of the broad spectrum antibiotic streptozotocin indicated that it is a substituted *N*-methyl-*N*-nitrosourea, and the maintenance of the biological activity could be related to the stability of the *N*-nitroso group.

A physicochemical parameter which could be correlated with biological activity, chemical structure, or reactivity would be useful in molecular modification to obtain a better drug. Although the mechanism of action of the *N*-alkyl-*N*-nitrosoureas is not known for certain, it is plausible that they may act as alkylating agents since a diazoalkane may be a solvolytic intermediate (6) or that they may act *via* a redox mechanism. The polarographic half-wave potential ($E^1/_2$) is a good electrochemical measure of the ease of reduction of such compounds.

Polarographic studies of *N*-nitrosoureas have been limited (5, 7) and have primarily been used to study the degradation of streptozotocin (5) and *N*-methyl-*N*-nitrosourea (7). This is in contrast to the detailed work on the polarographic reductions of various *N*-nitrosamines which have been studied by many investigators (8–14).

The purposes of this investigation were to characterize polarographically the reduction of a series of *N*-alkyl-*N*-nitrosoureas, to develop analytical methods, and to test for correlations of half-wave potentials with solvolytic rate constants and with substituent constants.

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metabolite Ro 5-2180 is excreted as the intact compound. No measurable amounts of intact diazepam have yet been found to be excreted in human urine, and the 3-hydroxy compound Ro 5-6789 (oxazepam) (13, 14) is a major urinary metabolite of diazepam in man.

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Polarography of Various *N*-Alkyl-*N*-nitrosoureas

By EDWARD R. GARRETT and ANTHONY G. CUSIMANO

The irreversible polarographic reductions of various *N*-alkyl-*N*-nitrosoureas were studied from a pH of 1.1–6.7. Below pH 3.2, the limiting current, $i_{lim.}$, of *N*-methyl-*N*-nitrosourea (NNMU) is controlled by diffusion. Above this pH, adsorption or chemical reaction is the electrode-controlling process and buffer effects are observed. Similar results were obtained for *N*-ethyl-*N*-nitrosourea (NNEU) and 1,3-dimethyl-1-nitrosourea (SRI-1384), except that the buffer effects were greatly diminished for the latter compound. The acetate buffer effects on $i_{lim.}$ have been quantified and have been attributed either to adsorption of the nitrosourea (NNMU) and the buffer components onto the microelectrode, or to a reaction between these species. The polarographic method offers a sensitive (1×10^{-6} *M*) and reliable (0.91 per cent standard error among days) assay procedure for the nitrosoureas. All of the compounds studied in this series were more easily reduced than the parent methyl compound. A correlation between the change in the half-wave potentials and the Taft substituent constants (σ^*) was noted for simple alkyl groups such as methyl, ethyl, butyl, etc. A correlation between the half-wave potentials and the apparent first-order rate constants (k, sec.$^{-1}$) for hydroxyl ion catalyzed solvolysis was noted for several of the compounds studied.

THE ANTILEUKEMIC and antiviral activities of various *N*-nitrosoureas have been reported (1–4). They have a high degree of lipoid solubility, are essentially nonionized, and are not bound to any extent by plasma proteins.

Degradation studies (5) of the broad spectrum antibiotic streptozotocin indicated that it is a substituted *N*-methyl-*N*-nitrosourea, and the maintenance of the biological activity could be related to the stability of the *N*-nitroso group.

A physicochemical parameter which could be correlated with biological activity, chemical structure, or reactivity would be useful in molecular modification to obtain a better drug. Although the mechanism of action of the *N*-alkyl-*N*-nitrosoureas is not known for certain, it is plausible that they may act as alkylating agents since a diazoalkane may be a solvolytic intermediate (6) or that they may act *via* a redox mechanism. The polarographic half-wave potential ($E^1/_2$) is a good electrochemical measure of the ease of reduction of such compounds.

Polarographic studies of *N*-nitrosoureas have been limited (5, 7) and have primarily been used to study the degradation of streptozotocin (5) and *N*-methyl-*N*-nitrosourea (7). This is in contrast to the detailed work on the polarographic reductions of various *N*-nitrosamines which have been studied by many investigators (8–14).

The purposes of this investigation were to characterize polarographically the reduction of a series of *N*-alkyl-*N*-nitrosoureas, to develop analytical methods, and to test for correlations of half-wave potentials with solvolytic rate constants and with substituent constants.

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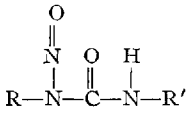
TABLE I.—NAMES AND STRUCTURES OF VARIOUS *N*-ALKYL-*N*-NITROSOUREAS



Compd.	Symbol	R	R′	Mol. Wt.
N-Methyl-*N*-nitrosourea	NNMU[a]	CH_3	H	103.1
N-Ethyl-*N*-nitrosourea	NNEU[a]	CH_3CH_2	H	117.1
N-Butyl-*N*-nitrosourea	NNBU[a]	$CH_3(CH_2)_3$	H	145.1
N-Isobutyl-*N*-nitrosourea	NNisoBU[a]	$CH_3CH(CH_3)CH_2$	H	146.1
N-Allyl-*N*-nitrosourea	NNAU[a]	CH_2=$CHCH_2$	H	129.1
N-Cyclohexyl-*N*-nitrosourea	NNCyU[a]	C_6H_{11}	H	171.2
N-Benzyl-*N*-nitrosourea	NNBcU[a]	$C_6H_5CH_2$	H	179.2
1,3-Dimethyl-1-nitrosourea	SRI-1384[b]	CH_3	CH_3	117.1
1,1′-Trimethylene bis(3-methyl-3-nitrosourea)	SRI-1631[b]	CH_3	$(CH_2)_3NHCON(NO)CH_3$	246.2
1,3-Bis (2-chloroethyl)-1-nitrosourea	SRI-1720[b]	$ClCH_2CH_2$	CH_2CH_2Cl	214.1
3-(*p*-Fluorophenyl)-1-methyl-1-nitrosourea	SRI-1746[b]	CH_3	*p*-F-C_6H_4	197.2
1-Methyl-1-nitroso-3-phenethylurea	SRI-1833[b]	CH_3	$CH_2CH_2C_6H_5$	207.2
1-Nitroso-1-phenethylurea	SRI-1834[b]	$C_6H_5CH_2CH_2$	H	193.2
1-(2-Chloroethyl)-nitroso-3-phenylurea	SRI-1879[b]	$ClCH_2CH_2$	C_6H_5	227.7

[a] Compounds synthesized in these laboratories (6). [b] Code number of the Southern Research Institute, Birmingham, Ala. (3).

EXPERIMENTAL

Materials and Equipment.—The *N*-alkyl-*N*-nitrosoureas (Table I) were synthesized either in these laboratories (6) or by the Southern Research Institute, Birmingham, Ala. (3). All other chemicals used were of analytical reagent grade. The buffer compositions were similar to those used by Malspeis and Hung (14). The pH values of all buffers except hydrochloric acid solutions were measured, before and after each run, with a Beckman expanded scale pH meter, model 76. The pH meter was standardized at 25.0° with Beckman standard buffers of pH 4 and 7. The pH values of hydrochloric acid buffers were calculated using the activity coefficients (15) at 25.0°. The ionic strength (μ) of each solution was adjusted to 0.200 with KCl.

A Sargent model XV polarograph was used. The electrolysis cell was a Sargent H-type which was immersed in a constant-temperature bath at 25.0° ± 0.1°.

Polarographic Procedure.—A master solution of the *N*-nitrosourea in distilled water was prepared at least every other day and was refrigerated when not in use. Degradation under these conditions was negligible. In those experiments where all of the compounds were studied, 8% alcohol by volume was used in preparing the master solution since some of the nitrosoureas, in particular the disubstituted derivatives, were difficultly soluble in water alone. An aliquot of the master solution was diluted with the appropriate buffer which was previously purged with nitrogen. No more than 1% alcohol by volume was present in the polarographed solutions. Alcohol did not have an effect on the polarograms.

The sample solutions were purged with nitrogen for 10 min., and their polarograms run over that of the appropriate blank solutions. Due to the instability of the nitrosoureas in phosphate buffers, this purging was limited to 3 min. The buffers were purged for 15–30 min. prior to the dilution of aliquots of the master solution.

Each polarogram was recorded at a constant mercury head and at a constant temperature of 25.0° ± 0.1°. The indicator electrode used was the dropping mercury electrode (DME), whereas a saturated calomel electrode (SCE) was the reference electrode. The DME had values of $m^{2/3}t^{1/6}$ of 2.780 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.766 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.0 v. *versus* SCE in HCl–KCl buffers, 2.799 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.786 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.0 v. *versus* SCE in tartrate buffers, 2.753 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.741 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.1 v. *versus* SCE in acetate buffers, and 2.741 mg.$^{2/3}$ sec.$^{-1/2}$ open circuit and 2.695 mg.$^{2/3}$ sec.$^{-1/2}$ at −1.4 v. *versus* SCE in phosphate buffers. These values were obtained at a column height of 62.2 cm.

Measurement of Half-Wave Potential and Limiting Current.—An arbitrary procedure was chosen to measure the half-wave potentials. The limiting currents of the polarographed solutions of the *N*-nitrosoureas were not exactly parallel to the currents of the blank solutions at the same applied potentials.

A line was drawn through the polarogram of the blank at the midpoints of the oscillations (curve A in Fig. 1). Another line was drawn through the midpoints of the oscillations for the sample polarogram in the region where the wave height halted its precipitous rise and before the hydrogen discharge wave (curve B in Fig. 1). The distances between these two lines were halved (curve C in Fig. 1). This third line intersected the polarogram at a potential which was designated the half-wave potential ($E_{1/2}$). No correction for resistance across the cell was necessary since the resistance was always less than 500 ohms.

The limiting currents, $i_{lim.}$ (Fig. 1), were estimated

from the differences between the currents for the sample solutions and the blank solutions at potentials 0.35 v. more negative than the half-wave potential. This was a reasonable estimate of the midpoint of the linear portion of the polarogram between the wave for the reduction of the *N*-nitrosourea and the hydrogen discharge wave.

PROCEDURES

Calibration Curve for NNMU.—A 10.3-mg. sample of NNMU is accurately weighed into a 25-ml. volumetric flask. It is dissolved and brought to volume with distilled water to give a 4×10^{-3} *M* master solution. This solution is refrigerated when not in use and is prepared freshly each day. Into several 25-ml. volumetric flasks are placed aliquots of the master solution ranging from 0.025–2.00 ml. Enough nitrogen purged distilled water is added to each flask to make 2.00 ml. and acetate buffer is added to volume. The acetate buffer is prepared so that the final solution is: $[HC_2H_3O_2]$ = 0.005 *M*, $[NaC_2H_3O_2]$ = 0.003 *M*, and [KCl] = 0.197 *M*. The polarogram of each standard solution is then run.

Assay Method.—The polarographic procedure is the same as that previously described. Each sample is purged with nitrogen for 10 min. and the polarogram is run over that of a blank solution. Samples of unknown concentrations of NNMU are prepared and run in a similar manner. The limiting currents are measured at −1.3 v. *versus* SCE.

The method may be modified somewhat to study the fast solvolysis of these compounds in alkaline or strong acid media at higher temperatures. In this case the degrading solution is kept in the electrolysis cell during the entire run, and the polarogram is obtained at specified time intervals.

RESULTS AND DISCUSSION

Wave Characteristics.—The *N*-alkyl-*N*-nitrosoureas (Table I) exhibited well-defined polarographic waves throughout the entire pH range. The polarographic parameters, $E_{1/2}$ and $i_{lim.}$, were easily measured and no maxima were observed. Typical polarograms of NNMU at various pH values and in various buffers are shown in Fig. 1. The curves are drawn through the midpoints of the oscillations. The half-wave potential shifted to more negative values with increased pH of the solution. Since the reduction of most organic compounds involves hydrogen ions, this shift is in accordance with the Nernst equation (16).

The irreversible nature of the reduction was evident from the results of the logarithmic analyses (16) of representative polarograms of NNMU.

Reversibility is characterized by a single straight line for the plot of the applied potential, *E versus* $\log[i/(i_{lim.} - i)]$, where *i* is the current at a specified potential, *E*, and $i_{lim.}$ is the limiting current. For a reversible electrode process the number of electrons (*n*) involved in the reduction can be directly obtained from the slopes of such plots (16). Reversible polarographic waves are symmetrically S-shaped.

Such logarithmic analyses yielded two straight line segments for pH 1.10 solutions. Single straight lines were obtained for solutions in the pH range

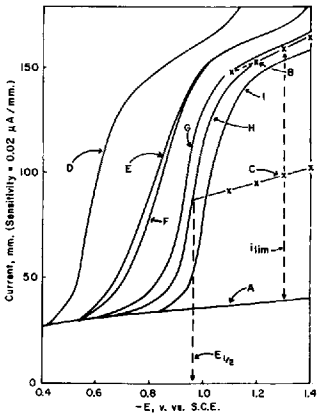


Fig. 1.—Typical polarograms of NNMU (1.1×10^{-4} *M*) in various buffers at 25.0° and at column height of 62.2 cm. Key: A, polarogram of blank solution; B, polarogram of sample solution; C, line through half-values of limiting current intersecting wave at half-wave potential ($E_{1/2}$); D, HCl–KCl buffer, pH 1.10; E, HCl–KCl buffer, pH 2.33; F, tartrate buffer, pH 2.52; G, acetate buffer, pH 3.70; H, acetate buffer, pH 4.81; I, phosphate buffer, pH 6.28.

2.0–4.8. However, the calculated values of *n* were less than unity. Typical values of *n* were 0.35–0.37 in HCl–KCl buffer (pH 2.33) and tartrate buffer (pH 2.78), and 0.54–0.59 in acetate buffers from pH 3.5–4.8.

The skew S-shaped curves (Fig. 1) are also representative of irreversibility where the electron transfer process is either slower or of the same order of magnitude as the electrode controlling process, *e.g.*, diffusion or adsorption.

Effect of pH on Half-Wave Potential and Limiting Current.—Typical data for the half-wave potentials and the limiting currents for NNMU at various pH values are presented in Table II. The half-wave potential-pH profile is shown in Fig. 2. The latter has two straight line segments. The intersection of these segments at a pH of 3.2 indicates a change in either the mechanism of the reduction reaction or in the electrode controlling process. The equations which describe the dependence of the half-wave potential on pH for NNMU are

$$-E_{1/2} = 0.165\ \text{pH} + 0.384 \quad \text{(pH 1.0 to 3.2)} \quad \text{(Eq. 1)}$$

$$-E_{1/2} = 0.030\ \text{pH} + 0.824 \quad \text{(pH 3.2 to 6.8)} \quad \text{(Eq. 2)}$$

Large changes in pH produced only small changes in the limiting current throughout the entire pH range (Table II). Malspeis and Hung (14) have reported that the wave height produced by the reduction of *N*-nitrosoephedrine decreased with pH. This was attributed to reduction controlled by both reaction rate and diffusion to the microelectrode. An electrode process in which the limiting current is essentially independent of pH is said to be controlled either by diffusion of the compound to the microelectrode or by adsorption onto the electrode surface (17). Malspeis and Hung (14) ascribed their results to a polarographic pKa with

TABLE II.—EFFECT OF pH ON LIMITING CURRENT ($i_{lim.}$) AND HALF-WAVE POTENTIAL ($E_{1/2}$) FOR REDUCTION OF NNMU AT 25.0°

Buffer	pH	$i_{lim.}$, μA [a]	$-E_{1/2}$, v. vs. SCE [b]
HCl–KCl	1.10	2.02	0.570
	1.38	2.00	0.605
	2.04	2.03	0.719
	2.33	2.05	0.781
Tartrate	2.51	2.05	0.815
	2.56	2.03	0.815
	2.67	2.04	0.828
	2.70	2.05	0.835
	2.80	2.02	0.841
Lactate	3.00	...	0.876
	3.18	...	0.906
	3.40	...	0.916
	3.77	...	0.934
	4.00	...	0.949
Acetate	3.50	2.04	0.925
	3.65	2.03	0.932
	3.72	...	0.942
	3.87	2.07	...
	3.94	2.05	0.948
	4.10	2.05	0.955
	4.53	...	0.958
	4.81	2.04	...
	5.10	2.03	0.974
Phosphate	5.52	2.02	0.996
	5.80	2.03	1.002
	6.28	2.01	1.010
	6.50	2.02	1.016
	6.70	...	1.020

[a] Limiting currents were measured at a potential 0.35 v. more negative than the half-wave potential. Each result is the average of three determinations. The deviations from the means were ±0.03. [NNMU] was 1×10^{-4} *M*. Height of mercury column was 58.0 cm. [b] Each result is the average of three determinations. The deviations from the means were ±0.003. [NNMU] was 1.1×10^{-4} *M*. Height of mercury column was 62.2 cm.

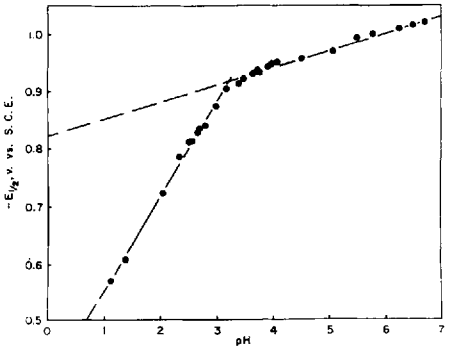


Fig. 2.—Effect of pH on half-wave potential ($E_{1/2}$) for reduction of NNMU (1.1×10^{-4} *M*) at 25.0° and at column height of 62.2 cm.

nitrosamine protonated at the microelectrode. Zahradnik (12) reported that the pKa values of *N*-nitroso derivatives of secondary aliphatic and heterocyclic amines ranged from −1.28 to −1.99 in 18.4% sulfuric acid at 22°. The absence of a spectrophotometric pKa was shown by no significant change in the absorbance and wavelength of maximum absorption of NNMU with pH at values above 1.10. There was no titratable pKa above pH 2.00 (6). There is no indication of a polarographic pKa for NNMU from a pH of 1.10–6.50.

Similar results were obtained for the reduction of *N*-ethyl-*N*-nitrosourea (NNEU) and 1,3-dimethyl-1-nitrosourea (SRI-1384). The equations describing the effect of pH on the half-wave potential for these compounds are for NNEU

$$-E_{1/2} = 0.153\ \text{pH} + 0.367 \quad \text{(pH 1.0 to 3.7)} \quad \text{(Eq. 3)}$$

$$-E_{1/2} = 0.027\ \text{pH} + 0.827 \quad \text{(pH 3.7 to 5.0)} \quad \text{(Eq. 4)}$$

and for SRI-1384

$$-E_{1/2} = 0.158\ \text{pH} + 0.330 \quad \text{(pH 1.0 to 4.3)} \quad \text{(Eq. 5)}$$

$$-E_{1/2} = 0.034\ \text{pH} + 0.808 \quad \text{(pH 4.3 to 7.0)} \quad \text{(Eq. 6)}$$

The similarities in the coefficients of the pH terms indicate that the mechanism of the reduction reaction or the electrode controlling process is the same for the three compounds and probably for all simple alkyl substituted nitrosoureas.

Effect of Height of Mercury Column on Limiting Current.—The dependence of the limiting current on the height of the mercury column is shown in

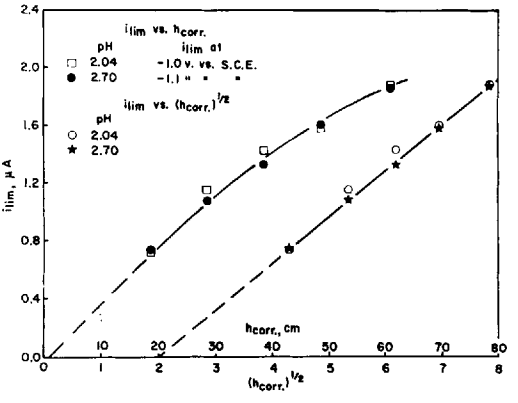


Fig. 3.—Effect of height of mercury column on limiting current for reduction of NNMU (9×10^{-5} *M*) in HCl–KCl (pH 2.04) and tartrate buffers (pH 2.70) at 25.0°.

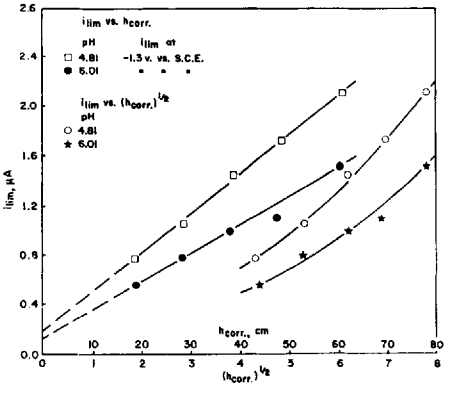


Fig. 4.—Effect of height of mercury column on limiting current for reduction of NNMU in acetate (pH 4.81, [NNMU] 9×10^{-5} *M*) and phosphate (pH 6.01, [NNMU] 8×10^{-5} *M*) buffers at 25.0°.

Figs. 3 and 4 for NNMU. In hydrochloric acid and tartrate buffers the limiting current was directly proportional to the square root of the corrected height of the mercury column (Fig. 3). Therefore, the control of the electrode process is consistent with diffusion of the nitrosourea to the microelectrode (17) in these buffers and at these pH values.

In acetate and phosphate buffers (Fig. 4) the limiting current was proportional to the corrected height of the mercury column and not to its square root. Thus, the control of the electrode process is consistent with adsorption of the nitrosourea onto the dropping mercury electrode surface (17) in these buffers and at these pH values.

These relations are consistent with the change in linearity in the half-wave potential-pH profile of NNMU (Fig. 2). Below a pH of 3.2, the electrode process is diffusion controlled, and above this pH adsorption may be the controlling process.

Effect of Buffer Concentration on Polarographic Parameters.—In tartrate buffers (0.001–0.01 *M* in each component) there was no significant change in the limiting current and half-wave potential of NNMU with buffer concentration at a particular pH. In acetate and phosphate buffers (0.001–0.01 *M* in each component) some decreases were observed in the limiting current and some positive shifts in the half-wave potential. A similar effect had been observed previously (5).

The parameters derived from polarograms in acetate buffers (0.010–0.200 *M* in each component) at several pH values are given in Table III. A typical plot of the limiting current against the concentration of acetic acid for various pH values is given in Fig. 5. Similar plots of varying slopes were obtained for the limiting current *versus* concentration of sodium acetate. It is apparent that the limiting current is dependent upon the concentrations of acetic acid, sodium acetate, and hydrogen ion in the acetate buffer region.

This functional dependence of the limiting current may be quantified in a manner similar to the procedure established for general acid-base catalysis (18). Therefore, we have

$$i_{lim.} = i_0 + a_{H^+}[H^+] + b_{HA}[HA] + c_{Ac^-}[Ac^-] \quad \text{(Eq. 7)}$$

where a_{H^+}, b_{HA}, and c_{Ac^-} are coefficients for the effect of a particular buffer species, i_0 is the limiting current in the absence of buffer species, and $[H^+]$, $[HA]$, and $[Ac^-]$ are the molar concentrations of hydrogen ion, acetic acid, and sodium acetate, respectively.

At constant pH, Eq. 7 reduces to

$$i_{lim.} = i_0 + a_{H^+}[H^+] + \{b_{HA} + c_{Ac^-}([Ac^-]/[HA])\}[HA] \quad \text{(Eq. 8)}$$

A plot of the limiting current *versus* the concentration of acetic acid is a straight line (Fig. 5) at each pH with

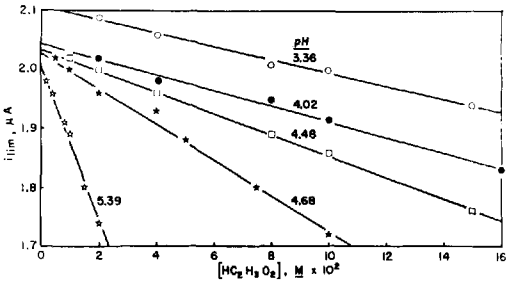


Fig. 5.—Effect of concentration of acetic acid on limiting current for reduction of NNMU (9×10^{-5} *M*) at higher buffer concentrations at 25.0° and at column height of 60.0 cm.

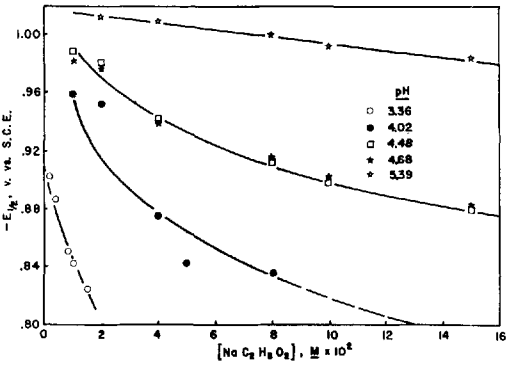


Fig. 6.—Effect of concentration of sodium acetate on half-wave potential for reduction of NNMU (9×10^{-5} *M*) at higher buffer concentrations at 25.0° and at column height of 60.0 cm.

Table III.—Effect of Buffer Concentration on Limiting Current and Half-Wave Potential for Reduction of NNMU (9×10^{-5} *M*) at Higher Concentrations of Acetic Acid and Sodium Acetate at 25.0° and at Column Height of 60.0 cm.

pH	$[HC_2H_3O_2]$, *M*	$[NaC_2H_3O_2]$, *M*	$i_{lim.}$, μA[a]	$-E_{1/2}$, v. *vs.* SCE[a]
4.48	0.010	0.010	2.02	0.989
	0.020	0.020	2.00	0.981
	0.040	0.040	1.96	0.943
	0.080	0.080	1.89	0.913
	0.100	0.100	1.86	0.898
	0.150	0.150	1.76	0.880
4.68	0.005	0.010	2.02	0.982
	0.010	0.020	2.00	0.977
	0.020	0.040	1.96	0.940
	0.040	0.080	1.93	0.916
	0.050	0.100	1.88	0.903
	0.075	0.150	1.80	0.882
	0.100	0.200	1.72	0.860
4.02	0.020	0.010	2.02	0.960
	0.040	0.020	1.98	0.953
	0.080	0.040	1.95	0.875
	0.100	0.050	1.92	0.843
	0.160	0.080	1.83	0.837
5.39	0.002	0.020	1.98	1.012
	0.004	0.040	1.96	1.010
	0.008	0.080	1.91	1.000
	0.010	0.100	1.89	0.992
	0.015	0.150	1.80	0.985
	0.020	0.200	1.74	0.970
3.36	0.020	0.002	2.09	0.903
	0.040	0.004	2.06	0.887
	0.080	0.008	2.01	0.850
	0.100	0.010	2.00	0.842
	0.150	0.015	1.94	0.825

[a] Each result is the average of three determinations. Limiting currents were measured at a potential 0.35 v. more negative than the half-wave potential.

$$\text{slope} = b_{HA} + c_{Ac^-}([Ac^-]/[HA]) \quad \text{(Eq. 9)}$$

and

$$\text{intercept} = i_0 + a_{H^+}[H^+] \quad \text{(Eq. 10)}$$

The values of the slopes of Eq. 8, when plotted against the buffer ratio in accordance with Eq. 9, gave a straight line with an intercept, b_{HA}, and a slope, c_{Ac^-}. Similarly, the values of the intercepts of Eq. 8, when plotted against the hydrogen ion concentration, gave a straight line with an intercept, i_0 and a slope, a_{H^+}. The same constants were evaluated similarly from a plot of the limiting current against the concentration of sodium acetate in accordance with an expression analogous to Eq. 8.

The resultant quantifiable expression for the limiting current in acetate buffers, [NNMU] = 9×10^{-5} M, is

$$i_{lim.}, \mu A = 2.02 + 210\,[H^+] - 0.740\,[HA] - 1.13\,[Ac^-] \quad \text{(Eq. 11)}$$

This expression is valid for acetate buffers up to 0.300 M in total buffer and where the concentration of NNMU is less than 0.16 mM. The expression is not valid below pH 3.2 for NNMU since buffer effects were not observed and would not be expected for a diffusion controlled process (17). Similar effects were found in phosphate buffers. It must be realized that the i_0 values will depend on the concentration.

The effect of concentration of sodium acetate on the half-wave potential of NNMU is shown in Fig. 6. The half-wave potential at a specific pH shifted nonlinearly in a positive direction as the buffer concentration increased. Similar buffer effects were observed for the antibiotic streptozotocin (5). It had been suggested that this effect might be due to complexation of the antibiotic with the buffer components in the region of the microelectrode since the half-wave potential of a complexed species depends upon the concentration of the complexing agent (16).

Mechanisms for Effects of Buffers on Polarographic Parameters.—Adsorption may be the electrode-controlling process (17, 19) for reduction in acetate and phosphate buffers. The shift in the half-wave potential to more positive values with increased buffer concentration (Table III) implies that such adsorption could be enhanced by the components of acetate buffers. Competition for adsorption sites may exist between the nonreducible buffer components and the nitrosourea. This would limit the numbers of molecules available for reduction and result in the observed decrease in limiting current with increased buffer concentration. Consistent with the hypothesis is the negative deviation from linearity of the plot of limiting current *versus* NNMU concentration at high acetate buffer concentrations (Fig. 7). The Ilkovic equation holds at low buffer concentrations for the same NNMU concentration range and the calibration curve is linear to 0.320 mM in NNMU for acetate buffers less than 0.020 M in total buffer.

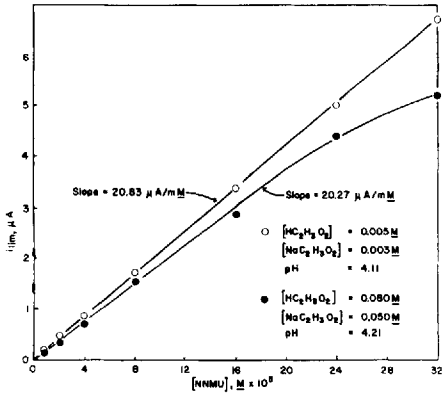
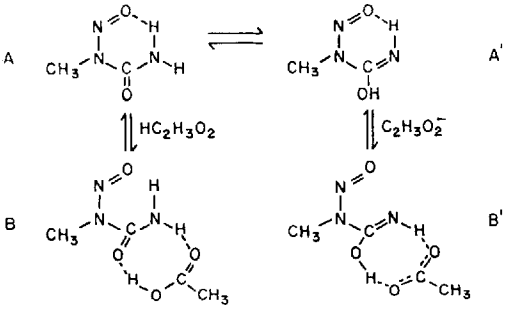


Fig. 7.—Effect of concentration of NNMU on limiting current at low and high acetate buffer concentrations at 25.0° and at column height of 60.0 cm.

In hydrochloric acid and tartrate buffers the limiting current was proportional to the concentration of NNMU to 0.320 mM.

Additional evidence for this competition with buffer, or other substance present in solution, for adsorption sites can be found in the literature. The reduction of nitromethane and nitroethane is easier in citric acid–disodium phosphate and acetate buffers than in either benzoate or phthalate buffers at the same pH (20). This is thought to be due to adsorption phenomena at the microelectrode. In the reduction of cystine (19) the wave was shifted to more negative potentials by the addition of thymol or camphor which prevents or counteracts the stereospecific adsorption of cystine necessary for its reduction.

An alternative explanation may be offered to account for these buffer effects. A catalytic current can range from an independent to a linear function of the height of the mercury column (17). Acetic acid or acetate ion may react with the nitrosourea to form a hydrogen bonded species, B or B′, respectively, in Scheme I. The possibility of



Scheme I

an equilibrium between NNMU and an intramolecularly hydrogen bonded species, A or A′, in Scheme I is also present.

On the basis of this hypothesis of complexation through hydrogen bonding of the buffer species with the nitrosourea (NNMU), it was predicted that the limiting current for the reduction of 1,3-dimethyl-1-nitrosourea (SRI-1384) would show greatly lessened dependence on concentration of the acetate buffer components. The reasoning was that this compound would have half the probability of forming B from A with acetic acid since a nonbonding methyl is substituted for one of the hydrogens on the urea. Also, the possibility of forming an analogous B′ from A′ with acetate ion would not exist since an

TABLE IV.—ANALYSIS OF VARIANCE FOR EFFECT OF CONCENTRATION OF NNMU ON LIMITING CURRENT IN ACETATE BUFFER[a] EVALUATED ON SEVERAL DAYS AT 25.0° AND AT COLUMN HEIGHT OF 60.0 cm.

Source of Variation	Degrees of Freedom	Sum of Sq.	Mean Sq.	F
Concn.	7	0.80162929	0.11451846	...
Days, factor A	2	0.00009963	0.000049815	0.842[b]
Error (*a*)	14	0.00082792	0.000059137	...
Replications, factor B	2	0.00000344	0.00000172	0.0007[c]
Interaction, AB	4	0.00123088	0.00030772	0.121[d]
Error (*b*)	42	0.10701813	0.00254805	...
Total	71	0.91080920	...	...

[a] $[HC_2H_3O_2] = 0.005\ M$, $[NaC_2H_3O_2] = 0.003\ M$, $[KCl] = 0.197\ M$. [b] 5% F = 3.74 for 2 and 14 degrees of freedom. 5% F = 3.23 for 2 and 42 degrees of freedom. [d] 5% F = 2.61 for 4 and 42 degrees of freedom.

TABLE V.—EFFECT OF SUBSTITUENTS ON POLAROGRAPHIC PARAMETERS FOR REDUCTION OF VARIOUS *N*-ALKYL-*N*-NITROSOUREAS[a] IN TARTRATE BUFFERS AT 25.0° AND AT COLUMN HEIGHT OF 60.0 cm.

Compd.	$-E_{1/2}$, v. *vs.* SCE pH = 2.51[e]	$-E_{1/2}$, v. *vs.* SCE pH = 2.80[f]	$i_{lim.}$, μA pH = 2.80[f]	Substituent Constant, σ^{*g}	$-\log(k, \text{sec.}^{-1})^h$
NNMU[b]	0.809	0.846	1.98	0.000	4.36
NNEU[b]	0.741	0.804	1.83	−0.100	4.38
NNBU[b]	0.631	0.646	1.56	−0.130	4.31
NNisoBU[b]	0.651	0.680	1.31	−0.125	4.31
NNAU[b]	0.682	0.692	1.40	...	4.10
NNBeU[b]	0.558	0.610	1.32	+0.215	3.97
NNCyU[b]	0.603	0.624	1.31	−0.150	2.70
SRI-1834[b]	0.530	0.570	1.20	+0.080	4.22
SRI-1384[b]	...	0.730	1.88	...	...
SRI-1631[b]	...	0.586	2.45	...	...
SRI-1720[b]	...	0.580	1.40	...	...
SRI-1746[b]	...	0.542	1.33	...	...
SRI-1833[c]	...	0.598	1.41	...	...
SRI-1879[d]	...	0.478 (1) 0.930 (2)	0.564 (1) 0.912 (2)	...	...

[a] All compounds were initially dissolved in 8% alcohol since some were difficultly soluble in water alone. Each result is the average of three determinations. Limiting currents were measured at a potential 0.35 v. more negative than the half-wave potential. [b] Polarogram was a single, well-defined wave. [c] Polarogram was a single wave, not well-defined. [d] Polarogram was a double, well-defined wave. Master solution became darkened after about 1 hr. at room temperature. [e] Concentration of *N*-nitrosourea was $8 \times 10^{-5}\ M$. [f] Concentration of *N*-nitrosourea was $9 \times 10^{-5}\ M$. [g] From *Reference 24*. At 35.0° and pH = 5.95 (6).

unsubstituted urea is demanded in the formation of B′.

The limiting currents for 1,3-dimethyl-1-nitrosourea ($8 \times 10^{-5}\ M$) in acetate buffers (0.010 M − 0.200 M in each component) were in order of increasing buffer concentration: 1.59, 1.60, 1.58, 1.51 μA at pH 4.27; 1.55, 1.52, 1.58, 1.57, 1.51 μA at pH 4.85; and 1.54, 1.52, 1.53, 1.52 μA at pH 5.50. This represents less than a 5% decrease in the limiting current with the same increasing concentrations of acetate buffer components that caused large (more than 10%) and systematic decreases in the limiting current of NNMU (Fig. 5, Table III).

In the same concentration ranges of acetate buffers as used for NNMU, the $-E_{1/2}$ values of 1,3-dimethyl-1-nitrosourea were 0.855, 0.826, 0.806, 0.800 v. *versus* SCE at pH 4.27; 0.902, 0.860, 0.837, 0.830, 0.823 v. *versus* SCE at pH 4.85; and 0.960, 0.946, 0.940, 0.938 v. *versus* SCE at pH 5.50. The shift in $E_{1/2}$ to more positive values with increased buffer concentration is similar but to a lesser extent than for NNMU (Table III).

The compound 1,3-dimethyl-1-nitrosourea was found to be more easily reduced than NNMU in acetate buffers at a particular pH. This is also consistent with the mechanism proposed in Scheme I. The presence of a 3-methyl group may inhibit the intramolecular hydrogen bonding of A by a statistical factor of 0.5 and permit more readily the reduction of the *N*-nitroso group.

A plausible mechanism for the reduction of the *N*-nitroso group in NNMU is through the hydroxylamino to an amino group. This route has been well-established for the reduction of *N*-nitrosamines (8–14).

Reliability of Polarographic Assay Method.—The effect of concentration of NNMU on the limiting current in acetate buffer ($[HC_2H_3O_2] = 0.005\ M$; $[NaC_2H_3O_2] = 0.003\ M$; $[KCl] = 0.197\ M$) was evaluated on 3 days at eight concentrations ranging from 0.004 mM in NNMU to 0.320 mM. Three replications were run at each concentration on each day. An analysis of variance (21) of these data was performed in the following manner. The regression coefficients were determined for each of the nine sets of data. From each regression equation a theoretical value of y ($i_{lim.}$, μA) was calculated for each fixed value of x (concentration, mM). The effect of concentration on the values of the limiting current was removed by taking the ratio $y_{exptl.}/y_{theoret.}$. The analysis of variance was then performed on the values obtained for this ratio for each concentration on each day. The results of the analysis of variance are shown in Table IV. The determinations of the limiting current did not vary significantly from day to day. The standard error

TABLE VI.—EFFECT OF SUBSTITUENTS ON POLAROGRAPHIC PARAMETERS FOR REDUCTION OF VARIOUS *N*-ALKYL-*N*-NITROSOUREAS[a] (6×10^{-5} *M*) IN PHOSPHATE BUFFER (pH = 6.03) AT 25.0° AND AT COLUMN HEIGHT OF 60.2 cm.

Compd.	$-E_{1/2}$, v. *vs.* SCE	$i_{lim.}$, μA	Max. Biological[e] Effectiveness (% ILS)
NNMU[b]	0.985	1.29	86
NNEU[b]	0.980	1.22	<40
NNBU[b]	0.963	1.16	<40
NNisoBU[b]	0.982	1.02	...
NNAU[b]	0.984	1.08	<40
NNBeU[c]	0.742 (1)	0.500 (1)	<25
	1.068 (2)	0.632 (2)	...
SRI-1384[b]	0.998	1.23	61
SRI-1631[c]	0.818 (1)	1.50 (1)	100
	1.083 (2)	0.660 (2)	...
SRI-1834[c]	0.754 (1)	0.534 (1)	...
	1.027 (2)	0.680 (2)	...
SRI-1720[c]	0.743 (1)	0.576 (1)	184
	1.050 (2)	0.706 (2)	...
SRI-1746[c]	0.730 (1)	0.690 (1)	<40
	1.068 (2)	0.536 (2)	...
SRI-1833[c]	0.785 (1)	0.808 (1)	...
	1.063 (2)	0.604 (2)	...
SRI-1879[c,d]	0.180 (1)	0.296 (1)	...
	0.953 (2)	0.724 (2)	...

[a] All compounds were initially dissolved in 8% alcohol since some were difficultly soluble in water alone. Each result is the average of three determinations. Limiting currents were measured at a potential 0.35 v. more negative than the half-wave potential. [b] Polarogram was a single, well-defined wave. [c] Polarogram was a double, well-defined wave. [d] Master solution became darkened after about 1 hr. at room temperature. [e] Reported as per cent increase in life span of mice treated with leukemia L1210 over that of controls (2).

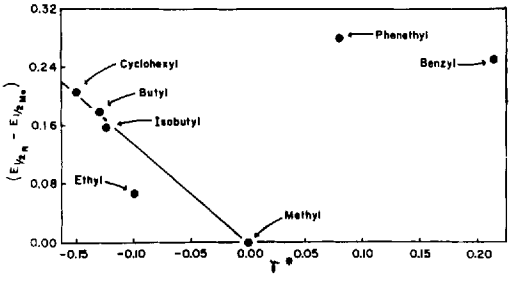


Fig. 8.—Effect of substituents on ease of reduction of various *N*-alkyl-*N*-nitrosoureas (8×10^{-5} *M*) in tartrate buffer (pH 2.51) at 25.0° and at column height of 60.0 cm.

for the difference between two-day means was 0.91%. The standard error for the difference between two concentration means was 1.31%. The regression equation obtained from the average values of the regression coefficients is

$$i_{lim.}, \mu A = 20.85\ [\text{NNMU}],\ M \times 10^3 + 0.062 \quad \text{(Eq. 12)}$$

The intercept is not significantly greater than zero.

The reproducibility of the assay method from day to day is good and the procedure is quite sensitive. A concentration of NNMU of 1×10^{-6} *M* could be detected. The polarographic method of assay is equally or more sensitive than other procedures for the assay of nitrosoureas (5, 6, 22, 23). Unlike the colorimetric method (5, 22, 23), it may be used in a pH region where the compounds are most stable, *i.e.*, between pH 3.0 and 5.0 (5, 22), and no corrections for drug instability are required. The polarographic method is less time consuming than the colorimetric method which requires a 45-min. incubation period at 50° (23). Although definite buffer effects due to adsorption or chemical reaction have been shown to occur in acetate buffers, a linear calibration curve is obtained up to concentrations of 0.320 m*M* in NNMU as long as the concentration of one of the buffer components does not exceed 0.010 *M*. Therefore, it is suggested that routine assays of these compounds be run in appropriate acetate buffers at 25.0°.

Effect of Substituents on Ease of Reduction of Various N-Nitrosoureas.—The effect of substituents on the ease of reduction of various alkyl substituted nitrosoureas is shown in Tables V and VI for several buffer systems. The data indicate that all of the compounds are more easily reduced than the parent methyl compound with more positive half-wave potentials. A reasonable correlation (Fig. 8) between the change in the half-wave potentials and the Taft (24) substituent constants (σ^*) is noted for the simple alkyl groups, *i.e.*, methyl, ethyl, butyl, etc., in tartrate buffer. Substituents containing an aromatic ring, *i.e.*, benzyl and phenethyl, did not follow this relationship. Thus, the ease of reduction of the nitrosourea due to substitution of another simple alkyl group for methyl seems to depend primarily on an inductive effect. However, it should be noted that the reduction reaction is facilitated by both electron donating groups (ethyl, butyl, etc.) and by electron withdrawing groups (benzyl and phenethyl). It is possible that steric factors may also be involved in the case of the last two compounds.

The most active compound from a biological standpoint is 1,3-bis-(2-chloroethyl)-1-nitrosourea (SRI-1720), which is one of the easiest to reduce.

In the diffusion controlled region (tartrate buffer) the limiting current decreased in approximate order of increasing molecular weights for the simple

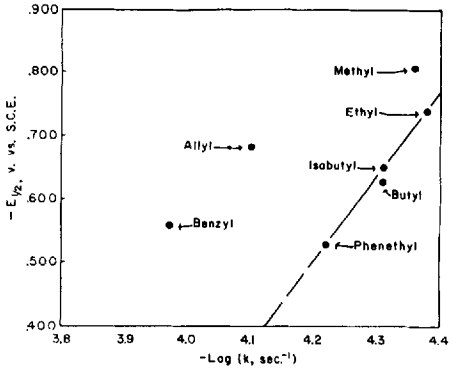


Fig. 9.—Relationship between half-wave potentials (pH 2.51) of various *N*-alkyl-*N*-nitrosoureas (8×10^{-5} *M*) at 25.0° and rate constants (k, sec.$^{-1}$ at 35.0° and pH 5.95) for hydroxyl ion catalyzed solvolysis.

alkyl substituted nitrosoureas, *i.e.*, NNMU, NNEU, NNBU, etc. Compounds with large groups in the R and R′ positions (Table V), *i.e.*, the disubstituted nitrosoureas, show deviations in this respect and imply that steric factors may affect the reduction reaction. Apparently both nitroso groups in SRI-1631 are equivalent and are reduced simultaneously, producing a current about twice that expected for a molecule of this size.

In the adsorption controlled region (phosphate buffer) the changes in the half-wave potentials and the limiting currents due to different substituents are not so pronounced as in the diffusion controlled region. However, many of the nitrosoureas, *i.e.*, NNBeU, SRI-1834, and those disubstituted derivatives with large groups in the R or R′ positions, exhibit a double wave in phosphate buffer (Table VI), indicating that the adsorbed species may be reduced more easily than the free form. Again steric factors may assume a role in the reduction of these particular compounds.

The relationship between the half-wave potentials and the apparent first-order rate constants (k, sec.$^{-1}$) for the solvolysis of various *N*-alkyl-*N*-nitrosoureas (6) in the neutral pH region is shown in Table V and in Fig. 9. In this case large discrepancies are noted when the substituents contain a double bond close to the electroactive site, *i.e.*, allyl and benzyl.

No correlation (Table VI) could be demonstrated between the half-wave potentials of the *N*-nitrosoureas and the available biological activity data (2). However, the biological data are limited and those which are available may not be entirely reliable due to the instability of these compounds in the neutral pH region.

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Rheological Study of Selected Pharmaceutical Semisolids

By JAMES C. BOYLAN

Rheograms were obtained with a Ferranti-Shirley cone and plate viscometer at 20, 25, 30, and 35° for 13 pharmaceutical semisolids. As the temperature was raised, all the products studied showed a decrease in viscosity, thixotropy, and yield value. For many of the semisolids there appears to be a straight line relationship between thixotropic area and temperature. For ointments whose base is predominately white ointment, the viscosity is reduced by a factor of 0.5 for every 5° rise in temperature. At 35° many of the products studied showed similar values for thixotropy and viscosity.

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Schulte and Kassem (1–6) have recently published an excellent series of papers dealing with the flow properties of several semisolid systems including silicone gels, triglyceride gels, polyethylene gels, polyethylene glycol gels, various vaselines, and carbohydrate gels. In addition,

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The author thanks Dr. R. R. Pfeiffer for helpful comments.

TABLE I.—SEMISOLIDS STUDIED AND THEIR INGREDIENTS[a]

Active ingredient	White Petrolatum U.S.P.	Petrolatum N.F.	White Ointment U.S.P.	Zinc Oxide Paste U.S.P.	Neomycin Sulfate Ointment U.S.P.	Ammoniated Mercury Ointment U.S.P.	Boric Acid Ointment	Sulfur Ointment U.S.P.	Bacitracin Ointment U.S.P.	Ichthammol Ointment N.F.	Cyclomethycaine Ointment	Cyclomethycaine Cream	Cyclomethycaine Jelly
White petrolatum U.S.P.	100.0	...	95.0	25.0	5.0	5.0	10.0	10.0	1.0	10.0	1.0	0.5	0.75
Petrolatum N.F.	...	100.0	...	50.0	90.3	87.4	80.7	76.0	53.0	80.0	80.1	...	...
Mineral oil U.S.P.	...	...	...	...	4.7	3.0	5.0	10.0	23.0	...	...	...	...
White wax U.S.P.	...	...	5.0	...	...	4.6	4.3	4.0	...	...	3.0	...	...
Anhydrous lanolin U.S.P.	...	...	...	...	...	...	...	...	23.0	10.0	15.0	...	...
Stearic acid U.S.P.	...	...	...	...	...	...	...	...	...	...	...	15.0	...
Cetyl alcohol N.F.	...	...	...	...	...	...	...	...	...	...	...	3.0	...
Polypropylene glycol	...	...	...	...	...	...	...	...	...	...	...	...	30.0
Sodium phosphate N.F. (dried)	...	...	...	...	...	...	...	...	...	...	...	0.4	0.3
Sodium lauryl sulfate U.S.P.	...	...	...	...	...	...	...	...	...	...	...	0.5	...
Polysorbate 80 U.S.P.	...	...	...	...	...	...	...	...	...	...	...	...	0.3
Polysorbate 60	...	...	...	...	...	...	...	...	...	...	...	1.0	...
Sorbitan monostearate	...	...	...	...	...	...	...	...	...	...	...	1.5	...
Hydroxypropyl methylcellulose 1500	...	...	...	...	...	...	...	...	...	...	...	...	3.3
Fluid benzoin	...	...	...	...	...	...	...	...	...	...	0.9	...	...
Glycerin U.S.P.	...	...	...	...	...	...	...	...	...	...	...	15.0	...
Purified water U.S.P.	...	...	...	...	...	...	...	...	...	...	...	63.1	65.3
Starch U.S.P.	...	...	...	25.0	...	...	...	...	...	...	...	...	...

[a] All figures are expressed as per cent w/w. Preservative materials are also present in some of the semisolids.

Van Ooteghem (7) has written a helpful review of the methods currently used to measure rheological properties of ointments.

The purpose of this paper is to report the rheological characteristics of several pharmaceutical semisolid products in the temperature range 20–35° and to show how these results can be valuable to the formulator and manufacturer of these products.

EXPERIMENTAL

Materials.—Except for white petrolatum, petrolatum, and white ointment, the semisolid products studied were in commercial 1-oz. tubes.[1] Table I lists the products studied and their component ingredients. The age of the semisolids varied from 4 to 21 months, with most of the samples being from 7 to 14 months in age. Storage was, in all cases, at room temperature.

Rheological Evaluation.—The viscometer used in this study was a Ferranti-Shirley cone and plate viscometer[2] equipped with a 1200-Gm. cm. spring, an automatic gap-setting device, an *x–y* recorder,[3] and a constant-temperature water bath.[4] The use of this viscometer has been described elsewhere (8). Calibration of the instrument was carried out using N.B.S. standard viscosity oils. The temperature region 20–35° was studied because this range includes the normal range of temperatures encountered during storage or application to the skin (see *Results and Discussion*). The water bath maintained the sample temperature at ±0.1° of the reported temperatures.

The sample to be evaluated (about 1 ml.) was gently squeezed from a tube onto the plate of the viscometer. The plate was immediately raised into position with the cone. The sample then remained undisturbed for 5 min. in this position (to allow temperature equilibration) before obtaining the rheogram. All rheograms were obtained using a truncated cone having an angle of 33 min., 26 sec., and a radius of 2 cm. The instrument was set at an upsweep time of 120 sec., a downsweep time of 120 sec., a maximum r.p.m. of 100, and a scale expansion of 4×. In the case of a few 20 and 25° determinations it was necessary to use a scale expansion of 5×.

The method of reporting a meaningful value for viscosity presented a problem. The products studied were plastic and pseudoplastic materials, all exhibiting thixotropy. It is normally necessary to use different equations when reporting viscosity values for plastic and pseudoplastic materials. Debate continues on whether a satisfactory equation exists for pseudoplastic materials, although the equation of Farrow *et al.* (9) seems to serve adequately for most purposes. Cross (10) has recently discussed the mathematical representation of pseudoplastic flow in some detail.

For the purposes of this study, the viscosity values reported were calculated as follows (11).

[1] Eli Lilly and Co., Indianapolis, Ind.
[2] Ferranti Electric Co., Plainview, Long Island, N. Y.
[3] Houston Instrument Co., Bellaire, Tex. (model HR-92).
[4] Brinkmann Instrument Co., Westbury, N. Y. (Haake model F).

TABLE II.—RHEOGRAMS: SCALE DEFLECTION IN dynes/cm.^{2} (UPCURVE)

	At 1074 sec.$^{-1}$ (100 r.p.m.)				At 120 sec.$^{-1}$ (11 r.p.m.)			
	20°	25°	30°	35°	20°	25°	30°	35°
White petrolatum U.S.P.	11,600	8,135	4,400	2,665	13,000	8,150	4465	2135
Petrolatum N.F.	14,470	6,000	5,600	3,065	14,735	7,465	4065	1600
White ointment U.S.P.	11,375	8,935	4,265	2,400	15,000	8,935	4265	1600
Zinc oxide paste U.S.P.	...	21,335	13,065	6,735	...	13,065	7000	2935
Neomycin sulfate ointment U.S.P.	14,470	8,000	4,200	2,665	10,265	5,150	3135	1735
Ammoniated mercury ointment U.S.P.	30,000	14,750	7,300	3,735	16,465	9,465	5135	2400
Boric acid ointment	21,865	12,665	6,665	3,465	13,000	7,335	4000	1500
Sulfur ointment U.S.P.	11,335	7,850	4,400	2,265	9,000	5,150	2665	1465
Bacitracin ointment U.S.P.	10,265	6,875	4,665	2,400	5,535	3,625	1465	665
Ichthammol ointment N.F.	21,470	14,750	7,935	5,200	11,735	8,150	3730	1865
Cyclomethycaine ointment	12,200	8,000	4,300	2,800	10,000	6,250	3200	2000
Cyclomethycaine cream	1,335	1,500	1,465	1,335	1,000	1,125	935	935
Cyclomethycaine jelly	11,865	12,500	10,800	10,000	8,000	8,000	5700	5265

The potentiometer scale reading was recorded for the maximum sample shear rate (100 r.p.m. in all cases). The viscosity (in poises) was then calculated from Eqs. 1 and 2.

$$R = \text{scale reading} \times \text{scale expansion} \quad \text{(Eq. 1)}$$

$$\eta = \frac{R}{100} \times C \quad \text{(Eq. 2)}$$

where η is the viscosity in poises, and C is a cone constant. The value of C is determined by Eq. 3

$$C = \left(\frac{3}{2\pi V}\right)\left(\frac{aT}{r^3}\right) \quad \text{(Eq. 3)}$$

where a is the cone angle in radians, T is the torque spring constant in dyne-cm./division (×5 scale), V is the angular velocity in radians/sec., and r is the radius of the cone in cm. The value of C for the truncated cone used in this study was 13.36.

The thixotropic area was measured directly in square inches with a compensating planimeter.[5]

Effect of Aging.—Rheological changes with time have been observed in several pharmaceutical systems (12–15). Consequently, it was necessary to ascertain whether any significant changes were occurring in the semisolids reported here. This was accomplished for a given material by obtaining rheograms of a second production lot of a different age and also by rerunning the original lot at a later date.

RESULTS AND DISCUSSION

Effect of Temperature.—The results obtained are shown in Table II and Figs. 1–14. As the temperature was raised from 20 to 35°, all products studied demonstrated a decrease in viscosity, thixotropy, and yield value. This was expected and agrees with the data of Schulte and Kassem (1–6) for other pharmaceutical semisolids.

Effect of Aging.—There was no evidence that the semisolids studied were undergoing any significant rheological changes during the course of this study. When stored properly, it was felt that if significant changes in the rheograms were going to occur, they would likely occur in the first few weeks after manufacturing. As was noted previously, the most recently prepared material was 4 months old.

Petrolatum.—Although the particular petrolatums studied differ slightly in both viscosity and thixotropy, the rheogram of each has a characteristic initial "spur" at low rates of shear (Table II, Figs. 1 and 2).

In their comprehensive paper on petrolatum from various sources, Schulte and Kassem (4) pointed out that petrolatum is a mixture of *n*, iso, and isocyclic paraffins. They show that the ratio of these constituents to each other determines the rheological properties of the particular petrolatum studied. *n*-Paraffins form the characteristic structure network and petrolatums with a low content of *n*-paraffins are considered good for ointments. The change of structure involution is greatly dependent upon the isoparaffin content. A gel network built up of large crystallites is more susceptible to mechanical strain than a gel whose network is built up of very small crystallites. In the presence of isoparaffins a finer gel structure forms. With a decrease in crystallite size, the viscosity of the petrolatum increases also.

White Ointment Series.—The addition of 5% white wax to white petrolatum when preparing white ointment increases the thixotropy somewhat and modifies the shape of the "spur," but has only a slight effect on the viscosity (Table II, Figs. 2 and 3).

Ammoniated mercury, sulfur, and boric acid ointments are composed of white ointment, mineral

[5] Keuffel and Esser Co. (model 3636).

oil, and medicament (Table I). Neomycin ointment and ammoniated mercury ointment basically differ only in the lack of white wax in the neomycin product (Table I). This lack, combined with the slight increase in mineral oil and white petrolatum in the neomycin ointment, results in a less viscous and less thixotropic product with a smaller yield value (Table II, Figs. 4 and 5). The base for sulfur ointment and boric acid ointment differ in that the sulfur ointment contains 5% more mineral oil (and, consequently, 5% less white petrolatum). As a result, the viscosity of sulfur ointment is less than that of boric acid ointment, although the thixotropy and the shape of the rheograms of the two products vary only slightly (Figs. 4 and 5).

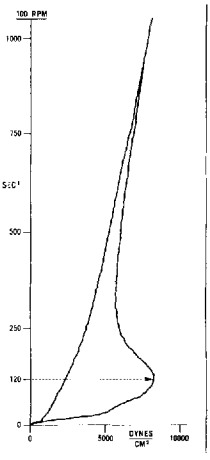

Fig. 1.—Rheogram of white petrolatum U.S.P. at 25°.

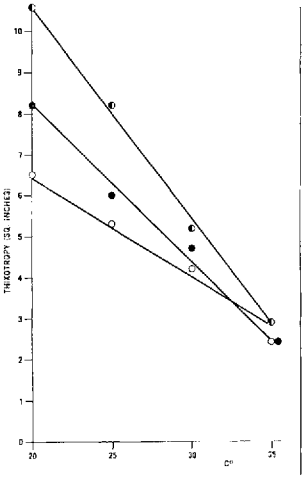

Fig. 2.—The effect of temperature on the thixotropy. Key: ○, white petrolatum; ●, petrolatum; ◐, white ointment.

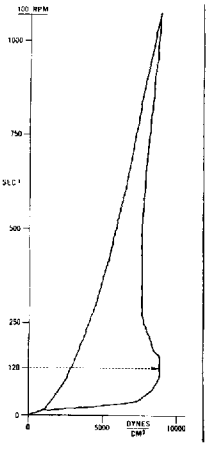

Fig. 3.—Rheogram of white ointment U.S.P. at 25°.

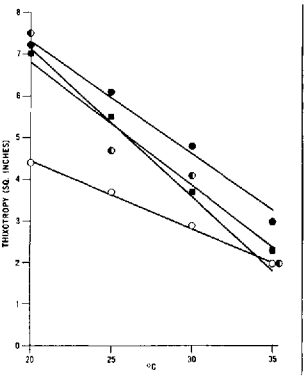

Fig. 4.—The effect of temperature on thixotropy of ointments. Key: ○, neomycin; ●, ammoniated mercury; ◐, boric acid; ■, sulfur.

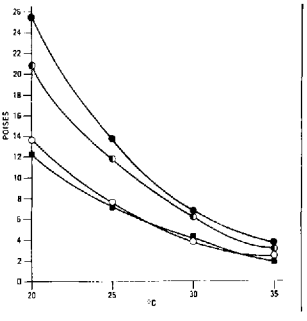

Fig. 5.—The effect of temperature on the viscosity of ointments. Key: ○, neomycin; ●, ammoniated mercury; ◐, boric acid; ■, sulfur.

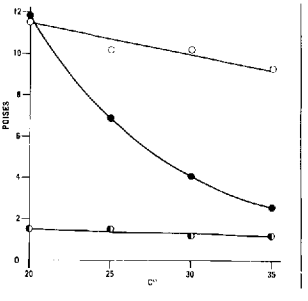

Fig. 6.—The effect of temperature on viscosity. Key: ○, cyclomethycaine jelly; ●, ointment; ◐, cream.

Figure 5 and cyclomethycaine[6] ointment in Fig. 6 illustrate a trend of importance to the pharmaceutical formulator—namely, that for every 5° rise in temperature the viscosity is reduced by a factor of 0.5. The viscosity of these ointments, containing predominately white ointment, demonstrate a definite first-order relationship between (loss of) viscosity and temperature (increase).

It may be seen from Figs. 2, 4, and 7 that there appears to be a linear relationship between thixotropic area and temperature (in the region 20–35°) for neomycin, bacitracin, ammoniated mercury,

[6] Marketed as Surfacaine by Eli Lilly and Co., Indianapolis, Ind.

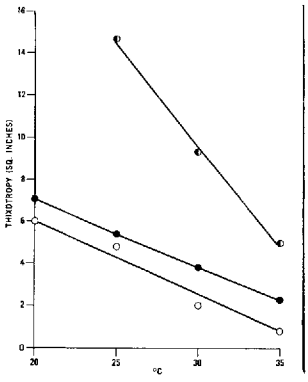

Fig. 7.—The effect of temperature on thixotropy. Key: ○, bacitracin ointment; ●, ichthammol ointment; ◐, zinc oxide paste.

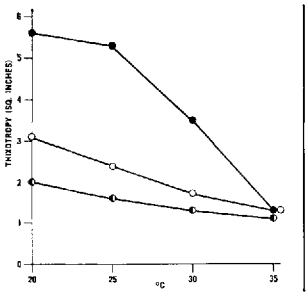

Fig. 8.—The effect of temperature on thixotropy. Key: ○, cyclomethycaine jelly; ●, ointment; ◐, cream.

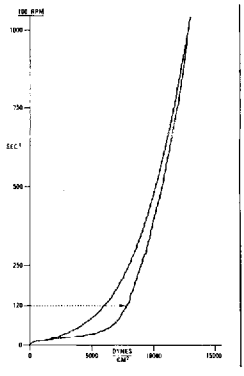

Fig. 9.—Rheogram of cyclomethycaine jelly at 25°.

sulfur, ichthammol, and white ointments, zinc oxide paste, and petrolatum.

Cyclomethycaine Series.—The cyclomethycaine series shows interesting and unique differences (Figs. 6, 8–11). Cyclomethycaine cream exhibits the least viscosity and thixotropy of any of the semisolids studied in this report. The rheograms for cyclomethycaine jelly retain their unique shape (Fig. 9) over the entire temperature range studied. In addition, it can be seen from Figs. 6 and 8 that there is relatively little change in thixotropy and only moderate change in viscosity for this product. This jelly is predominately a polypropylene glycol–water base (Table I) which would suggest a potential use as an all climate vehicle. Cyclomethycaine ointment follows the general pattern established by the other predominately white ointment base products.

Zinc Oxide Paste.—Zinc oxide paste, because it is designed as a protective and adsorptive material, is a deliberately stiff product with poor spreading and melting qualities. These characteristics are borne out by Figs. 7, 12, and 13. Using the Ferranti-Shirley cone and plate viscometer under the test conditions described, it was not possible to obtain data for zinc oxide paste at 20°.

Spreading.—The ease of application of a pharmaceutical semisolid to the surface of the skin is an

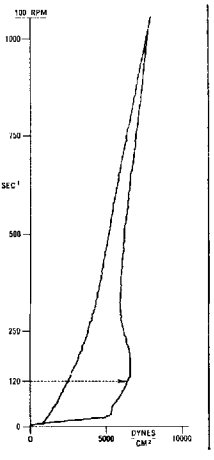

Fig. 10.—Rheogram of cyclomethycaine ointment at 25°.

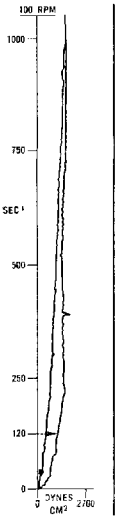

Fig. 11.—Rheogram of cyclomethycaine cream at 25°.

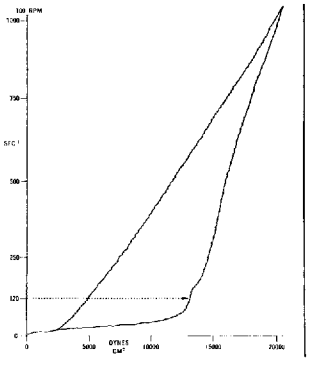

Fig. 12.—Rheogram of zinc oxide paste U.S.P. at 25°.

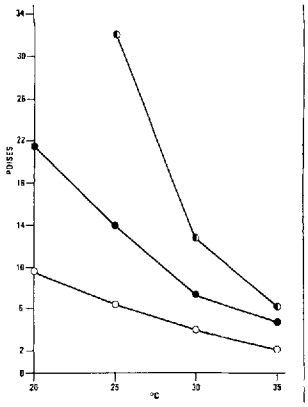

Fig. 13.—The effect of temperature on viscosity. Key: O, bacitracin ointment; ●, ichthammol ointment; ◐, zinc oxide paste.

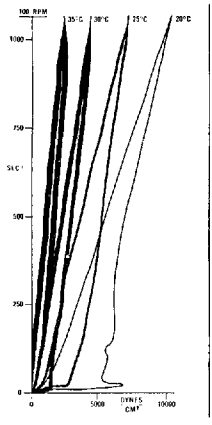

Fig. 14.—The effect of temperature on the rheogram of bacitracin ointment U.S.P.

important factor in consumer acceptance. Each individual applies ointment-like materials to the skin with a slightly different motion, stroke, and rate. Any rheological estimate of this process would, therefore, be limited accordingly. Henderson *et al.* (16) have approximated the rate of shear encountered when spreading an ointment on a surface by assuming a stroke averaging 6 cm., a rate of 4 strokes/sec., and an ointment layer thickness of 1 to 3 mm. Under these conditions they calculated a shear rate of 120 sec.$^{-1}$. If this value is used as a guide, recognizing its shortcomings, an estimate of the spreading resistance can be graphically shown by extending a dashed line through the rheograms at 120 sec.$^{-1}$. On the upcurve of nearly every rheogram 120 sec.$^{-1}$ occurs in a region of great resistance to flow. For example, in the instances of white petrolatum and white ointment (Figs. 1 and 3), 120 sec.$^{-1}$ represents the point of greatest resistance at low rates of shear. In nearly all the rheograms the area between 0 and 250 sec.$^{-1}$ appears to be an extremely important one when characterizing the spreading properties of pharmaceutical semisolids. A rheological investigation of this region has been undertaken and will be the subject of a forthcoming communication.

Relation to Body Temperature.—In his authoritative text, Rothman (17) discusses the temperature of the various regions of human skin under a wide variety of conditions. He clearly shows that there is a great variation in the temperature of human skin depending, *e.g.*, on the region of the body, the quantity and type of clothing worn, the degree of activity and state of health of the subject, environmental conditions, and whether the individual has eaten recently. Under these varying conditions a normal, healthy person's skin temperature will vary from 25 to 35°. If the extremities, especially the toes, are disregarded, the body surface temperature will probably fall between 30 and 35°.

At 35° many of the products studied had nearly the same thixotropy and nearly the same viscosity. This is as expected, since these products are designed to melt at or near the temperature of the human skin surface. An example of a product designed to melt at body temperature is bacitracin ointment U.S.P. (Lilly). As the temperature progresses from 20 to 35° the viscosity, thixotropy, and yield value of this product substantially decrease (Fig. 14). This figure, with minor modifications, is typical of the rheological changes the other semisolids in this study undergo in this temperature range. The curves shown in Fig. 14 also agree with the data of Schulte and Kassem (1–6) for other pharmaceutical semisolids.

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Statistical Procedures for Bioassays When the Condition of Similarity Does Not Obtain

By C. PHILIP COX and PAUL E. LEAVERTON*

Well-established statistical procedures are available for the analysis of dilution parallel line or slope ratio assays for which the condition of similarity obtains. Research scientists have long been aware that this condition is commonly violated, in log-dose response assays, for example, divergent rather than parallel lines may be obtained. The deviation cannot always be traced to deficient experimental techniques. In fact, as indications of differences in the response processes of the standard and test preparations, or as indications of impure test preparations, such findings may provide the most important inference from an assay experiment and immediately suggest further investigation into the causes of the differences. Statistical procedures have been developed to describe the phenomena in quantitative terms and, especially, to permit potency comparisons. The procedures may also have merit even in dilution assay situations where the condition of similarity may apparently be violated if appreciable differences between the responses of the standard and test preparations result from poorly matched doses.

THE TERM "analytical dilution assay" refers to an assay of a preparation of unknown potency which can be regarded as nothing but a dilution of the standard preparation in a diluent which does not contribute to the response by either chemical or physical properties. In such cases it follows that when response is plotted against log-dose the curves for the two preparations are the same apart from a constant relative displacement parallel to the log-dose axis. Furthermore, as was pointed out by Gaddum (1), the relative potency estimate obtained from an analytical dilution assay should not be dependent on the particular assay circumstances. The estimate should agree with one obtained by, for example, chemical determinations since both procedures should give estimates of the reciprocal of the dilution factor.

When the log-dose response curves are straight parallel lines well-established statistical procedures, such as those described by Bliss (2) and Finney (3), are available for obtaining the relative potency estimate. Estimation procedures for quadratic parallel curves have been described by Bliss (4) and Elston (5). Relatedly, Leaverton (6) has discussed methods, based on techniques described by Lewish (7), for fitting quadratic curves constrained to be strictly monotonic for use in bioassay contexts.

Similar statistical procedures are used if parallel log-dose response curves are obtained—for a particular response—even though the inert diluent assumption is not generally true. Such assays have been termed "comparative assays." Since both types of assay involve comparative experiments, however, there are grounds for using the general term "assay" for either type when distinction is not essential, the qualification "analytical dilution" or just "dilution" being used to distinguish the subclass of assays defined above.

As a prerequisite for estimation of relative potency in the above cases, statistical analyses incorporate tests for the relevance of the assumed mathematical model to the observed phenomena. In particular, an index sensitive to divergence, *i.e.*, to departure from parallelism, can be obtained from the sum of squares for interaction between preparations and log-doses in the analysis of variance. In research, as distinct from routine, bioassay situations it is usually not possible to assert that an unknown preparation is a dilution of the standard preparation; assays may, in fact, be initiated to examine just this question. As isolated from an animal or plant organism, for example, the unknown preparation may consist of a mixture of substances which may affect the response in different degrees. Accordingly, assay research workers, in pharmacology and endocrinology, for examples, have long been aware that assays where divergent rather than parallel lines are obtained are common, although the statistical significance of the divergence term may occasionally be masked because the assay is of low precision.

It should also be remembered that except in the somewhat unusual case that the log-dose response relationship is exactly rectilinear over a wide response range—as distinct from being approximately straight locally—it is an event with probability zero that doses will be so chosen to

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give exactly parallel lines even for an analytical dilution assay.

One early idea on statistical procedures appropriate for divergent line assays was put forward by Ing *et al.* (8), who suggested the computation of an average relative potency determined over a wide range of doses. Later, in Gibbs *et al.* (9), an estimate of the logarithm of the relative potency was computed at the 50% response level as a not strictly valid but nevertheless useful comparison. Neither of these suggested procedures, however, properly incorporate the basic fact that the relative potency is different for different doses or responses. And it was pointed out by Grimshaw and D'Arcy (10) that there was then no adequate method for quantitative assessment in such situations.

Discussions of the above introductory points and related statistical aspects may be found in Thompson (11), Leaverton (6), Cornfield (12), and Finney (13). In Cornfield (12), a procedure is presented for estimating the logarithm of the relative potency as a function of the log-dose in cases when linear log-dose response curves for both preparations can be estimated for each of a number of experimental subjects. Procedures are given here for divergent line assays obtained using the common experimental plan in which only one preparation-dose combination is observed on each of a number of experimental subjects.

SPECIFICATION

Let X_S and X_T denote log-doses of the standard and test preparations, respectively, and let Y denote the response.

Then linear log-dose response lines can be written as,

$$Y = \alpha_S + \beta_S X_S \qquad \text{(Eq. 1)}$$

$$Y = \alpha_T + \beta_T X_T \qquad \text{(Eq. 2)}$$

for the standard and test preparations, respectively.

The fact that, if $\beta_S \neq \beta_T$, the logarithm of the relative potency is a function of the log-dose or the response can now be expressed in any of the three ways *A*, *B*, and *C* described below.

A. Linear Relation Between Equipotent Log-Doses.—If log-doses X_S and X_T give the same response, Y, Eqs. 1 and 2 show that X_S and X_T are related by

$$\alpha_S + \beta_S X_S = \alpha_T + \beta_T X_T$$

that is,

$$X_S = -\frac{(\alpha_S - \alpha_T)}{\beta_S} + \frac{\beta_T}{\beta_S} X_T \qquad \text{(Eq. 3)}$$

B. Log (Relative Potency) as a Linear Function of Log-Dose.—If $\mu(X_T)$ denotes the logarithm of the relative potency at dose X_T, that is, $\mu(X_T)$ is the difference between equipotent log-doses, we have, at log-dose X_T,

$$\mu(X_T) = X_S - X_T$$

so that, from Eq. 3,

$$\mu(X_T) = \frac{(\alpha_S - \alpha_T)}{\beta_S} + \left(\frac{\beta_T}{\beta_S} - 1\right) X_T \qquad \text{(Eq. 4)}$$

C. Log (Relative Potency) as a Linear Function of Response.—If $\mu(Y)$ denotes the logarithm of the relative potency at response Y, $\mu(Y)$ is the difference between equally effective log-doses so that,

$$\mu(Y) = \frac{Y - \alpha_S}{\beta_S} - \frac{Y - \alpha_T}{\beta_T} \qquad \text{(Eq. 5)}$$

$$= -\left(\frac{\alpha_S}{\beta_S} - \frac{\alpha_T}{\beta_T}\right) + \left(\frac{1}{\beta_S} - \frac{1}{\beta_T}\right) Y \qquad \text{(Eq. 6)}$$

Presentation of the relations has been made in the above form to give expressions which are consistent with those obtaining in the usual parallel line assay case when $\beta_S = \beta_T$. In some applications, however, predictions in terms of the test rather than the standard preparation may be of interest. For example, it may commonly be required to estimate the log-dose, X_T, of the test preparation which will give a response equivalent to that obtained with a specified log-dose, X_S, of the standard. The expressions above and their developments below can readily be applied in such cases by simply interchanging the suffixes S and T so that Eq. 3, for example, would give,

$$X_T = -\frac{(\alpha_T - \alpha_S)}{\beta_T} + \frac{\beta_S}{\beta_T} X_S$$

Choice between the various alternative expressions may, therefore, be made according to the practical requirements of particular situations. Care, however, is required in any application because, apart from the simple indication that the test preparation is not a dilution of the standard preparation, the equations in themselves cannot readily be interpreted to give information about modes of action in a context more general than that of the particular assay. Thus, although Eq. 3 represents a calibration relationship between equipotent log-doses, it cannot be assumed that the parameters in such a relation determined from one laboratory species will remain constant for application to another species. In the absence of information about modes of action, therefore, the relationships should preferably be regarded as concise local descriptions of the observed phenomena. The relations are, of course, applicable in repetitions of the original assay circumstances of which accurate and detailed specifications are accordingly desirable. This latter aspect is particularly important for interlaboratory studies, as was recently emphasized by Youden (14).

ESTIMATION

Estimation procedures for the above relationships will first be considered for an assay in a completely randomized design in which one observation is obtained from each of N experimental subjects and r responses are observed at each of n_S log-dose levels of the standard preparation and n_T log-dose levels of the test preparation, so that,

$$N = r(n_S + n_T) \qquad \text{(Eq. 7)}$$

The assumptions will be made that (a) the log-dose response lines for each preparation are straight lines over the range of doses tested; (b) residual errors are normally and independently distributed with population mean zero and variance σ^2, this variance being the same for both preparations.
The following notation will be used:

S refers to the standard and T to the test preparation,
x_{iS} is the ith log-dose value for S, $i = 1, 2, \ldots, n_S$,
x_{jT} is the jth log-dose value for T, $j = 1, 2, \ldots, n_T$,
y_{ikS} and y_{jkT} are the kth response observations at the ith log-dose of S and the jth log-dose of T, $k = 1,2,\ldots,r$, and correspondingly
$\bar{y}_{iS}$ and $\bar{y}_{jT}$ are the mean responses at these log-doses.

By regression analyses described in standard texts estimates, a_S, b_S, a_T, and b_T, of the regression parameters, α_S, β_S, α_T, and β_T, in Eqs. 1 and 2 are first calculated. Thus, for S, with

$$\bar{x}_S = \frac{1}{n_S} \sum_{i=1}^{n_S} x_{iS} \qquad \text{(Eq. 8)}$$

$$\bar{y}_S = \frac{1}{n_S} \sum_{i=1}^{n_S} \bar{y}_{iS} = \frac{1}{rn_S} \sum_{i=1}^{n_S} \sum_{k=1}^{r} y_{ikS} \qquad \text{(Eq. 9)}$$

so that $\bar{y}_S$ and $\bar{y}_T$ are the means of the mean responses at the individual log-dose values, the estimates are

$$a_S = \bar{y}_S - b_S\bar{x}_S \qquad \text{(Eq. 10)}$$

$$b_S = \frac{\sum_{i=1}^{n_S} (x_{iS} - \bar{x}_S)(\bar{y}_{iS} - \bar{y}_S)}{\sum_{i=1}^{n_S} (x_{iS} - \bar{x}_S)^2} \qquad \text{(Eq. 11)}$$

In practice, of course, b will be calculated using the well-known identity that for any number, n, of pairs (x_i, y_i) with means $\bar{x}$ *and* $\bar{y}$,

$$\Sigma(x_i - \bar{x})(\bar{y}_i - \bar{y}) = \Sigma xy - \frac{1}{n}(\Sigma x)(\Sigma y) \qquad \text{(Eq. 12)}$$

The estimate s^2 of the residual variance σ^2 is calculated by pooling the mean squares for deviations from regression obtained from the two regression analyses. This estimate will have $N - 4$ degrees of freedom and is compounded from the deviations of the mean responses at individual log-dose levels and the deviations between individual responses at each of the log-dose levels, a procedure which is valid under the assumptions (a) and (b) above.

Estimates of the quantities defined in the Eqs. 3, 4, and 6 above can now be calculated as follows.

From Eq. 3, the estimate of the true value of X_S is $\hat{X}_S$ where,

$$\hat{X}_S = -\frac{1}{b_S}(a_S - a_T - b_T X_T) \qquad \text{(Eq. 13)}$$

or equivalently in terms of means as defined in Eqs. 8 and 9,

$$\hat{X}_S - \bar{x}_S = -\frac{1}{b_S} \times \{\bar{y}_S - \bar{y}_T - b_T(X_T - \bar{x}_T)\} \qquad \text{(Eq. 14)}$$

$$= -\frac{1}{b_S}(\bar{y}_S - \hat{Y}_T) \qquad \text{(Eq. 15)}$$

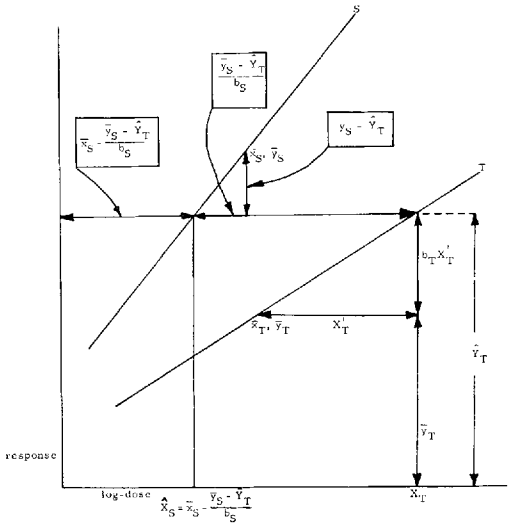

Fig. 1.—The relation between equipotent log-doses.

where

$$\hat{Y}_T = \bar{y}_T + b_T(X_T - \bar{x}_T) \qquad \text{(Eq. 16)}$$

is the response predicted from the line for the test preparation at log-dose X_T. A geometrical construction which illustrates how the above quantities are used to obtain the log-dose estimate $OA = \hat{X}_S$ from the specified value $OB = X_T$ is given in Fig. 1.

Next from Eq. 4 $M(X_T)$, the estimate of $\mu(X_T)$ is given by

$$M(X_T) = -\frac{1}{b_S}\{a_S - a_T - (b_T - b_S)X_T\} \qquad \text{(Eq. 17)}$$

which may alternatively be written as

$$M(X_T) = \bar{x}_S - X_T - \frac{1}{b_S} \times \{\bar{y}_S - \bar{y}_T - b_T(X_T - \bar{x}_T)\} \qquad \text{(Eq. 18)}$$

$$= \bar{x}_S - \bar{x}_T - \frac{\bar{y}_S - \bar{y}_T}{b_S} + \left(\frac{b_T}{b_S} - 1\right)(X_T - \bar{x}_T) \qquad \text{(Eq. 19)}$$

Third, from Eq. 6 $M(Y)$, the estimate of $\mu(Y)$ is given by

$$M(Y) = -\frac{1}{b_S b_T} \times \{a_S b_T - a_T b_S - (b_T - b_S)Y\} \qquad \text{(Eq. 20)}$$

or

$$M(Y) = \bar{x}_S - \bar{x}_T - \frac{1}{b_S b_T} \times \{\bar{y}_S b_T - \bar{y}_T b_S - (b_T - b_S)Y\} \qquad \text{(Eq. 21)}$$

INTERVAL ESTIMATION OF THE EQUIPOTENT DOSE

By an adaptation of the procedure Fieller (15, 16) for interval estimation from a ratio, a fiducial interval will now be derived for the true value of $\hat{X}_S$ calculated from Eqs. 13 or 14 for a single specified

X_T value. For this, Fieller's argument can be applied to a variate constructed as

$$u = a_S + b_S X_S - a_T - b_T X_T \quad \text{(Eq. 22)}$$

where X_S is the true or population value for the log-dose of the standard preparation corresponding to a log-dose X_T of the test preparation.

Under the stated assumptions, u is a linear combination of the normally distributed variates a_S, b_S, a_T, and b_T and so is itself normally distributed. In virtue of Eq. 3 it is also true that the population or expected value of u is zero. It follows that, if the estimated variance of u is $d_u s^2$, where d_u is a known constant coefficient determined by the construction of u from the original observations, the quantity $u^2/d_u s^2$ is distributed according to the F distribution with 1 and $N - 4$ degrees of freedom.

Hence, if F_c is the tabulated value from this distribution such that

$$P[F \leq F_c] = 1 - \alpha$$

we have

$$P\left[\frac{u^2}{d_u s^2} \leq F_c\right] = 1 - \alpha$$

By Fieller's theorem it now follows that solutions of

$$u^2 - F_c d_u s^2 = 0 \quad \text{(Eq. 23)}$$

which from Eq. 22 is a quadratic equation in the X_S corresponding to a given value of X_T, will give values defining a $100(1 - \alpha)\%$ fiducial interval for the required X_S value.

To obtain d_u, it is first convenient to write u from Eq. 22 in the equivalent form,

$$u = \bar{y}_S + b_S(X_S - \bar{x}_S) - \bar{y}_T - b_T(X_T - \bar{x}_T) \quad \text{(Eq. 24)}$$

in which all the estimates are statistically independent. Hence,

$$V(u) = V[\bar{y}_S + b_S(X_S - \bar{x}_S)] + V[\bar{y}_T + b_T(X_T - \bar{x}_T)]$$

which can be estimated as $d_u s^2$ where

$$d_u s^2 = \left[\frac{1}{n_S} + \frac{(X_S - \bar{x}_S)^2}{r\Sigma(x_S - \bar{x}_S)^2} + \frac{1}{n_T} + \frac{(X_T - \bar{x}_T)^2}{r\Sigma(x_T - \bar{x}_T)^2}\right] s^2 \quad \text{(Eq. 25)}$$

It is now convenient to introduce a more concise notation, wherein a prime is used to denote values "corrected for their means." Thus, we write

$$X_S' = X_S - \bar{x}_S, \quad X_T' = X_T - \bar{x}_T \quad \text{(Eq. 26)}$$

and

$$\Sigma_S' = r\sum_1^{n_S}(x_{iS} - \bar{x}_S)^2, \quad \Sigma_T' = r\sum_1^{n_T} \times (x_{jT} - \bar{x}_T)^2 \quad \text{(Eq. 27)}$$

Then, from Eqs. 24 and 25

$$u = \bar{y}_S - \bar{y}_T + b_S X_S' - b_T X_T' \quad \text{(Eq. 28)}$$

and

$$d_u = \frac{1}{n_S} + \frac{1}{n_T} + \frac{X_S'^2}{\Sigma_S'} + \frac{X_T'^2}{\Sigma_T'} \quad \text{(Eq. 29)}$$

Inserting these values into Eq. 23 and collecting terms then gives the quadratic equation for the unknown X_S' as,

$$AX_S'^2 + 2BX_S' + C = 0 \quad \text{(Eq. 30)}$$

where

$$A = b_S^2 - \frac{F_c s^2}{\Sigma_S'} \quad \text{(Eq. 31)}$$

$$B = b_S\{\bar{y}_S - \bar{y}_T - b_T X_T'\} \quad \text{(Eq. 32)}$$

$$= b_S(\bar{y}_S - \hat{Y}_T) \quad \text{(Eq. 33)}$$

from Eq. 16, and

$$C = (\bar{y}_S - \bar{y}_T - b_T X_T')^2 - F_c s^2\left(\frac{1}{n_S} + \frac{1}{n_T} + \frac{X_T'^2}{\Sigma_T'}\right) \quad \text{(Eq. 34)}$$

By the usual formula for the roots of a quadratic equation the lower and higher limits, X_{SL} and X_{SH}, of the fiducial interval are then given by

$$X_{SL}', X_{SH}' = \frac{-B \mp \sqrt{B^2 - AC}}{A} \quad \text{(Eq. 35)}$$

that is,

$$X_{SL} = \bar{x}_S - \frac{1}{A}\{B + \sqrt{B^2 - AC}\},$$

$$X_{SH} = \bar{x}_S - \frac{1}{A}\{B - \sqrt{B^2 - AC}\} \quad \text{(Eq. 36)}$$

In practice, the computations can be simplified by first calculating the quantity $\hat{X}_S' = \hat{X}_S - \bar{x}_S$ from the point estimate $\hat{X}_S$ of the equipotent dose. Then, substituting $-b_S\hat{X}_S'$ for $\bar{y}_S - \bar{y}_T - b_T X_T'$ in Eqs. 32 and 34 leads to an expression for the interval as,

$$X_{SL}', X_{SH}' = \frac{1}{A}\left[b_S^2 X_S' \mp \sqrt{F_c s^2\left\{A\left(\frac{1}{n_S} + \frac{1}{n_T} + \frac{X_T'^2}{\Sigma_T'}\right) + \frac{b_S^2\hat{X}_S'^2}{\Sigma_S'}\right\}}\right] \quad \text{(Eq. 37)}$$

By analogy with the usual calculations for parallel line assays [Finney (3)], an approximate formula, which is often sufficiently accurate, can now be easily obtained. For this we note that the quantity s^2/Σ_S' in Eq. 31 is the variance of b_S, and, if b_S^2 is very much larger than its variance, *i.e.*, if the square of the coefficient of variation of b_S is very small, we have, from Eq. 31

$$A = b_S^2\left(1 - \frac{F_c s^2}{b_S^2\Sigma_S'}\right) \simeq b_S^2$$

As an empirical working rule, following Finney (3), it may be suggested that the approximation will give sufficiently accurate results if $A/b_S^2 > 0.95$, or, equivalently, if $20\,F_c < b_S^2\Sigma_S'/s^2$. In such cases it can easily be checked that the fiducial interval defined above becomes,

$$X_{SL}, X_{SH} = \hat{X}_S \mp \sqrt{\frac{F_c s^2}{b_S^2}\left(\frac{1}{n_S} + \frac{1}{n_T} + \frac{X_T'^2}{\Sigma_T'}\right)} \quad \text{(Eq. 38)}$$

TABLE I.—FOUR-POINT ASSAY OF LUTEINIZING HORMONE IN SWINE PITUITARY TISSUE BY ASCORBIC ACID DEPLETION METHOD

	Standard (N.I.H.)		Test	
	0.4 mg.	1.6 mg.	0.0625 mg.	0.2500 mg.
	77	55	64	60
	81	45	71	54
	80	47	70	54
	78	52	80	61
	80	48	72	54
Total	396	247	357	283
Mean	79.2	49.4	71.4	56.6

The fiducial intervals calculated as above apply when the $\hat{X}_S$ value corresponding to only one X_T value is required. Commonly, however, the procedure may be required for an unspecified number of X_T values. The theoretical treatment by Scheffé (17) then indicates that the interval should be calculated by substituting for F_c, as defined above, the value $4F_c'$ where F_c' is the tabulated value from the F-distribution with 4 and $N - 4$ degrees of freedom such that,

$$P[F \leq F_c'] = 1 - \alpha$$

EXAMPLE

The data in Table I were obtained from a four-point assay of luteinizing hormone (LH) in swine pituitary tissue [Melampy and Hendricks (18)] by the ascorbic acid depletion (AAD) method. The responses are in units of mcg. AAD/100 mg. rat ovary tissue.

When the doses are, as here, conveniently chosen so that the ratio of the higher to the lower dose is the same for both preparations, the log-dose transformations can be chosen to give a log-dose metameter which takes simple integral values. Thus, in Table I, where the dose-ratio is 4, the transformations from doses z_S and z_T to metameters x_S and x_T such that,

$$x_S = \frac{1}{\log 4}\{\log z_S - \log 0.4\} \qquad \text{(Eq. 39)}$$

$$x_T = \frac{1}{\log 4}\{\log z_T - \log 0.0625\} \qquad \text{(Eq. 40)}$$

give $x_S = x_T = 0$ at the two lower doses and $x_S = x_T = 1$ at the two upper doses.

The usual assay analysis of variance then gives:

	d.f.	Mean Squares
Between preparations	1	0.45
Common regression	1	2486.45
Divergence	1	281.25
Residual	16	16.15

Since the $\alpha = 0.05$ critical F-value for 1 and 16 degrees of freedom is 4.49 it can be seen (*a*) from the between preparations term that closely similar response levels were achieved and (*b*) from the divergence term that the slopes of the regression lines for the standard and test preparations were significantly different.

We are therefore in a situation for which the preceding procedures are appropriate with $n_S = n_T = 2$ doses for each preparation and $r = 5$ responses at each preparation-dose combination. Conventional regression calculations, as indicated by Eqs. 8, 9, and 11 and similar equations for the test preparation, then give

$$\bar{x}_S = \bar{x}_T = {}^1/_2$$

and

$$\bar{y}_S = (396 + 247)/10 = 64.3$$

$$\bar{y}_T = (357 + 283)/10 = 64.0$$

Again, from Eq. 27

$$\Sigma_S' = \Sigma_T' = 5\left(\frac{1}{4} + \frac{1}{4}\right) = \frac{5}{2}$$

and it follows from Eqs. 11 and 12, or because the interval between the two values of x_S is unity, that

$$b_S = -(79.2 - 49.4) = -29.8$$

and similarly,

$$b_T = -(71.4 - 56.6) = -14.8$$

The equation for the prediction of an X_S value corresponding to a specified X_T value can now be written down from Eq. 14 as

$$\hat{X}_S - \frac{1}{2} = \frac{1}{29.8}\left\{64.3 - 64.0 + 14.8\left(X_T - \frac{1}{2}\right)\right\}$$

$$= \frac{1}{29.8}(-7.1 + 14.8\,X_T)$$

that is,

$$\hat{X}_S = 0.26 + 0.50\,X_T \qquad \text{(Eq. 41)}$$

With $F_c = 4.49$, for $\alpha = 0.05$, and $s^2 = 16.15$

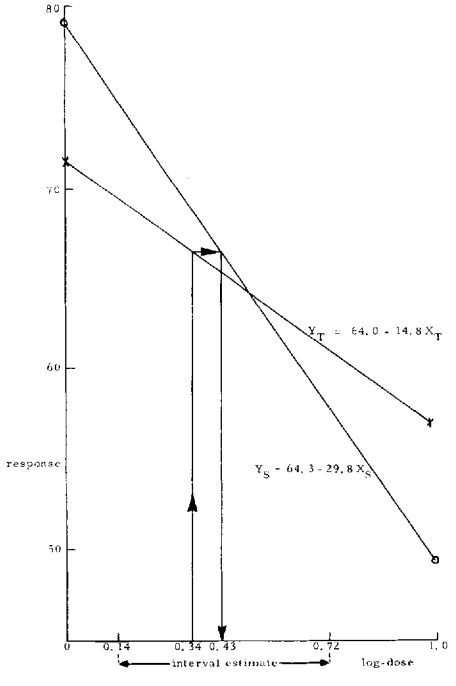

Fig. 2.—Assay of LH in swine pituitary tissue (18).

(16 d.f.) the fiducial interval can now be calculated. For this, from Eq. 31,

$$A = 29.8^2 - \frac{2}{5}(4.49)(16.15)$$
$$= 888.04 - 29.01$$
$$= 859.03$$

Hence, from Eq. 37

$$X_{SL}', X_{SH}' = \frac{1}{859.03}\left[29.8^2\hat{X}_S' \mp \sqrt{(4.49)(16.15)\left\{859.03\left(\frac{1}{2}+\frac{1}{2}+\frac{2X_T'^2}{5}\right)+\frac{2(29.8^2)}{5}\hat{X}_S'^2\right\}}\right] \quad \text{(Eq. 42)}$$

$$= 1.03\hat{X}_S' + \sqrt{0.0844\,(1 + 0.40\,X_T'^2 + 0.42\,\hat{X}_S'^2)} \quad \text{(Eq. 43)}$$

Now, in particular, suppose it is desired to estimate the dose of the standard preparation equivalent to 0.1 mg. of the test preparation. First, from Eq. 40, the specified value of X_T is

$$X_T = \frac{1}{\log 4}\{\log 0.1/0.0625\} = 0.3390$$

Hence, from Eq. 41,

$$\hat{X}_S = 0.26 + (0.50)(0.3390) = 0.4295$$

and, from Eq. 39,

$$\log(z_S/0.4) = 0.4295 \log 4$$

from which the estimated equipotent dose is

$$\hat{Z}_S = 0.73 \text{ mg.}$$

Noting that $X_T' = 0.3390 - 0.50 = -0.1610$ and $X_S' = -0.0705$, the corresponding 95% fiducial interval is obtained from Eq. 43 as,

$$X_{SL}', X_{SH}' = -(1.03)(0.0705) \mp \sqrt{0.0844\,\{1 + (0.40)(0.1610^2) + 0.42(0.0705)^2\}}$$
$$= -0.365, +0.220$$

From Eqs. 26 and 39 the interval for the equipotent dose can then be calculated as

$$Z_{SL}, Z_{SH} = 0.48, 1.09 \text{ mg.}$$

Alternatively, since for these data $A/b_S^2 = 0.97$, the approximate formula in Eq. 38 may be used to give,

$$X_{SL}, X_{SH} = 0.4295 \mp \sqrt{\frac{(4.49)(16.15)}{29.8^2}(1 + 0.4X_T'^2)}$$
$$= 0.14, 0.72$$

and on subtraction of $\bar{x}_S = 0.5$, values are obtained which closely agree with those obtained above for X_{SL}' and X_{SH}'.

The results are illustrated in Fig. 2. In this the width of the interval estimate serves to emphasize the fact that an experiment designed to give sufficient precision for a parallel line assay will give poor precision for an estimate of an equipotent dose if divergence has to be admitted as the more realistic situation.

For simplicity of exposition, the above development and the example have been carried through for a constant number r of response observations at each preparation-dose level combination. The extension to the unequal numbers case readily follows, *mutatis mutandis*, using the standard statistical procedures for dealing with unequal numbers in single classification experiments. Additionally, assays based on designs other than the completely randomized design can be treated by the basic techniques described above.

QUADRATIC RESPONSE CURVES

The principles described above are also applicable when one or both of the log-dose response relationships can be described by quadratic curves about which the responses of individual experimental units are normally distributed. As expected, however, more computation is required.

If the two quadratic relationships are

$$\bar{y}_S = a_S + b_S X_S + c_S X_S^2 \quad \text{(Eq. 44)}$$

and

$$\bar{y}_T = a_T + b_T X_T + c_T X_T^2 \quad \text{(Eq. 45)}$$

the log-dose of the standard preparation equivalent to a specified log-dose X_T of the test preparation can be estimated as one solution of the quadratic equation,

$$c_S\hat{X}_S^2 + b_S\hat{X}_S + a_S - a_T - b_T X_T - c_T X_T^2 = 0 \quad \text{(Eq. 46)}$$

Identification of the appropriate root can be made without difficulty because the two dose-response curves must be monotonic (though not necessarily in the same sense) in the region of interest and because the specified X_T and its correspondent $\hat{X}_S$ should be within the dose ranges over which the curves themselves were estimated.

In many practical cases extreme accuracy will not be required of such estimation procedures and, particularly if numerous equipotent doses are required, it may be more convenient to read them from graphs of the two curves.

Fiducial intervals for the estimate defined in Eq. 46 can also be obtained on the above principles as the appropriate solutions of Eq. 23 with u equal to the expression on the left of Eq. 46. Solution of a fourth degree equation is required in this case. The interpretation of solutions of quartic equations in a similar inverse estimation problem has been discussed by Williams (19).

RELATIVE POTENCY AS A FUNCTION OF DOSE

Divergent line assays may occur in some contexts where it may be of interest to estimate the relative potency itself, although this quantity is now of more restricted use than in the simple case when it is constant. For example, if we now find that 1 mg. of the test preparation is equipotent to ρ(1 mg.) of the standard preparation it is no longer true that 1 Gm. of the test and 1000ρ(1 mg.) of the standard preparations are equipotent.

When, however, the relative potency itself is required it can be estimated as a function of log-dose from Eq. 19. Since $\mu(X_T) = X_S - X_T$, where X_S is the equipotent log-dose of the standard preparation, an interval estimate for $\mu(X_T)$ can be obtained by subtracting X_T from the interval estimate previously determined for X_S. Alternatively, we may proceed directly by applying Fieller's procedure, *via* Eq. 23 with

$$u = b_S\{\mu(X_T) - \bar{x}_S + \bar{x}_T\} + (\bar{y}_S - \bar{y}_T) - (b_T - b_S)X_T' \quad \text{(Eq. 47)}$$

As a result if, for convenience, we write $\lambda = \mu(X_T) - \bar{x}_S + \bar{x}_T$ the interval can be determined by adding $(\bar{x}_S - \bar{x}_T)$ to each root of the quadratic equation

$$A\lambda^2 + 2B\lambda + C = 0 \quad \text{(Eq. 48)}$$

where

$$A = b_S^2 - \frac{F_c s^2}{\Sigma_S'} \quad \text{(Eq. 49)}$$

$$B = -b_S^2\{M(X_T) - \bar{x}_S + \bar{x}_T\} + \frac{F_c s^2}{\Sigma_S'} \quad \text{(Eq. 50)}$$

$$C = b_S^2\{M(X_T) - \bar{x}_S + \bar{x}_T\}^2 - F_c s^2\left\{\frac{1}{n_S} + \frac{1}{n_T} + X_T'^2\left(\frac{1}{\Sigma_S'} + \frac{1}{\Sigma_T'}\right)\right\} \quad \text{(Eq. 51)}$$

RELATIVE POTENCY AS A FUNCTION OF RESPONSE

The point estimate $M(Y)$ of $\mu(Y)$, the log relative potency at response Y can be calculated from Eq. 21, but exact interval estimation is not so straightforward as in the previous cases. In many practical cases, however, it will be sufficient to use approximate fiducial intervals which can be obtained as follows.

It is a well-known result that, if

$$r = \frac{u}{v}$$

is a ratio of two variates, u and v, which are statistically independent, and if the coefficient of variation of the denominator, v, is small, then

(coefficient of variation of r)^2 = (coefficient of variation of u)^2 + (coefficient of variation of v)^2 (Eq. 53)

That is, if $d_u s^2$ and $d_v s^2$ are the estimated variances of u and v, respectively, $V(r)$, the estimated variance of the ratio is given by,

$$V(r) = r^2 s^2\left(\frac{d_u}{u^2} + \frac{d_v}{v^2}\right) \quad \text{(Eq. 54)}$$

To apply this in the present context we have, from Eq. 21

$$M(Y) - \bar{x}_S + \bar{x}_T = \left\{\frac{(Y - \bar{y}_S)}{b_S} - \frac{(Y - \bar{y}_T)}{b_T}\right\} \quad \text{(Eq. 55)}$$

The difference between two independent ratios appears on the righthand side and hence

$$V\{M(Y) - \bar{x}_S + \bar{x}_T\} = V\left(\frac{Y - \bar{y}_S}{b_S}\right) + V\left(\frac{Y - \bar{y}_T}{b_T}\right) \quad \text{(Eq. 56)}$$

and now, applying Eq. 54

$$V\left(\frac{Y - \bar{y}_S}{b_S}\right) = \left(\frac{Y - \bar{y}_S}{b_S}\right)^2 s^2\left\{\frac{\frac{1}{n_S}}{(Y - \bar{y}_S)^2} + \frac{\frac{1}{\Sigma_S'}}{b_S^2}\right\} = \frac{s^2}{b_S^2}\left\{\frac{1}{n_S} + \frac{(Y - \bar{y}_S)^2}{b_S^2\Sigma_S'}\right\} \quad \text{(Eq. 57)}$$

The variance of $(Y - \bar{y}_T)/b_T$ can be similarly calculated and, on the assumption that the quantity $M(Y) - \bar{x}_S + \bar{x}_T$ in Eq. 55 is normally distributed, the approximate fiducial limits of $\mu(Y)$ are then,

$$\mu_L(Y),\ \mu_H(Y) = \bar{x}_S - \bar{x}_T \mp \sqrt{F_c s^2\left\{\frac{1}{n_S} + \frac{1}{n_T} + \frac{(Y - \bar{y}_S)^2}{b_S^2\Sigma_S'} + \frac{(Y - \bar{y}_T)^2}{b_T^2\Sigma_T'}\right\}} \quad \text{(Eq. 58)}$$

SLOPE RATIO ASSAYS

Suppose that the two dose-response lines in a slope ratio assay are

$$Y_S = a_S + b_S z_S \quad \text{(Eq. 59)}$$

$$Y_T = a_T + b_T z_T$$

where z_S and z_T represent doses, and the intercepts a_S and a_T are estimates of the parameters α_S and α_T and, instead of α_S being equal to α_T as in the regular slope ratio assay case, we now have $\alpha_S \neq \alpha_T$. The dose Z_S which is equipotent with a specified dose Z_T of the test preparation is then estimated as

$$\hat{Z}_S = -\frac{1}{b_S}(a_S - a_T - b_T Z_T) \quad \text{(Eq. 61)}$$

It can now be seen that this is directly analogous to Eq. 13 for the previous case, except that we now have doses Z_S and Z_T instead of log-doses X_S and X_T, so that *mutatis mutandis*, the above procedures can readily be applied in slope-ratio assay situations.

DISCUSSION

Finney (1965) has recently given an interesting general discussion of the role of the concept of constant relative potency, or equivalently of the condition of similarity, in bioassay. It should be noted that the estimation procedures in the present paper are referred to situations when the condition of similarity does not obtain. Such situations are common in research situations for which the condition would often be an unrealistically ideal assumption.

Relatedly, although techniques have been presented for estimating relative potency as a function of dose (concentration) or response, it is considered that these are of less importance and value than those described for the estimation of equipotent doses. It is suggested that this latter is the more basic concept for bioassay in general because, even when relative potency is constant, applications of its estimation are often, in effect, made toward determinations of equipotent doses.

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Drug Standards

Standardization of Papain Activity

Report of a Collaborative Study

By EDGAR A. LAZO-WASEM

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PAPAIN, a crude or purified proteolytic enzyme derived from the tropical plant *Carica papaya*, has been used in the pharmaceutical and food industries for over half a century. Twenty years ago, a monograph for papain was included in the eighth edition of the "National Formulary" (1). The then official assay procedure consisted of a limit test based on digestion of beef muscle.

After deletion of papain from the "National Formulary," many procedures came into use for the standardization of commercial papain. For pharmaceutical and food grade papain, the most widely used procedures have been milk-clotting (2), casein digestion (3), and digestion of hemoglobin (4, 5). For crystalline papain, most laboratories have, at least recently, relied on the initial rate of hydrolysis of synthetic peptide substrates such as *N*-benzoyl-L-arginine ethyl ester hydrochloride.

In an attempt to bring about unification in methods of assay throughout United States laboratories, a committee was established within the Quality Control Section of the Pharmaceutical Manufacturers Association in the fall of 1962. This group was to study current prevailing methods and recommend a generally acceptable method for use throughout the industry. This report describes the findings and recommendations of that committee.

PLAN OF STUDY AND RESULTS

Member firms of the Pharmaceutical Manufacturers Association, representing manufacturing suppliers and pharmaceutical firms marketing papain in dosage forms, were invited to supply their procedures. The methods received involved either milk-clotting, casein digestion, or hemoglobin digestion. From the procedures received, three assays based on the above principles were prepared and forwarded to eight laboratories for collaborative study. "Standard" and "unknown" papain preparations were also forwarded, and thus an effort was initiated whereby

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After the study reported here was underway, it was learned that efforts toward uniformity of enzyme assays, including papain, were being made by the International Commission for the Standardization of Pharmaceutical Enzymes, Fédération Internationale Pharmaceutique. Since then this writer has been kept informed of the efforts of this predominantly European group, the initial studies of which have been excellently summarized in the commission's First Report (6). For papain, the commission has endorsed a method based on the initial rate of hydrolysis of a synthetic substrate, *N*-benzoyl-L-arginine ethyl ester hydrochloride, for both crystalline papain and less purified preparations. A comparison of the unit of activity reported here with that adopted by the commission will be the subject of a future report.

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the potency of the preparations using each procedure was compared. An analysis of intra- and inter-laboratory variation showed all three procedures to be equally reliable. The milk method (visual observation of the clotting of milk by the enzyme, an initial step in the proteolysis of casein) was found to require personal experience by the analyst to correctly time the corresponding subjective end point, but was found to be the simplest of the methods tried, and thus, worthy of further work. Difficulty was encountered at some laboratories in the duplication of the hemoglobin procedure studied, and thus no further study with this procedure was recommended. The milk-clotting and casein digestion methods were adopted as choice methods. Details of the two adopted assay procedures, as used by the committee, are described below.

Casein Digestion Assay

Reagents.—*Sodium Phosphate, 0.05 M.*—Dissolve 7.1 Gm. of anhydrous disodium phosphate in sufficient water to make 1000 ml. Add a drop of toluene as preservative.

Citric Acid, 0.05 M.—Dissolve 10.5 Gm. of citric acid monohydrate in sufficient water to make 1000 ml. Add a drop of toluene as preservative.

Casein Substrate.—Disperse 1 Gm. of Hammersten type casein in 50 ml. of 0.05 *M* sodium phosphate. Place in a boiling water bath for 30 min. with occasional stirring. Cool to room temperature and add 0.05 *M* citric acid to pH 6.0 ± 0.1. Solution must be stirred rapidly and continuously during addition of the 0.05 *M* citric acid to prevent precipitation of the casein. Dilute to 100 ml. with water. Prepare fresh daily.

Phosphate–Cysteine Disodium Ethylenediaminetetraacetate Buffer Solution.—Dissolve 3.55 Gm. of disodium phosphate in 400 ml. of water in a 500-ml. volumetric flask. Add 7.0 Gm. of disodium ethylenediaminetetraacetate and 3.05 Gm. of cysteine hydrochloride monohydrate. Adjust to pH 6.0 ± 0.1 with 1 *N* HCl or 1 *N* NaOH solutions, and dilute to volume with water. Prepare fresh daily.

Trichloroacetic Acid, 30% (TCA).—Dissolve 30 Gm. of reagent grade trichloroacetic acid in water and dilute to 100 ml. with water.

Standard Test Dilution (Standard).—Accurately weigh 100 mg. of N.F. papain reference standard in a 100-ml. volumetric flask and add buffer solution to dissolve. Dilute to volume with buffer solution. Further dilute 2 ml. of this solution to 50 ml. with buffer solution. Use within 30 min. after preparation.

Assay Test Dilution (Unknown).—Accurately weigh an amount of sample containing an activity equivalent to 100 mg. of reference standard and proceed exactly as in the preparation of the *Standard Test Dilution.*

Procedure.—Into each of 12 test tubes (18 × 150 mm.) pipet 5.0 ml. of casein substrate. Place in a 40° water bath and allow 10 min. to reach bath temperature. Into each of two of the tubes (tests are run in duplicate except for the blanks) labeled S_1, pipet 1 ml. of standard and 1 ml. of buffer solution, mix by swirling, note zero time, stopper, and replace in the bath. Into each of two other tubes labeled S_2, pipet 1.5 ml. of standard and 0.5 ml. of buffer solution, and proceed as before. Repeat this procedure for two tubes labeled S_3, to which 2 ml. of standard is added, and for two tubes labeled U_2, to which 1.5 ml. of unknown and 0.5 ml. of buffer solution are added. After exactly 60 min., add to all 12 tubes 3 ml. of 30% TCA and shake vigorously. With the four tubes to which no standard or unknown solutions were added, prepare blanks by pipetting, respectively: 1 ml. standard plus 1 ml. buffer solution, 1.5 ml. standard plus 0.5 ml. buffer solution, 2 ml. standard, and 1.5 ml. unknown plus 0.5 ml. buffer solution. Replace all tubes in the 40° bath for 30–40 min. to allow to fully coagulate the precipitated protein. Filter through Whatman No. 40 or equivalent filter paper, discarding the first 3 ml. of filtrate (filtrates must be completely clear). Read the absorbance at 280 mμ of the filtrates of all solutions against their respective blanks. Plot the readings for S_1, S_2, and S_3 against the enzyme concentration of each corresponding level. By interpolation from this curve, taking into consideration dilution factors, calculate the potency of the sample in units/mg.

Calculation of Potency.—

$$C \times \frac{100}{\text{sample wt. (mg.)}} \times \frac{50}{2} \times \frac{10}{1.5} \times A = \text{units/mg.}$$

where C = mg./ml. obtained from the standard curve, and A = activity of reference standard in units/mg.

Milk-Clotting Assay

Reagents.—*pH 4.5 Buffer Solution Concentrate.*—Mix 2 vol. of 1 *M* acetic acid with 1 vol. of 1 *M* sodium hydroxide. Check pH and adjust.

Dilute Buffer Solution.—Dilute 2 vol. of the pH 4.5 concentrated buffer solution to 15 vol. with water.

Milk Substrate.—Mix thoroughly 50 Gm. of milk powder with 215 ml. of dilute buffer solution in a Waring blender or similar device. Add a trace of octyl-alcohol (2-ethyl-1-hexanol) to decrease foaming during the blending operation. Filter through cheesecloth into a clean bottle. Add a few drops of toluene as a preservative. Allow to stand a few hours before use. Use the day of preparation. Keep refrigerated.

Phosphate–Cysteine–Disodium Ethylenediaminetetraacetate Buffer Solution (P–C–EDTA Buffer).—Dissolve 3.55 Gm. of disodium phosphate in 400 ml. of water in a 500-ml. volumetric flask. Add 7.0 Gm. of disodium ethylenediaminetetraacetate and 3.05 Gm. of cysteine hydrochloride monohydrate. Adjust to pH 6.0 with 1 *N* HCl or 1 *N* NaOH solutions, and dilute to volume with water. Prepare fresh daily.

Standard Test Dilution (Standard).—Accurately weigh 100 mg. of N.F. papain reference standard in a 50-ml. volumetric flask and add P–C–EDTA buffer solution to dissolve. Bring to volume with more P–C–EDTA buffer solution. Use within 30 min. after preparation.

Assay Test Dilution (Unknown).—Accurately weigh about 100 mg. of sample and dilute with P–C–EDTA buffer solution to obtain an activity approximately equivalent to that of the standard test dilution.

Procedure.—Pipet 25 ml. of milk substrate into each of a series of test tubes (25 × 150 mm.), close with rubber stoppers, place in a water bath at 40 ± 0.5°, and allow the contents of the tubes to reach bath temperature. (The assay is quite sensi-

tive to temperature. Variations of 1.0° can introduce errors of about 10%.) At zero time, pipet 2 ml. of standard and discharge the contents into one of the tubes. Stopper the tube, shake briefly but gently, so as to avoid the inclusion of air bubbles, and return to the bath. Using a stopwatch, measure the time from the addition of the standard test dilution until clotting of the milk begins. Roll the tube gently back and forth in a horizontal position while in the bath. (Less than 1 min. prior to clotting, the milk will appear to thicken somewhat and will no longer drain readily from the walls of the tube.) Watch the smooth film of milk closely from this point on. The end point is the almost instantaneous appearance of a granular character in the milk film. The time required for the end point to be reached should not be less than 100 sec. and not more than 150 sec. If not, the test should be repeated using a higher or lower enzyme concentration level. Once the right time limit of activity has been found, the test should be repeated at least once, and the average time recorded. Repeat the determination using the assay test dilution in the same volume as used for the standard.

Calculation of Potency.—Determine the relative potency of the sample in terms of the reference standard preparation by the equation:

$$\frac{w^S \times t^S}{w^U \times t^U} \times D = \text{units/mg.}$$

where

w^S = mg. of reference standard added to milk,
t^S = time in seconds for standard to produce clotting,
w^U = mg. of sample added to milk,
t^U = time in seconds for sample to produce clotting,
D = activity of reference standard in units/mg.

A second collaborative study further comparing milk-clotting and casein digestion again showed total equivalence of the two procedures. Interlaboratory variation in determining relative potency of two preparations by either method was below 5%. At this point, the committee felt that in view of the objective end point of the casein procedure (spectrophotometric measurement of released products of casein digestion), this method was the one of choice.

After selection of the assay procedure, the committee turned its attention to the selection of a suitable reference standard preparation. This study involved assay of preparations of varying potency, type, and origin.

The adopted standard preparation was tested for its tyrosine-releasing activity when allowed to act on a standard casein substrate. Definition of a unit of activity was done essentially following the conditions recommended by the International Union of Biochemistry, which states that an enzyme unit should be defined as the amount which catalyzes transformation of a stated amount (1 μm.) of substrate per unit time under defined conditions.

After experimental study at various laboratories, the committee agreed on the following definition of a papain unit: one unit represents the activity which releases the equivalent of 1 mcg. of tyrosine from a standard casein substrate, under specified conditions and at the enzyme concentration which liberates 40 mcg. of tyrosine per ml. of test solution. Using this unit definition, the preselected reference standard was found to contain 6600 units per mg. This N.F. papain reference standard is being made available through the offices of the National Formulary, American Pharmaceutical Association, Washington, D. C. The method of assay has been adopted by the Food Chemicals Codex. The committee agreed that papain for pharmaceutical use should have a potency of not less than 6000 papain units in each mg., when tested by the above described method.

SUMMARY AND CONCLUSIONS

1. Prevailing methods in the U. S. pharmaceutical and food industry for papain standardization have been studied by a Committee of the Pharmaceutical Manufacturers Association. Of the methods evaluated, that which measures hydrolysis of casein under standardized conditions was endorsed.

2. A preparation to be used as the reference standard in conjunction with the assay procedure was selected.

3. The papain reference standard has been submitted to the National Formulary for storage and distribution. The casein digestion method of assay has been adopted by the Food Chemicals Codex.

4. It is hoped that as many segments of industry as possible will begin using the recommended method and unit for routine quality control and for the labeling of papain activity.

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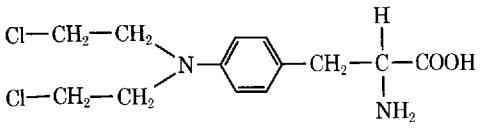
Qualitative and Quantitative Tests for Melphalan

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

L - 3 - p - [BIS(2 - chloroethyl)amino]phenylalanine; $C_{13}H_{18}Cl_2N_2O_2$; mol. wt. 305.21. The structural formula of melphalan may be represented as



Physical Properties.—Melphalan occurs as an off-white to buff-colored powder with a faint odor. It is slightly soluble in methanol and in alcohol, and practically insoluble in water. It is soluble in dilute mineral acids.

Identity Tests.—Transfer 1 ml. of a 0.01% alcoholic solution of melphalan into a glass-stoppered test tube and add 1 ml. of U.S.P. phthalate buffer, pH 4.0, 1 ml. of a 5 in 100 solution of 4-(*p*-nitrobenzyl)pyridine (NBP) in acetone, and 1 ml. of saline T.S. Heat on a water bath at 80° for 20 min. and cool the solution quickly. Add 10 ml. of alcohol and 1 ml. of 0.1 *N* potassium hydroxide: a violet to red-violet color is produced.

A 1 in 100,000 solution of melphalan in alcohol exhibits an ultraviolet absorbance maximum at about 260 mμ [absorptivity (*a*) about 72] and a minimum at about 226 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of melphalan in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests.—Dry about 1 Gm. of melphalan, accurately weighed, *in vacuo* at 105° to constant weight: it loses not more than 7% of its weight.

Char about 1 Gm. of melphalan, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%.

Determine the nitrogen content by the U.S.P. XVII nitrogen determination, method II, using about 325 mg. of melphalan, accurately weighed, and 0.1 *N* sulfuric acid for the titration. Each milliliter of 0.1 *N* sulfuric acid is equivalent to 1.401 mg. of nitrogen (N). The amount of nitrogen found is not less than 8.90% and not more than 9.45% calculated on a dried sample weight.

Received April 5, 1966, from the Drug Standards Laboratory, AMERICAN PHARMACEUTICAL ASSOCIATION FOUNDATION, Washington, D. C.

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Burroughs Wellcome & Co., Inc., Tuckahoe, N. Y., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

Assay.—Transfer about 200 mg. of melphalan, accurately weighed, into a titrating beaker and dissolve in 20 ml. of 0.5 *N* sodium hydroxide. Cover the beaker with a watch glass and boil the solution for 30 min., adding water as necessary to maintain the volume. Cool, neutralize to phenolphthalein T.S. with acetic acid, add 1 ml. excess acetic acid, and titrate potentiometrically with 0.1 *N* silver nitrate. Each milliliter of 0.1 *N* silver nitrate is equivalent to 15.26 mg. of $C_{13}H_{18}Cl_2N_2O_2$. The amount of melphalan found is not less than 93% and not more than 100.5% calculated on the dried basis. (*Note.*—Based on the assay result, melphalan is factored to 100% for use in the tablet formulation.)

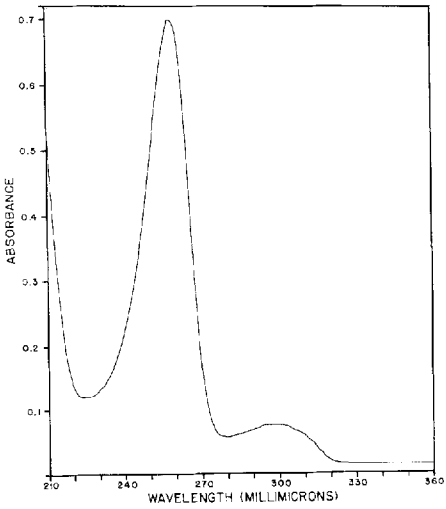

Fig. 1.—Ultraviolet absorption spectrum of melphalan in alcohol (10 mcg./ml.). Beckman model DK-2A spectrophotometer.

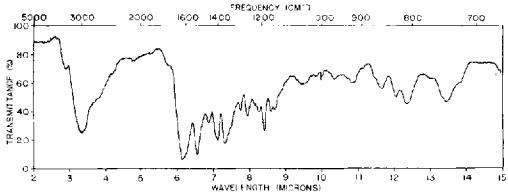

Fig. 2.—Infrared spectrum of melphalan in potassium bromide disk (0.5%). Perkin-Elmer model 21 spectrophotometer; sodium chloride prism.

DOSAGE FORMS OF MELPHALAN

Melphalan Tablets

Identity Tests.—Transfer 2 ml. of the assay solution (5 mg. of melphalan in 100 ml.) into a glass-stoppered test tube and add 1 ml. of U.S.P. phthalate buffer, pH 4.0, 1 ml. of a 5 in 100 solution of 4-(*p*-nitrobenzyl) pyridine (NBP) in acetone, and 1 ml. of saline T.S. Heat on a water bath at 80° for 20 min. and cool the solution quickly. Add 10 ml. of alcohol and 1 ml. of 0.1 *N* potassium hydroxide: a violet to red-violet color is produced.

Assay.—*Standard Preparation.*—Transfer about 10 mg. of melphalan reference standard, accurately weighed, to a 100-ml. volumetric flask, dissolve in alcohol, dilute to volume with alcohol, and mix. Transfer 10.0 ml. of this solution to a second 100-ml. volumetric flask, dilute to volume with alcohol, and mix.

Procedure.—Weigh and finely powder not less than 20 melphalan tablets. Transfer to a 100-ml. volumetric flask an amount of powdered tablets, accurately weighed, equivalent to about 5 mg. of melphalan. Add 10 ml. of water, swirl the sample, then add 10 ml. of alcohol. Warm on a steam bath for about 2 min. with intermittent shaking and cool the solution. Add alcohol to volume and mix. Centrifuge a portion of the mixture, transfer 10.0 ml. of the clear liquid to a 50-ml. volumetric flask, make to volume with alcohol, and mix. Concomitantly, determine the absorbance of this solution and that of the *Standard Preparation* in 1-cm. cells, at the maximum at about 260 mμ with a suitable spectrophotometer, using alcohol as the blank. Calculate the quantity, in mg., of $C_{13}H_{18}Cl_2N_2O_2$ in the portion of the tablets taken by the formula $0.5C\ (A_u/A_s)$, where C is the exact concentration, in mcg./ml., of melphalan in the *Standard Preparation*, calculated on the dried basis, A_u is the absorbance of the solution from the tablets, and A_s is the absorbance of the *Standard Preparation*. The amount of melphalan found is not less than 93.0% and not more than 107.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminologies for solubility, melting range, reagents, etc., have been used wherever feasible.

Melphalan,[1] synthesized by Bergel and Stock (1), is an orally active alkylating agent of the nitrogen mustard class which is useful in the treatment of multiple myeloma. Early literature references to this compound may be found under the synonym sarcolysin.

Identity Tests.—The colorimetric identification of melphalan is based on the procedure of Petering and Van Giessen (2) for the determination of alkylating agents. An additional identification test for melphalan drug is obtained by comparing the absorbances of the alcoholic solution at 226 mμ (minimum) and at 260 mμ (maximum). The ratio A_{226}/A_{260} is about 0.15.

Quantitative Methods.—Argentimetric determination of melphalan gave an average value of 95.7 ± 0.2%.[2] The titration was conducted using a silver electrode and a calomel electrode modified to contain saturated potassium sulfate solution. A rapid, precise measure of the chloride content may be determined by the oxygen flask method included for butyl chloride, N.F. XII, First Supplement. Analysis of commercial melphalan tablets by the spectrophotometric method gave an average value of 101.7 ± 1.5%.[2]

REFERENCES

(1) Bergel, F., and Stock, J. A., *J. Chem. Soc.*, **1954**, 2409.
(2) Petering, H. G., and Van Giessen, G. J., *J. Pharm. Sci.*, **52**, 1159(1963).

[1] Marketed as Alkeran by Burroughs Wellcome & Co. Inc., Tuckahoe, N. Y.
[2] Maximum deviation from the mean value.

Technical Articles

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By NICHOLAS H. BATUYIOS

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IN TERMS of economics and product stability, direct tablet compression offers distinct advantages over double compression, known also as "slugging," and the wet granulating method (1). Also, direct compression should produce tablets of faster dissolution rates because no colloidal binders, (*e.g.*, starch and gelatin) are used to envelop the granules.

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TABLE I.—WEIGHT VARIATION

Random Sample Size	Target Wt.	Sample Mean	S. D.	Coeff. of Variation
100	175.0	174.5[a]	1.1	0.63

[a] The mean ±3 standard deviations included every tablet weight of the sample.

The materials were mixed in a Twin-Shell blender for approximately 15 min. Then the blend was compressed at a weight of 125 mg. on a Stokes 551 tablet machine at various speeds using $^{9}/_{32}$-in. special flat-faced beveled edge, engraved punches. At a speed up to 3500 tablets/min., tablets of excellent quality were produced. When 100 tablets were weighed collectively and individually, it was found that no tablet varied more than ±4.5% from the mean.

TABLE II.—ANALYTICAL DATA

Compression Period	Time Interval Tablets Removed During Compression Run, hr.	Assay Results of Single Tablets, % of Label Claim					Av. of Single Tablets Assay	Assays from Powder of 10 Crushed Tablets
1st day	2	101.5	102.1	101.5	102.9	100.2	101.6	100.8
	4	97.1	96.2	95.2	94.3[a]	102.9	97.1	99.1
	6	97.7	98.9	96.0	99.0	97.5	97.8	96.6
2nd day	2	100.9	102.9	102.7	101.7	99.9	101.6	100.1
	4	99.2	101.2	99.2	97.3	99.8	99.3	98.5
	6	99.2	101.6	101.6	100.0	99.0	100.3	98.9
3rd day	2	102.1	101.5	97.7	102.5	102.5	101.3	96.9
	4	103.5	102.5	102.9	105.5	105.9[b]	104.1	98.6
	6	99.9	98.7	103.3	102.1	100.1	100.8	97.0

[a] Low. [b] High.

There are several tablet diluents commercially available that can be compressed directly (1–3). Because of high cost, physical and chemical properties, and poor tableting characteristics, they have only limited use.

In this laboratory, it was found that anhydrous lactose[1] U.S.P. XVII, tablet grade, possesses excellent flow and compression properties. It produces highly elegant tablets on a high-speed rotary tablet machine without requiring induced feeding and/or metered hoppers. It contains no more than 1.0% free and bound moisture, and its price is comparable to spray-dried lactose.

EXPERIMENTAL

Anhydrous Lactose Particle Size

	Sieve Analysis Mesh Size	%
Retained on:	40	0
	60	13.3–14.1
	80	22.2–24.5
	100	15.4–16.7
	200	26.5–28.7
Through:	200	16.7–21.6

Formulations

Several small experimental batches indicated that anhydrous lactose U.S.P. XVII, tablet grade, has good tableting characteristics. Therefore, in order to fully determine its tableting properties, a placebo batch of approximately 500,000 tablets of the following composition was made:

Formulation I

	%
Anhydrous lactose U.S.P., tablet grade	89.25
Starch U.S.P.	10.00
Calcium stearate U.S.P.	0.75

[1] Marketed by Sheffield Chemical.

Samples of tablets were placed at 50°, 80% R. H. at R. T. and sunlight for 6 weeks. No significant change with respect to color, hardness, and disintegration time was found.

When results indicated that a satisfactory placebo tablet could be made, then the following active formulations of 200,000 tablets each were made:

Formulations II, III, IV, and V

	per tablet, mg. II	III	IV	V
McN-JR-2498[2] hydrochloride	0.5	1.0	2.0	5.0
Anhydrous lactose U.S.P. XVII	155.7	155.2	154.2	151.2
Starch U.S.P.	17.5	17.5	17.5	17.5
Calcium stearate U.S.P.	1.3	1.3	1.3	1.3
	175	175	175	175

The active ingredient was passed through a 100-mesh screen and mixed with the inert materials in a 2-cu. ft. Twin-Shell blender for 30 min. The blend was compressed on a Stokes B-2 tablet machine at a speed of 44 r.p.m. A set of four special, flat oval, engraved punches was used (only four punches were available). No attempt was made to control the humidity during the compression of the four formulations which was done over a period of 2 months.

In all formulations tablets of excellent quality were obtained. All formulations were subjected to various tests, and the results were satisfactory. Formulation III, containing 1 mg. active ingredient per tablet, was tested more extensively, and the results are reported below. The solubility of McN-JR-2498 hydrochloride in water is 5 mg./ml.

Test Methods

Moisture Contents.—Moisture content was determined on a Cenco moisture balance operating at

[2] A No. code designation for trifluperido

120 v. with a 125-w. infrared lamp. The test was continued until a constant weight was reached at 50°.

Hardness.—Hardness was determined by the Strong-Cobb Arner hardness tester.

Friability.—Friability was measured with a Roche Friabilator using a 4-min. cycle and at least a 6.0-Gm. tablet sample.

Disintegration.—Disintegration time was determined using water and the U.S.P. apparatus; no disks were used.

Dissolution Rates.—The dissolution rate was determined in a 1-L. three-necked round-bottom flask fitted with a mechanical stirrer and a 7.5-cm. Teflon stirring paddle. The stirring paddle was adjusted to 2.5–3.5 cm. above the bottom of the flask and the stirring rate was maintained at 50 r.p.m. The flask contained 750 ml. of fluid and was immersed in a constant-temperature water bath maintained at 37° ± 0.1°. Each run consisted of 15 tablets and a Swinny hypodermic adapter filter was used for sampling. The fluids used were U.S.P. simulated gastric fluid without pepsin and U.S.P. simulated intestinal fluid without pancreatin.

Sieve Analysis.—Sieve analysis was done on a Cenco-Meinzer sieve shaker operating at 115 v. 50/60 cycles and at a setting of No. 5. Harshaw Scientific 5-in. diameter sieves were employed. A 100-Gm. sample per determination was used, and the range reported represents three determinations.

Table III.—Dissolution Rates

% McN-JR-2498 Released in Modified, Simulated Gastric Fluid			
Time, min.	Initial	3 Mo. at R. T.	3 Mo. at 60°
1	...	10.1	14.5
2	...	24.1	26.1
4	...	43.3	39.8
6	...	63.5	66.4
8	...	78.3	81.5
10	...	88.4	91.6
15	...	96.4	100.6
20	...	98.7	102.2
25	...	97.9	102.4
30	100.0	99.2	103.9

% McN-JR-2498 Released in Modified, Simulated Intestinal Fluid	
Time, hr.	Initial
1/2	25.3
1	31.9
2	42.0
2 3/4	47.4
5	61.8
7	72.0

DISCUSSION

The placebo batch showed that this anhydrous lactose formulation can be run on a high-speed tablet machine, using 9/32-in. special, flat-faced beveled edge, engraved punches, producing excellent tablets. Also, the four batches containing up to 5 mg. of active ingredient per tablet produced tablets

Table IV.—Chemical and Physical Stability Data

	Time, wk.							
	Initial	1	2	3	4	5	6	12
Chemical								
(McN-JR-2498, %)								
R.T.	100.2	...	...	...	...	...	...	102.4
	100.6	...	...	...	...	...	...	100.5
40°	...	...	...	...	101.8	...	...	100.0
	...	...	...	...	102.0	...	...	99.2
60°	...	...	...	...	100.0	...	...	98.0
	...	...	...	...	103.0	...	...	97.2
80°	...	...	...	...	98.7	...	...	...
	...	...	...	...	99.3	...	...	...
Physical								
R.T.								
Moisture, %	0.9	...	...	...	...	...	...	...
Color	White	...	...	...	...	...	White	White
Disint. time, min.	3–4	...	...	...	...	...	3–4	3–4
Hardness	9–10	...	...	...	...	...	9–10	9–10
Friability, %	0.08	...	...	...	...	...	0.08	0.08
50°[a]								
Color change	...	...	...	...	NSC[b]	...	NSC	NSC
Disint. time, min.	...	...	...	...	3–4	...	3–4	3–4
Hardness	...	...	...	...	8–9	...	8–9	9–10
Friability, %	...	...	...	...	0.08	...	0.07	0.0
80% R.H., R.T.[a] (open container)								
Color change	...	NC[c]	NC	NC	NC	...	...	...
Disint. time, min.	...	3–3.5	2–3	2–3	2–3.5	...	...	...
Hardness	...	8–9	8–9	8–9	8–9	...	...	...
Friability, %	...	...	...	0.21	0.20	...	...	...
Weight increase, %	...	...	...	1.5	1.5	...	...	...
Sunlight (color change)								
Amber glass	...	NC	NC	NC	NC	...	...	...
Flint glass	...	NC	NC	NC	NC	...	...	...

[a] The 50° sample and the 80% R. H. sample were tested immediately upon removal from their respective stations and after standing at R. T. for approximately 2 hr. The results were the same. [b] NSC, no significant change. [c] NC, no change.

of excellent quality using special, flat oval, engraved punches.

The criteria used in evaluating the tablets were appearance, distribution of active ingredient, weight uniformity, friability, binding, sticking, capping, hardness, disintegration time, dissolution rates, and the effects of elevated temperatures, high humidity, and sunlight. No attempt was made to control the relative humidity during compression which took place over a period of approximately 2 months.

The data in Table I show excellent tablet weight uniformity. Table II shows that the active material was uniformly distributed and that no separation occurred during a 3-day compression period. Table III shows that the dissolution rates in simulated gastric fluid were not affected by high temperature. In simulated intestinal fluid the tablets disintegrated in 3–4 min., but the dissolution rates were slow due to poor solubility of the active compound at pH 7.5 (0.06 mg./ml.). Table IV shows that the tablets were not affected chemically or physically by high temperature, high humidity, and direct sunlight. Assays not reported here on tablets exposed to sunlight and high humidity showed good stability. The tablet color did not change significantly after 12 weeks at 50°. A very slight off-white color developed which could be detected only when the room temperature and the 50° samples were observed together.

SUMMARY

1. Placebo and active tablets were made by direct compression using anhydrous lactose U.S.P. XVII, tablet grade, as a diluent.

2. A placebo batch of approximately 500,000 tablets was run at a speed of 3500 tablets/min. on a Stokes 551 tablet machine resulting in excellent tablets. Special $^{9}/_{32}$-in. flat beveled edge, engraved punches were used.

3. Four batches of 200,000 tablets each, containing 0.5, 1, 2, and 5 mg. active material per tablet, were made using a set of four special, flat oval, engraved punches, on a stokes B-2 tablet machine at a speed of 44 r.p.m.

4. The placebo and active tablets were excellent as shown by the elegance, small tablet weight variation, uniform distribution of the active ingredient, fast disintegration and dissolution rates, good hardness, low friability, and lack of binding, sticking, and capping.

5. No induced feeding and/or metered hoppers were required.

6. Physical and chemical stability studies showed that high temperature, high humidity, and direct sunlight had no effect on the formulations.

REFERENCES

(1) Milosovich, G., *Drug Cosmetic Ind.*, **92**, 557(1963).
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(3) Brownley, C. A., and Lachman, L., *J. Pharm. Sci.*, **53** 452(1964).

Notes

Quantitative Determination of Iodochlorhydroxyquin by Infrared Analysis

By T. URBÁNYI, D. SLONIEWSKY, and F. TISHLER

A quantitative infrared procedure for the determination of iodochlorhydroxyquin and its intermediates is described. The method is based on measurements of absorption in the 14.4 and 14.9 μ regions of a carbon disulfide solution of the compound. By measurements at other wavelengths in the infrared region, 5,7-diiodo-8-hydroxyquinoline, 5-chloro-8-hydroxyquinoline, and 5,7-dichloro-8-hydroxyquinoline, present as impurities, can also be quantitatively determined.

THE OFFICIAL U.S.P. XVII procedure (1) for the determination of iodochlorhydroxyquin, based on halogen content, suffers from the fact that the method frequently does not distinguish between the parent compound and its intermediates, which may occur as contaminants. The thin-layer chromatographic procedure of Korzun, Brody, and Tishler (2) offers a semiquantitative method for the determination of iodochlorhydroxyquin; however, 5,7-dichloro-8-hydroxyquinoline, which was found in many of the commercial samples examined, cannot be separated from iodochlorhydroxyquin by this method. Until now, phase solubility has been the only quantitative technique available for determining the absolute purity of iodochlorhydroxyquin. This procedure, although accurate and specific, is time consuming.

Bigeard *et al.* (3) have recently developed an infrared spectrophotometric method for the semiquantitative determination of iodochlorhydroxyquin

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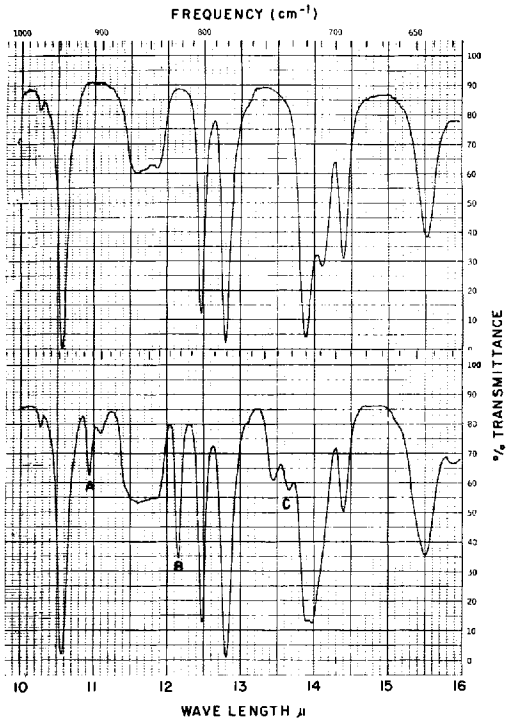

Fig. 1.—Infrared spectrum of iodochlorhydroxyquin (upper curve). Iodochlorhydroxyquin adulterated with: A, 5,7-diiodo-8-hydroxyquinoline; B, 5-chloro-8-hydroxyquinoline; C, 5, 7-dichloro-8-hydroxyquinoline (lower curve).

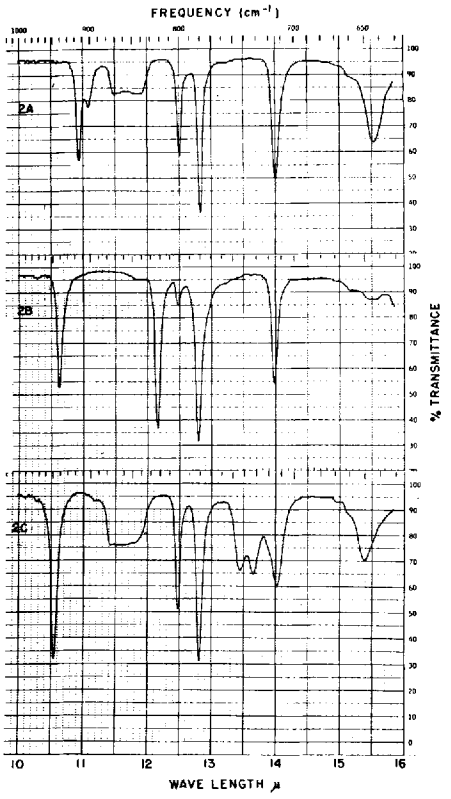

Fig. 2.—Infrared spectrum of: 2A, 5,7-diiodo-8-hydroxyquinoline; 2B, 5-chloro-8-hydroxyquinoline; 2C, 5,7-dichloro-8-hydroxyquinoline.

and its intermediates. The method, however, not only suffers from the typical experimental problems which arise in the potassium bromide disk technique, but also requires an infrared spectrophotometer capable of measurements in the far infrared region.

The modified infrared method described below fulfills the desired requirements for an analytical method—namely, specificity, rapidity, accuracy, and precision.

EXPERIMENTAL

All compounds used in this study were pure as determined by either phase solubility or by thin-layer chromatography. The infrared spectra were recorded on a Beckman I.R. 5 spectrophotometer and a Perkin-Elmer I.R. 621 spectrophotometer using reagent grade carbon disulfide as the solvent.

A calibration curve for the iodochlorhydroxyquin was prepared by dissolving various concentrations (2–10 mg./ml.) of the compound in carbon disulfide. The per cent transmittance at 14.4 and 14.9 μ was determined *versus* a carbon disulfide blank using 3-mm. cells equipped with sodium chloride plates. The ratios $[(T_{14.4\ \mu}/T_{14.9\ \mu}) \times 10]$ were calculated and the values plotted on the ordinate axis of 1-cycle semilogarithmic paper *versus* concentration. In a similar manner, calibration curves were prepared for 5,7-dichloro-8-hydroxyquinoline $[(T_{13.45\ \mu}/T_{15.25\ \mu}) \times 10]$, 5-chloro-8-hydroxyquinoline $[T_{12.15\ \mu}/TA\mu) \times 10]$,[1] and 5,7-diiodo-8-hydroxyquinoline $[(T_{10.95\ \mu}/TB\mu) \times 10]$.[1] A 0.5% solution of the sample in carbon disulfide was used in the spectrophotometric analysis of the synthetic mixtures and commercial samples. The phase solubility analyses were carried out in a nitrogen atmosphere using redistilled benzene as the solvent (4).

[1] A and B represent the transmittance readings at wavelengths 12.15 and 10.95 μ which are determined by the base line technique for the respective compounds.

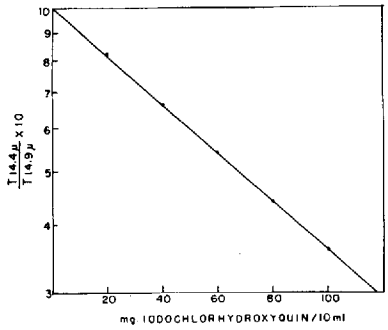

Fig. 3.—Linearity plot for iodochlorhydroxyquin.

DISCUSSION

A qualitative infrared examination of samples of iodochlorhydroxyquin U.S.P. currently on the market indicated the presence of the following impurities: 5,7-diiodo-8-hydroxyquinoline, 5,7-dichloro-8-hydroxyquinoline, and 5-chloro-8-hydroxyquinoline.

TABLE I.—ANALYSIS OF SYNTHETIC MIXTURES USING THE BECKMAN I.R. 5

Compd.	Mixture 1		Mixture 2	
	Added, %	Found, %	Added, %	Found, %
5-Chloro-8-hydroxyquinoline	3.0	2.85	5.0	5.2
5,7-Dichloro-8-hydroxyquinoline	3.0	2.80	5.0	5.0
5,7-Diiodo-8-hydroxyquinoline	3.0	2.70	5.0	5.5
Iodochlorhydroxyquin	91.0	89.0	85.0	84.0

TABLE II.—COMPARISON OF INFRARED AND PHASE SOLUBILITY METHODS

Manufacturer	Iodochlorhydroxyquin Content		
	I.R. 5, %	I.R. 621, %	Phase Solubility, %
Synthetic mixture[a]	89	90	90
A[b]	97	99	100
B	96	97	98
C[c]	60[d]	...	70
D	38	...	38
E	30	...	...

[a] Synthetic mixture contains 9% impurity. [b] Samples A and B are typical production batches prepared at Summit, N. J., and Basle, Switzerland. [c] Samples C, D, and E represent material sold as U.S.P. iodochlorhydroxyquin. [d] Insoluble material present.

5-Iodo-8-hydroxyquinoline was not observed in any samples and is not considered to be an important contaminant. The same technique described below can be used, however, for its determination.

The infrared spectrum between 10 and 16 μ for phase solubility pure iodochlorhydroxyquin is shown in the upper curve of Fig. 1. A sample of pure iodochlorhydroxyquin adulterated with 20%, 5,7-dichloro-8-hydroxyquinoline, 10% 5-chloro-8-hydroxyquinoline, and 20% 5,7-diiodo-8-hydroxyquinoline is shown in the lower curve of this figure. The infrared spectra of the three intermediates, 5,7-diiodo-8-hydroxyquinoline (2A), 5-chloro-8-hydroxyquinoline (2B), and 5,7-dichloro-8-hydroxyquinoline (2C) are shown in Fig. 2. It is quite obvious from Fig. 1 that the intermediates have a great effect on the spectrum of the pure material, especially at 10.95 μ (diiodo-), 12.15 μ (monochloro-), 13.45 μ (dichloro-), and 14.4 μ (iodochlorhydroxyquin).

Examination of the spectra of solutions of iodochlorhydroxyquin shows that the intensity of the absorption band occurring at 14.4 μ decreases as the amounts of impurities present increase. It can be seen from Fig. 2 that the three intermediates exhibit no significant absorption at this wavelength. Semilogarithmic plots of the ratio of per cent transmittance at 14.4 μ/per cent transmittance at 14.9 μ *versus* concentration of iodochlorhydroxyquin are linear. Figure 3 shows this relationship. Although similar plots are not shown for the three intermediates, they also exhibited linearity.

Even though the plots are linear over a wide range of concentration, it was observed that the most reproducible results were obtained in the range of 40–60% transmittance. It was found that samples containing these intermediates in substantial amounts were not soluble to the extent of 1% in carbon disulfide. They did give clear solutions, however, at a concentration of 0.5%. In order to maintain the absorption of the 14.4 μ band in the transmittance range of 40–60%, a light path of 3 mm. was employed.

In order to determine the accuracy of the procedure, synthetic mixtures were prepared and analyzed spectrophotometrically as described. The results appear in Table I.

Samples of U.S.P. quality iodochlorhydroxyquin from various commercial sources were analyzed using a Beckman I.R. 5 spectrophotometer and a Perkin Elmer I.R. 621 spectrophotometer. These results were compared with phase solubility analyses and are shown in Table II.

It can be seen from Table II that the accuracy of the method is increased if a more accurate and better resolving infrared spectrophotometer is used. In the case of the I.R. 5 spectrophotometer the accuracy is ±3% and with the I.R. 621 spectrophotometer, ±1%.

Analysis of samples C, D, and E by the current U.S.P. XVII procedures for chlorine and iodine and identity showed that two of the three samples passed all tests and could be considered of U.S.P. quality, even though they contain 60% or less of iodochlorhydroxyquin as determined by infrared measurements.

Since it has been demonstrated that iodochlorhydroxyquin may contain considerable amounts of intermediates as impurities and still meet present U.S.P. specifications, it is apparent that a more specific method of determination of purity of iodochlorhydroxyquin is necessary. The infrared procedure described provides a rapid, highly specific, and quantitative method for this purpose.

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Quantitative Determination of Hexachlorophene in Ointments Containing Salicylic Acid and Methyl *p*-Hydroxybenzoate

By RHYS BRYANT, DONALD E. MANTLE, and DAVID S. YODER

Hexachlorophene in ointments is determined spectrophotometrically by the "difference" method after selective removal of all interfering components.

THE PRESENTLY available colorimetric (1, 6–10, 14, 15) and spectrophotometric (2–5, 11, 12) methods for quantitative estimation of hexachlorophene [2,2′-methylenebis(3,4,6-trichlorophenol)] are unsuitable for the determination of hexachlorophene in ointment preparations containing both salicylic acid and methyl *p*-hydroxybenzoate.

The colorimetric method using 4-aminoantipyrine requires the color complex to be in aqueous solution (6). In the case of sulfur-containing ointments, the sulfur remains suspended[1] in the aqueous solution and causes considerable interference (*cf. Reference 13*).

Attempts to remove salicylic acid from both ether and chloroform extracts of the ointments with sodium bicarbonate resulted in emulsion formation. The emulsions were extremely stable and permitted only a 50% recovery of hexachlorophene.

To measure the absorbance of hexachlorophene at 257.5 mμ, where the absorbance of salicylic acid is independent of pH change (5), would result in interference due to methyl *p*-hydroxybenzoate, which absorbs (5) at 257 mμ in pH 7.5 buffer.

A procedure for hexachlorophene assay which overcomes the above difficulties is reported here. The organic components of the ointments are extracted into ether from a methanol–aqueous sodium chloride suspension which suppresses emulsion formation. The salicylic acid is removed selectively into pH 8 buffer, and the methyl *p*-hydroxybenzoate saponified. The hexachlorophene may then be measured spectrophotometrically by the "difference" method (2, 5), with no interference from other components.

EXPERIMENTAL

Apparatus.—A Cary model 14M was used in this work.

Reagents.—All solutions are aqueous unless otherwise specified. The hexachlorophene standard solution is prepared by dissolving 100 mg. of hexachlorophene U.S.P. in 100 ml. of methanol.

Procedure.—A quantity of ointment containing 2 mg. of hexachlorophene was weighed into a 125-ml. separator and shaken with 15 ml. of methanol. Fifteen milliliters of 7% sodium chloride was added to the solution which, after cooling to room temperature, was extracted with 25 ml. of ether. The aqueous-methanol layer was re-extracted with 25 ml. of ether in a second 125-ml. separator. Each ether extract was washed by allowing 1 ml. of 7% sodium chloride to pass through the ether layer without shaking. After draining and discarding the sodium chloride layer, 1 ml. of 35% sodium chloride and 10 ml. of pH 8 Clark and Lubs buffer were added to each ether extract. Each separator was shaken gently, and the aqueous layers were combined in a single 125-ml. separator. After extracting the aqueous layer with 25 ml. of ether, the aqueous portion was discarded. The three ether extracts obtained were combined into a 100-ml. volumetric flask and evaporated to dryness. The residue was dissolved in 5 ml. of methanol and 10 ml. of 1 *N* sodium hydroxide and the solution heated at 75° for 30 min. The solution was cooled, diluted to volume with methanol, and filtered. Exactly 20 ml. of the solution was placed in a 25-ml. volumetric flask and diluted to volume with methanol. This solution was placed in the sample cell of the spectrophotometer. Another 20 ml. of the solution was placed in a second 25-ml. volumetric flask and diluted to volume with 1 *N* methanolic hydrochloric acid. This solution was placed in the reference cell of the spectrophotometer and the maximum differential ultraviolet absorbance of hexachlorophene measured at 320 mμ. The concentration of hexachlorophene was determined by comparison with a standard hexachlorophene solution which had been carried through the above procedure beginning with the methanol–1 *N* sodium hydroxide treatment.

DISCUSSION

The presence of fairly large quantities of surfactant in ointments resulted in incomplete extraction of hexachlorophene due to formation of stable emulsions. When the extractions were preceded by treatment with sodium chloride–methanol, a quantitative separation of the hexachlorophene was obtained. Attempts to separate the salicylic acid using sodium carbonate or sodium bicarbonate were unsuccessful due to emulsion formation and concomitant loss of hexachlorophene.

The saponification of methyl *p*-hydroxybenzoate is necessary for the quantitative estimation of the hexachlorophene. The maximum absorbance of methyl *p*-hydroxybenzoate both at pH 0.8 (the pH of the final methanolic acid solution) and at pH 11.5 (the pH of the final alkaline methanol solution) is 298 mμ, and the hexachlorophene absorption is seen only as a shoulder on the larger methyl *p*-hydroxybenzoate peak. Sodium *p*-hydroxybenzoate in methanolic solution at pH 11.5, when read using the "difference" method against the acid solution at pH 0.8, absorbs at 286 mμ. This hypsochromic shift is sufficient to separate completely the *p*-hydroxybenzoate absorption from that of the hexachlorophene, which can then readily be measured. The use of acidified 90% methanol suggested in U.S.P. XVII (16) was precluded for the following reason: it was necessary to hydrolyze the methyl *p*-hydroxybenzoate with strong alkali. Neutralization of the solution with acidified 90% methanol gave a final solution of pH 7.7. At this pH, the higher absorbance of the *p*-hydroxybenzoate inter-

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[1] The reviewer of the manuscript suggests the use of 2-oz. stainless steel syringes fitted with disposable membrane holders for the removal of sulfur by membrane filtration. This is an excellent suggestion, although it was not used in this case.

fered with the hexachlorophene absorbance. The wavelength of hexachlorophene absorbance is pH dependent. In the system reported here (pH 0.8/11.5), the absorbance was at 320 mμ. Using the U.S.P. XVII buffer–acid system, the absorbance is at 312 mμ, causing slight interference with the *p*-hydroxybenzoate absorbance.

RESULTS

The recovery of standard hexachlorophene carried through this method was 99 ± 2%. The accuracy and reproducibility of the spectrophotometric "difference" method have already been well documented (5).

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(16) "United States Pharmacopeia," 17th rev., Mack Publishing Co., Easton, Pa., 1965, p. 281.

Identification of 1-, 2-, 3-, and 4-Chlorophenothiazine Isomers

By I. B. EISDORFER, R. J. WARREN, W. E. THOMPSON, and J. E. ZAREMBO

The infrared and ultraviolet spectral data for four monochloroisomers of phenothiazine are presented and discussed. From these data it is possible to make a positive and rapid identification of any of the isomers with a minimum amount of sample. The method can be used to identify the isomers alone or in combination.

THE PRESENCE of isomers in the preparation of chlorinated phenothiazines is always a possibility. The problem of determining the presence of an isomer and identifying the specific isomer present is a frequent analytical problem. A simple, rapid method for identifying the 1-, 2-, 3-, and 4-chlorophenothiazine isomers is presented.

EXPERIMENTAL

Reagents. — 1 - Chlorophenothiazine, 2-chlorophenothiazine, 3-chlorophenothiazine, and 4-chlorophenothiazine. All chemicals are of analytical grade as prepared at Smith Kline & French Laboratories.

Spectrophotometers.—The infrared spectra were recorded with a Perkin-Elmer model 21 double beam spectrophotometer with a sodium chloride prism. The phenothiazines studied were prepared as mineral oil mulls.

The ultraviolet spectra were recorded with a Cary model 14 recording spectrophotometer using matched fused silica cells with a 1-cm. light path.

RESULTS AND DISCUSSION

Infrared Spectra.—Figure 1 shows the infrared spectra obtained for the four chlorophenothiazine isomers. The area of greatest interest is the region between 1000 and 650 cm.$^{-1}$. This is the region which contains absorption bands due to C–H out-of-plane deformations in aromatic ring systems. Each of the isomers has its own unique pattern in this area owing to the particular position of the chlorine atom on the ring. The infrared pattern here is specific enough to distinguish one isomer from another, and the absorption bands are so located as to permit detection of one or more isomers in the presence of another.

Spectrum A, which is that of the 1-chloro isomer, shows strong absorption bands between 770 and 700 cm.$^{-1}$ which are assignable to 3 and 4 adjacent free hydrogen atoms in an aromatic ring (1). Spectrum B is that of the 2-chloro isomer with absorption

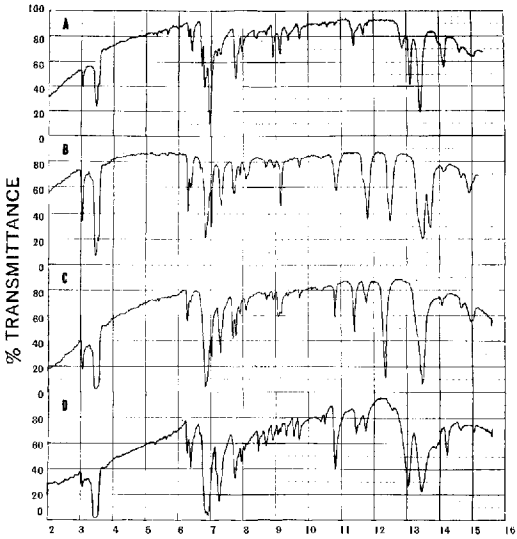

Fig. 1.—Key: A, 1-chlorophenothiazine; B, 2-chlorophenothiazine; C, 3-chlorophenothiazine; D, 4-chlorophenothiazine.

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THE PRESENCE of isomers in the preparation of chlorinated phenothiazines is always a possibility. The problem of determining the presence of an isomer and identifying the specific isomer present is a frequent analytical problem. A simple, rapid method for identifying the 1-, 2-, 3-, and 4-chlorophenothiazine isomers is presented.

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Infrared Spectra.—Figure 1 shows the infrared spectra obtained for the four chlorophenothiazine isomers. The area of greatest interest is the region between 1000 and 650 cm.$^{-1}$. This is the region which contains absorption bands due to C–H out-of-plane deformations in aromatic ring systems. Each of the isomers has its own unique pattern in this area owing to the particular position of the chlorine atom on the ring. The infrared pattern here is specific enough to distinguish one isomer from another, and the absorption bands are so located as to permit detection of one or more isomers in the presence of another.

Spectrum A, which is that of the 1-chloro isomer, shows strong absorption bands between 770 and 700 cm.$^{-1}$ which are assignable to 3 and 4 adjacent free hydrogen atoms in an aromatic ring (1). Spectrum B is that of the 2-chloro isomer with absorption

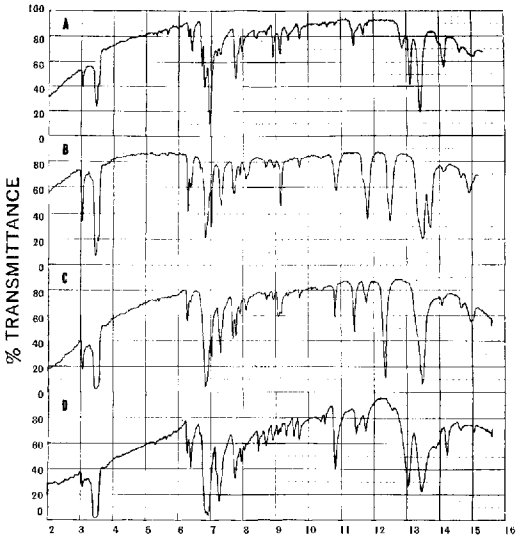

Fig. 1.—Key: A, 1-chlorophenothiazine; B, 2-chlorophenothiazine; C, 3-chlorophenothiazine; D, 4-chlorophenothiazine.

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bands at 750, 800, 825, and 920 cm.$^{-1}$ which are assignable to a 2-substituted phenothiazine (2). Spectrum C is the 3-chloro isomer with absorption bands at 750, 810, 850, 875, and 925 cm.$^{-1}$ which are all assignable to 4 and 2 adjacent free hydrogen atoms and one free hydrogen atom on an aromatic ring (1). Spectrum D is the 4-chloro isomer with strong absorption bands at 740 and 765 cm.$^{-1}$ which are in agreement with out-of-plane bending vibrations of 3 and 4 adjacent free hydrogen atoms on an aromatic ring system (1). From these infrared data it is possible not only to identify each isomer alone but also in combination with the other three isomers. An illustration of how these data may be used to identify the isomers in combination is shown in Fig. 2. Spectrum A is that of 2-chlorophenothiazine. Spectrum B is a mixture containing 6% of 3-chlorophenothiazine. Spectrum C is the spectrum of a 10% mixture of the 3-isomer in 2-chlorophenothiazine. There is no absorption whatever at 11.45 μ in the 2-chloro isomer but in the spectra of both mixtures an absorption band at 11.45 μ is noted. This band is due to the presence of the 3-chloro isomer (see spectrum C in Fig. 1). Preparation of a series of standards or mixtures of known concentrations makes it possible to prepare a calibration curve from which quantitative data and minor components could be derived.

The infrared spectra of the four isomers complements the ultraviolet data which follow and is very valuable indeed in identifying chromatographic fractions from gas chromatography or spots from thin-layer or paper chromatography. It also provides a rapid means of identification of isomers in a given preparation of chlorinated phenothiazines.

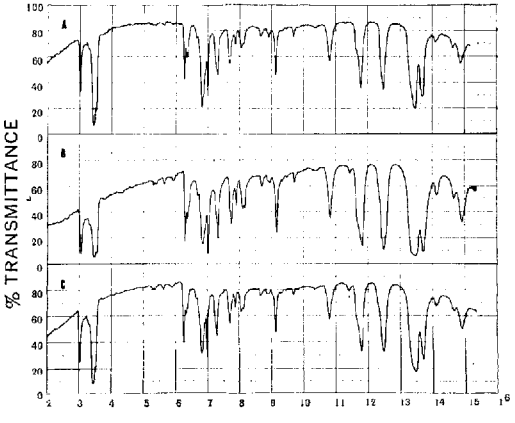
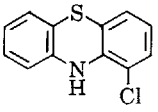
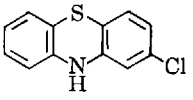
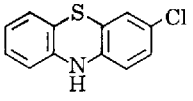
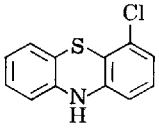


Fig. 2.—Key: A, 2-chlorophenothiazine; B, 6% 3-chlorophenothiazine in 2-chlorophenothiazine; C, 10% 3-chlorophenothiazine in 2-chlorophenothiazine.

Ultraviolet Spectra.—Table I lists the ultraviolet maxima and corresponding log ϵ values for the four

TABLE I.—U.V. SPECTRAL DATA FOR CHLOROPHENOTHIAZINES[a]

Compd.	λ, mμ	Log ε
 1-Chlorophenothiazine	318	3.62
	257	4.63
 2-Chlorophenothiazine	320	3.69
	256	4.71
 3-Chlorophenothiazine	323	3.67
	258	4.68
 4-Chlorophenothiazine	325	3.65
	259.5	4.66

[a] U. V. spectra run in 95% ethanol.

chloro isomers. The effect of the position of the chlorine on the ring can readily be seen in the various wavelength shifts. The infrared data complemented by these ultraviolet data for the isomers make possible a quantitative estimation of the amount of various isomers present in a given preparation.

SUMMARY

Data are given to show that 1-, 2-, 3-, and 4-chlorophenothiazines each possess a unique infrared and ultraviolet spectrum. This information provides a basis for determining the presence or absence of isomers in a given preparation of chlorinated phenothiazine.

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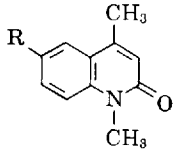
Nitration of 1,4-Dimethyl-2-quinolone and 1,2-Dimethyl-4-quinolone

By I. NABIH and M. NASR

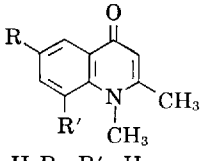
Nitration occurred at position 6 in 1,4-dimethyl-2-quinolone and at 6 and 8 in 1,2-dimethyl-4-quinolone. Structures were confirmed by alternate synthesis.

EXTENSIVE work has been carried out on the nitration of quinolines (1). In the case of quinolones, nitration of 1 - methyl - 2 - quinolone with nitric and sulfuric acids has given 6-nitro-1-methyl-2-quinolone, which on further nitration gave 3,6,8 - trinitro - 1 - methyl - 2 - quinolone (2, 3). 1-Methyl-4-quinolone has been treated with 68% nitric acid to give 3 - nitro - 1 - methyl - 4 - quinolone, and further nitration led to 3,6-dinitro-1-methyl-4-quinolone (4). 1,4-Dimethyl-2-quinolone (I) has been treated with an excess of fuming nitric acid to give a mixture of compounds tentatively assigned the structures of 6-nitro-1,4-dimethyl-2-quinolone (III) and 3,6,8-trinitro-1, 4-dimethyl-2-quinolone (5). The purpose of the present work was to establish structure III, and also to carry out similar studies on the nitration of 1,2-dimethyl-4-quinolone (II) and to establish the structures of the nitration products. Their assignments were mainly dependent upon using compounds of accurate structural assignment as possible starting materials.

Thus, compound III was identified through the preparation of 6-nitro-2-hydroxy-4-methylquinoline (6), which upon treatment with methyl iodide and potassium methoxide gave 6-nitro-1,4-dimethyl-2-quinolone which is identical with III (as shown by mixed melting point).



I, R = H
III, R = NO_2
IV, R = NH_2



II, R = R′ = H
V, R = R′ = NO_2
VI, R = NO_2, R′ = H

NMR spectra were determined for both I and II. The vinyl hydrogen at the 3-position appears in both spectra as a singlet at 3.65 τ and 4.1 τ, respectively, while the aromatic hydrogens in I appeared at 2.8 τ. They split into separate peaks in the spectrum of III at 3.2 τ and 2.3 τ. These correspond to the hydrogen atoms at the 5-position and 7- and 8-positions as shown from their integration values, but the signal arising from the vinyl hydrogen at the 3-position (3.65 τ) remained unchanged, an indication of nonsubstitution at that position.

Reduction of III with stannous chloride and hydrochloric acid led to 6-amino-1,4-dimethyl-2-quinolone (IV).

Nitration of 1,2-dimethyl-4-quinolone (II) in sulfuric–nitric acid medium led to substitution at both the 6- and 8-positions to give V, the structure of which was assigned by alternative synthesis.

6-Nitro-1,2-dimethyl-4-quinolone (VI) was prepared by the action of dimethyl sulfate in an alkaline solution of 6-nitro-2-methyl-4-hydroxyquinoline (7), and further nitration of VI gave 6,8-dinitro-1,2-dimethyl-4-quinolone, which was identical with V. 8-Nitro-2-methyl-4-hydroxyquinoline (8), when subjected to further nitration, led to the 6,8-dinitro derivative, which upon treatment with dimethyl sulfate in an alkaline medium gave V.

EXPERIMENTAL

All melting points are uncorrected and measured on Kofler hot stage.

6 - Nitro - 1,4 - dimethyl - 2 - quinolone (III).—*Method A.*—To a solution of 1,4-dimethyl-2-quinolone (9) (3 Gm., 0.017 mole) in 10 ml. of concentrated sulfuric acid was added 5 ml. of nitric acid (d. 1.42) mixed with 15 ml. of concentrated sulfuric acid, while the temperature was kept at 0°. The reaction mixture was kept for 24 hr. at room temperature, and when poured onto crushed ice, a yellow precipitate formed, m.p. 223–225°. Recrystallization from ethanol gave 3.46 Gm. (90% yield) of III, m.p. 225–227°. [Lit. m.p. 228.5–229° (7).]

Anal.—Calcd. for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.59; N, 12.84. Found: C, 60.51; H, 4.75; N, 12.89.

Method B.—To a mixture of 6-nitro-4-methyl-2-hydroxyquinolone (8) (2.04 Gm., 0.01 mole) in 10 ml. of 2 *N* potassium methoxide and 15 ml. of absolute methyl alcohol was added 4 Gm. of methyl iodide. The mixture was heated under reflux for 3 hr., then allowed to cool overnight. The precipitated potassium iodide was separated, the filtrate was diluted with water and made alkaline by addition of sodium hydroxide solution, and a yellow substance precipitated, m.p. 224–225°. Recrystallization from ethanol gave m.p. 225–227° which was undepressed on admixture with an authentic sample, prepared by *Method A*.

Anal.—Calcd. for $C_{11}H_{10}N_2O_3$: C, 60.55; H, 4.59; N, 12.84. Found: C, 60.75; H, 4.52; N, 12.96.

6 - Amino - 1,4 - dimethyl - 2 - quinolone (IV).—To a solution of 6-nitro-1,4-dimethyl-2-quinolone (1.88 Gm., 0.01 mole) in glacial acetic acid (15 ml.), was added a solution of stannous chloride (7.5 Gm.) in concentrated hydrochloric acid (10 ml.). The mixture was refluxed for 3 hr., then kept overnight at room temperature. The precipitated stannic chloride was filtered off, and the filtrate was boiled for 20 min. with 50% aqueous sodium hydroxide (25 ml.). The solid so obtained, m.p. 275–277°, was recrystallized from ethanol, m.p. 279–280°.

Anal.—Calcd. for $C_{11}H_{12}N_2O$: C, 70.21; H, 6.39; N, 14.89. Found: C, 70.08; H, 6.35; N, 14.66.

1,2-Dimethyl-4-quinolone (II).—To a solution of

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2-methyl-4-hydroxyquinoline (15.9 Gm., 0.4 mole) in 150 ml. of aqueous potassium hydroxide (5%), was added dimethyl sulfate (8 ml.). After shaking the mixture for 30 min., potassium hydroxide solution (50 ml.) was added with continuous shaking during an additional 30 min. The reaction mixture was kept for 48 hr. at room temperature. The product was extracted with chloroform, then recrystallized several times from benzene to give 2.59 Gm. (15% yield) of II in the form of light-red needles, m.p. 158–160°. [Lit. m. p. 156–160° (10).]

6,8 - Dinitro - 1,2 - dimethyl - 4 - quinolone (V).—To a solution of 1,2-dimethyl-4-quinolone (0.173 Gm.) in sulfuric acid (5 ml.) was added a mixture of nitric acid (1 ml.) and sulfuric acid (3 ml.) by the same method as described for 1,4-dimethyl-2-quinolone (I). The product obtained, m.p. 258°, when recrystallized from ethanol, gave 0.17 Gm. (65% yield) of V, m.p. 259–261°.

Anal.—Calcd. for $C_{11}H_{12}N_3O_5$: C, 49.62; H, 4.51; N, 15.78. Found: C, 50.02; H, 4.45; N, 15.98.

6 - Nitro - 1,2 - dimethyl - 4 - quinolone (VI).—To a solution of 6-nitro-2-methyl-4-hydroxyquinoline (7) (2.04 Gm.) in 50 ml. of aqueous potassium hydroxide (5%) was added dimethyl sulfate (3.3 Gm.). The mixture was stirred at 70° for 30 min. and kept at room temperature overnight. The formed yellow precipitate, m.p. 215°, was recrystallized from hot benzene to give 1.27 Gm. (40% yield) of VI, m.p. 218–220°.

Anal.—Calcd. for $C_{11}H_{10}N_2O_5$: C, 60.55; H, 4.58; N, 12.88. Found: C, 60.37; H, 4.46; N, 12.75.

6,8 - Dinitro - 1,2 - dimethyl - 4 - quinolone (V).—To a solution of 6-nitro-1,2-dimethyl-4-quinolone (VI) (1.5 Gm.) in concentrated sulfuric acid (10 ml.) at 10° was added a mixture of nitric acid (d. 1.42, 1 ml.) and concentrated sulfuric acid (3 ml.). After standing for 24 hr. at room temperature, the mixture was poured onto crushed ice, the isolated product, m.p. 257°, was recrystallized from ethanol to give 1.13 Gm. (90% yield) of V, m.p. 259–261°. It was identical in melting point with an authentic sample of V, and showed no depression of mixed melting point.

Anal.—Calcd. for $C_{11}H_{12}N_3O_5$: C, 49.62; H, 4.51; N, 15.78. Found: C, 49.78; H, 4.62; N, 15.92.

6,8 - Dinitro - 2 - methyl - 4 - hydroxyquinoline.—A solution of 8-nitro-2-methyl-4-hydroxyquinoline (8) (2.04 Gm., 0.01 mole) in concentrated sulfuric acid (10 ml.) was treated with a mixture of nitric acid and sulfuric acid, as described above. The isolated product, m.p. 258°, was recrystallized from ethanol to give 1.85 Gm. of 6,8-dinitro-2-methyl-4-hydroxyquinoline (75% yield), m.p. 247–249°.

Anal.—Calcd. for $C_{10}H_7N_3O_5$: C, 48.19; H, 2.81; N, 16.86. Found: C, 48.21; H, 3.04; N, 16.85.

When this compound was treated with dimethyl sulfate in an alkaline medium, as for the preparation of 6-nitro-1,2-dimethyl-4-quinolone, the isolated product was identical with V, with no depression of the mixed melting point.

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The present note deals with the preparation of a series of *N*-aminoalkyl-α-aminoacids and their corresponding ethyl esters for submitting to pharmacological screening. In addition, these compounds were useful intermediates for the synthesis of 3-substituted sydnones, as we have recently reported (7).

The esters in question were prepared by alkylation with α-bromo esters of suitable *N*-aminoalkyl-amines. Because of the side reactions resulting from the competitive aminolysis of the ester group (8), the authors studied the synthesis procedure in detail, and found it an advantage to use

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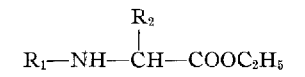
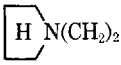
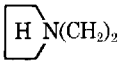
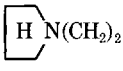
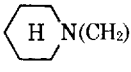
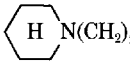
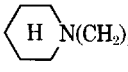
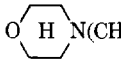
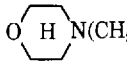
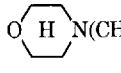
TABLE I.—*N*-AMINOALKYL-α-AMINOESTERS



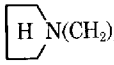
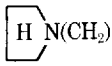
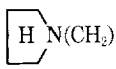
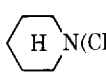
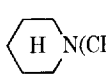
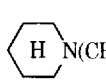
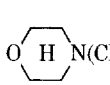
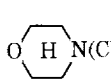
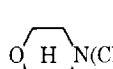
Compd.	R_1	R_2	Reflux Time, hr.	Yield,[a] %	B.p., °C., mm.	Formula	Anal., % Calcd.	Found
I[b]	$(C_2H_5)_2N(CH_2)_2$	H	1	75	77–80 (0.5)	$C_{10}H_{22}N_2O_2$	C, 59.37 H, 10.96 N, 13.85	58.94 10.81 13.54
II	$(C_2H_5)_2N(CH_2)_2$	CH_3	2	55	73–76 (0.8)	$C_{11}H_{24}N_2O_2$	C, 61.07 H, 11.18 N, 12.95	60.89 11.20 12.75
III[c]	$(C_2H_5)_2N(CH_2)_2$	C_6H_5	1	54	125–126 (0.3)	$C_{16}H_{26}N_2O_2$	C, 69.03 H, 9.41 N, 10.06	68.95 9.26 9.87
IV		H	1	51	100–103 (1.0)	$C_{10}H_{20}N_2O_2$	C, 59.97 H, 10.07 N, 13.99	59.56 9.92 14.14
V		CH_3	2	67	92–94 (1.0)	$C_{11}H_{22}N_2O_2$	C, 61.65 H, 10.35 N, 13.07	61.11 10.17 12.80
VI[d]		C_6H_5	1	74	125–127 (0.2)	$C_{16}H_{24}N_2O_2$	C, 69.53 H, 8.75 N, 10.14	69.00 8.85 10.13
VII		H	1	87	99–102 (0.5)	$C_{11}H_{22}N_2O_2$	C, 61.65 H, 10.35 N, 13.07	62.03 10.26 13.31
VIII		CH_3	2	64	98–101 (1.0)	$C_{12}H_{24}N_2O_2$	C, 63.12 H, 10.60 N, 12.27	62.94 10.39 12.45
IX[e]		C_6H_5	1	78	147–149 (0.5)	$C_{17}H_{26}N_2O_2$	C, 70.31 H, 9.02 N, 9.65	70.76 9.20 9.50
X		H	0.5	42	94–98 (0.2)	$C_{10}H_{20}N_2O_3$	C, 55.53 H, 9.32 N, 12.95	55.76 9.29 13.16
XI		CH_3	2	50	106–108 (1.0)	$C_{11}H_{22}N_2O_3$	C, 57.36 H, 9.63 N, 12.17	57.04 9.70 12.42
XII		C_6H_5	1	31	155–158 (0.3)	$C_{16}H_{24}N_2O_3$	C, 65.72 H, 8.27 N, 9.58	66.08 8.32 9.56
XIII	$(CH_3)_2N(CH_2)_3$	H	1	38	76–78 (1.0)	$C_9H_{20}N_2O_2$	C, 57.41 H, 10.71 N, 14.88	57.24 10.58 14.55
XIV	$(CH_3)_2N(CH_2)_3$	CH_3	2	69	72–74 (1.0)	$C_{10}H_{22}N_2O_2$	C, 59.37 H, 10.96 N, 13.85	58.87 10.85 13.66

[a] Distilled once. [b] Lit. (8) b.p. 78° (1.1 mm.), yield 45%. [c] Lit. (2) b.p. 156–158° (1.5 mm.), yield 52%. [d] Lit. (2) b.p. 163–166° (1.5 mm.), yield 80%. [e] Lit. (2) b.p. 172–176° (2 mm.), yield 76%.

ether as the solvent and 1 mole of triethylamine as scavenger of the hydrobromic acid formed during the reaction. By contrast, the use of boiling benzene and excess *N*-aminoalkyl-amine as acid scavenger (9, 10), as also the use of α-chloro esters instead of the more reactive α-bromo derivatives, were found to give rise to *N*,*N*′-diaminoalkylglycinamides as by-products. Table I gives the reaction conditions, yields, and physical and analytical properties of the esters prepared. All the compounds are colorless oils which may easily be purified by vacuum distillation.

The *N*-aminoalkyl-α-aminoacids were obtained by hydrolyzing the above esters with 20% sodium hydroxide at 60–70°. Brown products difficult to crystallize were obtained at the refluxing tempera-

TABLE II.—*N*-AMINOALKYL-α-AMINOACID DIHYDROCHLORIDES

Compd.	R_1	R_2	Yield,[a] %	M.p.,[b] °C.	Recrystn. Solvent	Formula	Anal., % Calcd.	Found
XV	$(C_2H_5)_2N(CH_2)_2$	H	78	143–144	Ethanol	$C_8H_{20}Cl_2N_2O_2$	C, 38.87 H, 8.15 Cl, 28.69 N, 11.33	38.33 8.26 28.60 11.42
XVI	$(C_2H_5)_2N(CH_2)_2$	CH_3	86	193–195	Ethanol–acetone	$C_9H_{22}Cl_2N_2O_2$	C, 41.38 H, 8.49 Cl, 27.12 N, 10.72	41.57 8.40 26.98 10.73
XVII[c]	$(C_2H_5)_2N(CH_2)_2$	C_6H_5	74	229–230	Ethanol–methanol	$C_{14}H_{24}Cl_2N_2O_2$	C, 52.01 H, 7.48 Cl, 21.93 N, 8.67	51.58 7.37 22.18 8.71
XVIII		H	81	190–191	Ethanol, 98%	$C_8H_{18}Cl_2N_2O_2$	C, 39.19 H, 7.40 Cl, 28.93 N, 11.43	39.01 7.46 28.83 11.23
XIX		CH_3	95	189–190	Ethanol–acetone	$C_9H_{20}Cl_2N_2O_2$	C, 41.70 H, 7.78 Cl, 27.36 N, 10.81	40.91 7.86 26.95 10.81
XX		C_6H_5	84	219–220	Ethanol	$C_{14}H_{22}Cl_2N_2O_2$	C, 52.34 H, 6.90 Cl, 22.07 N, 8.72	52.30 7.01 22.03 8.56
XXI		H	86	172–173	Ethanol	$C_9H_{20}Cl_2N_2O_2$	C, 41.70 H, 7.78 Cl, 27.36 N, 10.81	41.39 7.90 27.12 10.56
XXII		CH_3	95	210–211	Ethanol	$C_{10}H_{22}Cl_2N_2O_2$	C, 43.92 H, 8.12 Cl, 25.96 N, 10.25	44.11 8.09 26.05 10.03
XXIII[d]		C_6H_5	80	224–225	Ethanol	$C_{15}H_{24}Cl_2N_2O_2$	C, 53.73 H, 7.22 Cl, 21.15 N, 8.36	53.70 7.47 21.09 8.29
XXIV		H	92	190–192	Methanol	$C_8H_{18}Cl_2N_2O_3$	C, 36.79 H, 6.95 Cl, 27.15 N, 10.72	36.39 7.04 27.00 10.54
XXV		CH_3	95	223–224	Methanol	$C_9H_{20}Cl_2N_2O_3$	C, 39.29 H, 7.33 Cl, 25.75 N, 10.18	39.66 7.32 25.78 9.95
XXVI[d]		C_6H_5	93	231–232	Acetic acid	$C_{14}H_{22}Cl_2N_2O_3$	C, 49.85 H, 6.57 Cl, 21.02 N, 8.31	49.35 6.67 20.71 8.33
XXVII	$(CH_3)_2N(CH_2)_3$	H	92	190–191	Ethanol–methanol	$C_7H_{18}Cl_2N_2O_2$	C, 36.05 H, 7.78 Cl, 30.40 N, 12.01	35.77 7.71 30.22 11.96
XXVIII	$(CH_3)_2N(CH_2)_3$	CH_3	83	227–228	Ethanol, 95%	$C_8H_{20}Cl_2N_2O_2$	C, 38.87 H, 8.15 Cl, 28.69 N, 11.33	39.06 8.30 28.48 11.25

[a] Crude product. [b] The compounds melt with decomposition. [c] Reported as the hydrobromide by Szarvasi, E., and Neuvy, L., *Bull. Soc. Chim. France*, **1957**, 1019. [d] Reported as the free base by Kawahara, S., and Katsuno, K., *Yakugaku Zasshi*, **82**, 912(1962).

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Preliminary data on the pharmacological screening which was performed in accordance with the techniques previously described (11), have shown that some members of both series possess a certain degree of antispasmodic, local anesthetic, and antitussive activity.

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Boiling points are uncorrected. Melting points are corrected and were taken on a Büchi capillary melting point apparatus. The intermediates were commercial products or else obtained according to the procedures reported in the literature.

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N - (3 - Dimethylaminopropyl)-alanine Ethyl Ester (XIV).—Ethyl α-bromo-propionate (54.3 Gm., 0.3 mole) dissolved in ether (60 ml.) was added dropwise to a solution of 3-dimethylamino-1-propylamine (30.6 Gm., 0.3 mole) and triethylamine (30.3 Gm., 0.3 mole) and ether (150 ml.), stirring and cooling moderately to room temperature. The mixture was stirred for 1 hr., then refluxed for 2 hr., and allowed to stand overnight. The precipitated triethylamine hydrobromide was filtered off and the solvent removed under reduced pressure. The residue was then distilled, b.p. 72–74° (1 mm.), giving a colorless oil (41.9 Gm.).

N - (2 - Pyrrolidinylethyl) - glycine Dihydrochloride (XVIII).—A mixture of IV (20 Gm., 0.1 mole), sodium hydroxide (6 Gm., 0.15 mole), and water (24 ml.) was cautiously heated to 65°, with efficient stirring. At this temperature hydrolysis continued spontaneously, without necessitating further heating. The solution so obtained was washed with ether and acidified to pH 1 by cautious addition of concentrated hydrochloric acid. The reaction mixture was evaporated to dryness *in vacuo* and the residue was extracted with 300 ml. of boiling ethanol in portions. The combined alcoholic extracts were then distilled and the residue (19.8 Gm.) was crystallized from 98% ethanol. After drying at 90° *in vacuo*, colorless crystals were obtained, m.p. 190–191° dec.

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By LEO GREENBERG

Metered doses of a quaternary ammonium, glycol, alcohol formulation were dispensed automatically by motorized apparatus at 15-min. intervals around the clock for 1 month, and the effects of such treatment on the microbial flora of the air in a heavily trafficked area were studied. Data indicate that, despite large variations in number and types of microorganisms found in the air, especially during periods of heavy traffic, continued aerosol treatment was capable of sharply reducing mean population values and in altering the flora from a predominantly bacterial population to one dominated by members of the *Penicillium–Aspergillus* group of fungi.

In recent years, the subjects of microbiological air pollution and air sanitation have gained considerable importance, and much information is now available concerning the immediate and latent effects caused by inhalation and retention of foreign airborne particles and bacteria. Present knowledge *indicates* that particles approximately 1–5 μ in diameter are most effective for penetration and retention in the deep pulmonary spaces (1), and in addition, larger particles bearing many organisms may infect open wounds. In light of recent experiences with hospital-associated staphylococcic infections, much attention has been devoted to the removal or inactivation of biological particles of all sizes from the air used in critical spaces.

To accomplish these ends, a widely diversified group of chemical agents and methods has been proposed, including the use of the gaseous fumigants formaldehyde, β-propiolactone, and ethylene oxide. For more limited and routine use, numerous commercial products designed to reduce air contamination have been developed, and are designed for aerosolization either by mechanical spray or by means of propellants such as the freons. Among the agents utilized for such purposes have been various alcohols, glycols, volatile oils, phenols, and quaternaries.

In general, such commercial aerosols have been

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To accomplish these ends, a widely diversified group of chemical agents and methods has been proposed, including the use of the gaseous fumigants formaldehyde, β-propiolactone, and ethylene oxide. For more limited and routine use, numerous commercial products designed to reduce air contamination have been developed, and are designed for aerosolization either by mechanical spray or by means of propellants such as the freons. Among the agents utilized for such purposes have been various alcohols, glycols, volatile oils, phenols, and quaternaries.

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TABLE I.—COLONY COUNTS OF MICROORGANISMS COLLECTED BY AIR SAMPLING IN TEST LABORATORY

Organism	(Start) No Treatment, 2 Wk.[a]	Aerosol Treatment				(Finish) No Treatment 2 Wk.[a]
		Wk. 1	Wk. 2	Wk. 3	Wk. 4	
Bacteria	2176	1559	820	463	148	413
Cocci	587	280	124	38	7	37
Gram-positive spore-formers	1306	1060	588	342	124	347
Other	283	219	108	83	17	29
Yeasts	80	84	49	1	9	13
Molds	398	463	771	666	669	906
Penicillium–Aspergillus	62	89	266	271	308	413
Alternaria	17	29	35	34	38	50
Others	219	345	470	361	323	443
Total colonies	2654	2106	1640	1130	826	1332

[a] Mean weekly value.

evaluated either by determining minimum inhibitory concentrations of active ingredients against standard suspensions of known microbial species or through the use of closed chamber techniques in which a synthetic atmosphere is produced which contains a known concentration of microbial forms, either vegetative or spores. *Serratia indica* and *Bacillus subtilis* var. niger (*Bacillus globigii*) are among the organisms more commonly employed for study (2). In either case, results obtained have little in common with actual conditions of use in a critical air space such as a hospital room or laboratory. The present study was undertaken to determine the effects of germicidal aerosol treatment on the microbial flora of the air in a heavily trafficked laboratory over an extended period of time.

MATERIALS AND METHODS

The laboratory chosen for study was approximately 30,000 cu. ft. with two windows at one end and a door at the opposite end. The only furniture at the beginning of the experiment consisted of several long, formica-topped tables. Table tops, floor, walls, ledges, and other exposed surfaces were cleaned by normal maintenance prior to the start of the experiment.

The aerosol formulation chosen for study contained 1.8% quaternary ammonium compounds as BTC-50[1] in an alcohol–glycol base, and a fluorinated hydrocarbon[2] propellant. A special metering valve was installed on all containers to provide for dispensing a 100-mg. spray dose at each activation. Quantitative studies indicated that such an arrangement produced a spray with a median particle size of 18 μ and that each 100-mg. spray contained approximately 12,000,000 aerosol particles.

To provide constant round-the-clock air treatment, a special dispenser was adapted to activate the container valve at 15-min. intervals. The unit was powered by a small, synchronous motor similar to that of an electric clock, operating on 115 v. 60 cycle a.c. Preliminary investigation determined that 15-min. intervals represented the most satisfactory spacing of spray bursts. Such spacing yielded barely measurable levels of germicidal particles at the end of the period but did not permit excessive build-up of concentrations in daily use.

Three units were installed on the walls of the laboratory 8 ft. above the floor level so spaced that the spray paths converged in the approximate center of the laboratory. Air samples were collected every 4 hr. during the day and at 6-hr. intervals during the night for 8 weeks. To determine pretreatment normal values, nonmedicated aerosol was employed during the first 2 weeks. Medicated aerosol was sprayed during the next 4 consecutive weeks and finally, during the next 2 weeks the nonmedicated spray was used again.

Air sampling in duplicate was done by means of a hospital contamination analysis kit[3] utilizing impingement to buffered gelatin broth and concentration and cultivation on filter disks. Samples were taken for a 5-min. period at an approximate flow rate of 12.5 L. of air per minute. Brain heart infusion agar was used for total aerobic bacterial counts while potato dextrose agar, acidified with sterile 10% tartaric acid to a pH of 3.5 was used for mold and yeast determinations (3).

Throughout the study, a constant traffic pattern was established; students entered and left at will during the hours of 10 a.m. and 4 p.m. Windows and doors were closed at other times, except for the entrance and exit of the sample taker. Parallel samples were taken in an adjoining laboratory to confirm that any gross changes in flora observed during the 8-week experiment were not simply seasonal variations in the general environment. Room temperature, relative humidity, and barometric pressure were recorded as each air sample was taken to determine if these factors seemed to influence the results.

Bacterial colony counts were made after 48-hr. incubation at 35° while yeast and mold counts were made after 7-day incubation at 32°. Following counting, simple microscopic and macroscopic criteria were used to single out the following groups of organisms: sarcina, staphylococcus types (micrococci), Gram-positive spore-forming bacilli, yeasts, and molds. Among those colonies identified as molds, members of the general *Penicillium*, *Aspergillus*, and *Alternaria* were identified routinely by conidial characteristics and *Mucor*, *Neurospora*, *Cladosporium* and other common forms diagnosed where possible by morphological characteristics.

[1] Supplied by Onyx Chemical Co.
[2] Marketed as Freon-12 by E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.
[3] Millipore Filter Corp., Bedford, Mass.

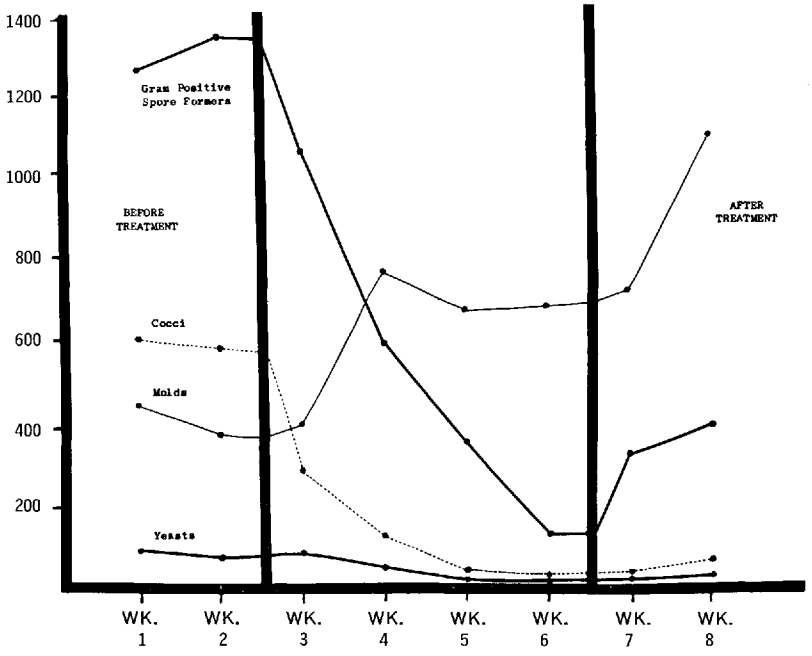

Fig. 1.—Average weekly number of colony isolates identified as Gram-positive spore-forming bacilli, cocci, molds, and yeasts.

RESULTS AND DISCUSSION

Monitoring the air during the preliminary 2-week period in both the test laboratory and the control laboratory indicated that the normal flora in this particular environment consisted of an over-all mean distribution as follows: bacteria, 82% (range 48–96%); yeasts, 3% (range 0–5%); molds, 15% (range 2–28%). As might well have been expected, mean values for total bacterial counts tended to rise sharply during periods of heavy traffic. However, values did not fall promptly on cessation of traffic. For example, during this 2-week period, the number of bacterial colonies isolated rose from 227 at 8 a.m. to 492 at 12 noon and fell only to 451 at 8 p.m. On more than half of the days sampled, total bacterial counts were higher 4 and 8 hr. after traffic periods than during traffic periods. A similar effect, although less marked, was found in total mold counts. This persistence was most evident among the penicillia and almost nonexistent with yeasts.

With the onset of air treatment, profound alterations in the microbial flora were observed which are summarized in Table I and graphically represented in Fig. 1. These changes were not found in values obtained in the control environment and may be regarded as being the result of the spray regimen rather than as normal or seasonal variations. Although there were marked fluctuations in experimental values at different times during the 4-week experimental period, the general trend was clearly toward a marked drop in total microbial species amenable to routine cultivation, and a pronounced conversion of the persistent flora from one dominated by Gram-positive spore-forming bacilli to one dominated by the *Penicillium–Aspergillus* group of molds. Among the bacteria, a relatively rapid decline in the presence of coccal forms apparently testifies to the sensitivity of such species to quaternary germicides, and the sharp quantitative drop in yeast isolates may be similarly explained.

No evident correlation could be discerned between the alterations in flora recorded during the course of the experiment and the temperature, barometric pressure, or relative humidity readings, although there remains with the experimenter the subjective impression that mold colony counts, particularly in the *Penicillium–Aspergillus* group, tended to increase during periods of relatively high humidity.

In conclusion, it seems evident that the use of germicidal aerosol is capable of changing both the quality and quantity of the microbial flora of the air even under conditions of free access and air exchange in the space evaluated. The use of a quaternary type disinfectant system is purely a personal choice, and it is probable that where air sanitation of critical spaces is desired and for one reason or another the quaternary ammonium type of germicide is undesirable, satisfactory results can be obtained with other formulations. The apparent success of the present system against coccal forms may indicate a clinical usefulness against pathogenic forms of staphylococci, currently of profound public health significance, and if such is the case, it is encouraging to note from the data that cessation of air sanitation under the conditions described did not result in the prompt re-establishment of the pretreatment flora.

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Opium Alkaloids III. Isolation of α-Allocryptopine

By EINAR BROCHMANN-HANSSEN and BENDIK NIELSEN

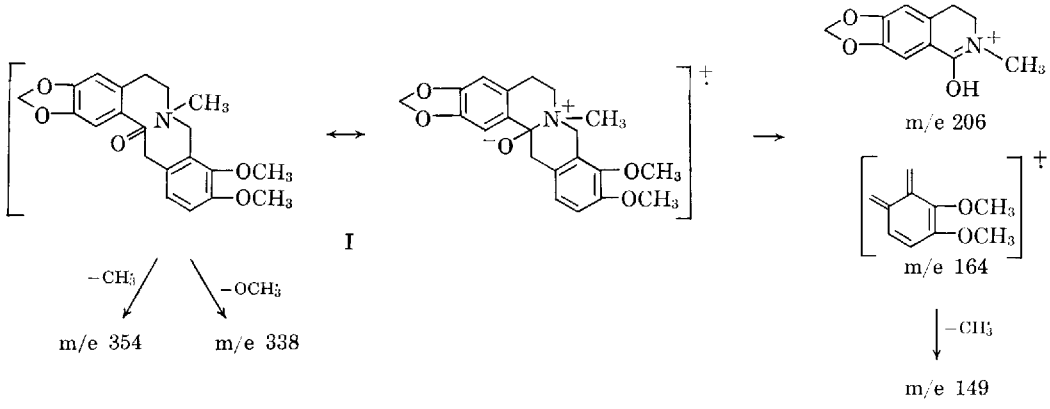
Allocryptopine has been isolated from opium in approximately 0.01 per cent yield as the α-form. The alkaloid has been identified by NMR and mass spectrometry and by comparison of its infrared spectrum with that of its allotropic modification, β-allocryptopine. The biosynthetic pathway leading to allocryptopine is discussed briefly.

RECENT STUDIES of the alkaloid composition of opium have revealed several new alkaloids, some of which have served to confirm modern theories on the biosynthetic pathways in the opium poppy (1–6). This report describes the isolation of allocryptopine which belongs to the protopine group of alkaloids.

Allocryptopine is present in many plants, especially in members of the *Papaveraceae*, and is generally found in concentrations of less than 0.1% (7). A notable exception is *Argemone squarrosa*, from which it has been isolated in quantities of approximately 1% (8). The alkaloid occurs in two allotropic modifications referred to as the α- and the β-forms with different crystal structures and melting points.

with low R_f value (between morphine and codeine) was scraped off and eluted with warm methanol. The alkaloid was purified by repeated crystallization of the picrate, m.p. 208°.[1] The base was liberated by passing through a column of neutral alumina and washing with chloroform. After evaporation of the solvent, the residue was crystallized from heptane, m.p. 160°.

Identification of α-Allocryptopine.—The NMR spectrum[2] in deuterochloroform revealed four aromatic protons in the region 3.0–3.4 τ, a methylenedioxy group at 4.08 τ, two *O*-methyl groups at 6.16 and 6.22 τ, and a *N*-methyl group at 8.15 τ. The high field absorption band of the *N*-methyl group is similar to that of protopine (10) and is probably caused by the shielding effect of the carbonyl group.



Major Fragments in Mass Spectrometry of Allocryptopine
Scheme I

EXPERIMENTAL

Isolation.—The mother liquor from the purification of morphine was extracted as described in a previous communication (2). The chloroform extract obtained at pH 1–1.5 (HCl), containing the weakly basic alkaloids, was separated into phenolic and nonphenolic bases with ether at pH 13, and the nonphenolic fraction subjected to preparative thin-layer chromatography (9). An alkaloid band

Mass spectrometry[3] gave a molecular ion with mass 369. Major fragments appeared in the mass spectrum at m/e 149, 164, 206, 338, and 354, corresponding to the expected cleavage of allocryptopine (I) as illustrated in Scheme I.

The I.R. spectrum[4] of the alkaloid base in chloroform solution was identical with that of authentic allocryptopine. The melting point agreed with that reported for α-allocryptopine (7). The β-form melts at 170° (7, 8). A mixed melting point with authentic β-allocryptopine gave a depression to 166°. When the melt was allowed to cool and crystallize, it melted at 170°. This is consistent with the observation of Soine and Willette (8). The amounts of

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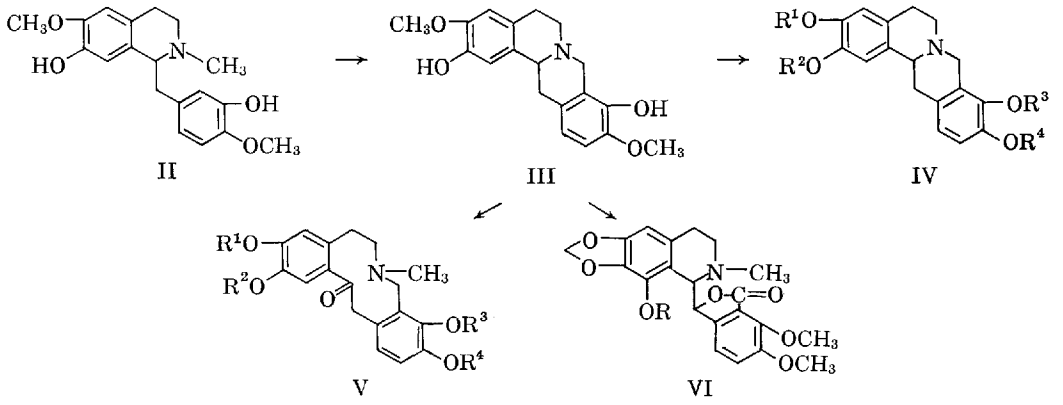
The authors are indebted to Mallinckrodt Chemical Works for supplying the opium mother liquor, to Dr. T. O. Soine for a generous supply of authentic β-allocryptopine, and to Dr. J. A. Martin and Dr. R. Ramage for helpful suggestions and discussions during the interpretations of the NMR and mass spectra.

[1] All melting points were determined with a Kofler micro-melting point apparatus.
[2] The instrument used was a Varian A-60 nuclear magnetic resonance spectrometer.
[3] Associated Electrical Industries, MS9.
[4] Unicam SP-200 infrared spectrophotometer.



Scheme II

α-allocryptopine in opium appears to be of the order of 0.01%.

Anal.[5]—Calcd. for $C_{21}H_{23}NO_5$: C, 68.28; H, 6.28; N, 3.79. Found: C, 68.24; H, 6.03; N, 3.99.

DISCUSSION

Reticuline (II) has been shown to be a precursor for protopine in several plant species (11, 12), and there is evidence that this biotransformation proceeds by way of scoulerine (III) (12), which has recently been isolated from opium (6). Scoulerine, like reticuline, represents an important branching point in the biosynthesis of opium alkaloids from which a number of tetrahydroprotoberberine (IV), protopine (V), and phthalideisoquinoline alkaloids (VI) may be derived (Scheme II). A thorough search may well reveal other members of these alkaloid groups in the opium poppy than those which have been reported so far.

[5] The analyses were carried out by the Microanalytical Laboratory, Department of Chemistry, University of California, Berkeley.

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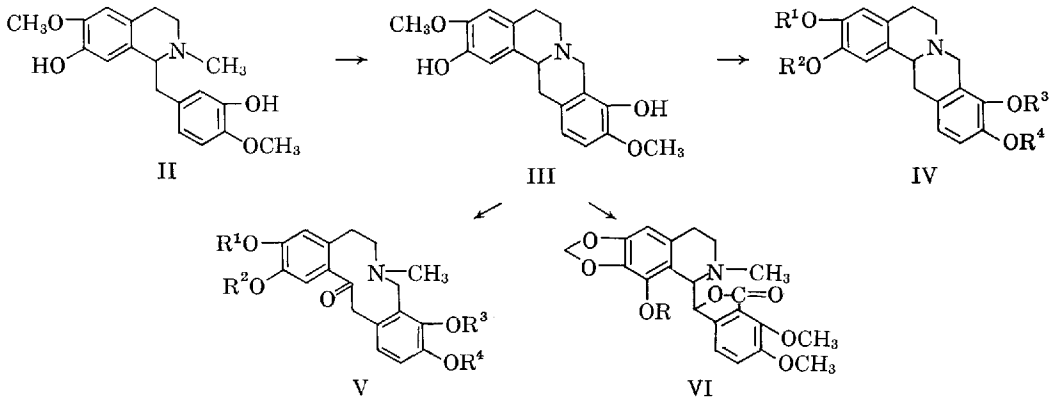
Communications

Nonspecificity of Published Assays for Chloramphenicol Solutions

Sir:

The authors have found that the accepted spectrophotometric method (1) for chloramphenicol-containing pharmaceuticals does not yield valid results when applied to a partially degraded aqueous solution of chloramphenicol. The Code of Federal Regulations designates as acceptable various analytical methods for the antibiotic in pharmaceuticals. The procedures for two microbiological methods and one spectrophotometric method are outlined in the code (1). In addition, Higuchi, Marcus, and Bias have developed a different microbiological method and have compared this with a chromatographic method for chloramphenicol (2).

Samples of an aqueous solution of chloramphenicol containing stabilizing agents were stored at 4°, 22°, and 32° for approximately 16 months. Table I shows the chloramphenicol content of



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Samples of an aqueous solution of chloramphenicol containing stabilizing agents were stored at 4°, 22°, and 32° for approximately 16 months. Table I shows the chloramphenicol content of

TABLE I.—COMPARISON OF METHODS[a]

Storage Temp.	Microbiological (Cup Plate)	Microbiological (Turbidimetric)	U. V. with Prior TLC Separation	Direct U. V.
Std.[b]	0.492 ± 0.011	0.507 ± 0.001	0.491 ± 0.003	0.505 ± 0.002
4°	0.517 ± 0.005	0.515 ± 0.001	0.497 ± 0.005	0.555 ± 0.000
22°	0.390 ± 0.013	0.390 ± 0.007	0.383 ± 0.001	0.556 ± 0.002
32°	Below 0.240	Not assayed	0.183 ± 0.002	0.566 ± 0.004

[a] Each figure represents the average of three determinations and is expressed as the per cent of chloramphenicol in the solution. The precision is expressed as average deviation from the values given. [b] Freshly prepared solution of the same formulation containing 0.50% chloramphenicol.

TABLE II.—ANALYSIS OF COMMERCIALLY AVAILABLE CHLORAMPHENICOL PRODUCTS[a]

	U. V. with Prior TLC Separation	Direct U. V.
Product *1*	0.189	0.242
Product *2*	0.166	0.221

[a] Each figure represents the average of two determinations and is expressed as the per cent of chloramphenicol in the solution. The label claim on these two products was 0.2% chloramphenicol.

these samples after 16 months as determined by two accepted microbiological methods (1), by the accepted ultraviolet method (1), and by an improved spectrophotometric method utilizing thin-layer chromatography. The solution was originally prepared to contain 0.55% chloramphenicol.

The improved spectrophotometric method consisted of quantitative separation of chloramphenicol from degradation products utilizing thin-layer chromatography followed by ultraviolet analysis. Standard 2 by 9 in. thin-layer chromatography plates were coated with silica gel $HF_{254 + 366}$ (Brinkmann Instruments, Inc.) and preconditioned before use by heating in an oven at 90° for 15 min. The developing medium was chloroform–isopropanol (4:1) and visualization of the antibiotic was accomplished using an ultraviolet light emitting at 2537 Å. Development time for the chromatogram was 55 min. The chloramphenicol was desorbed from the adsorbent by ethanol over a 12-hr. period and determined at 274 mμ on an ultraviolet spectrophotometer.

It can be seen from the data in Table I that all four methods of analysis agree closely on a freshly prepared aqueous solution of chloramphenicol. It is also evident from the above table that direct ultraviolet analysis of a partially degraded aqueous solution of chloramphenicol which is 16 months old yields high values. These figures are not in agreement with the two accepted microbiological methods which are specific for active antibiotic concentration. On the other hand, the results using prior separation by thin-layer chromatography followed by ultraviolet determination are in close agreement with those values obtained by the microbiological methods. In fact, if the 0.183 figure obtained by the thin-layer chromatography method on a 32° sample is used, it can be calculated that the chloramphenicol in this sample has undergone approximately 67% degradation. A determination by direct ultraviolet analysis on the same sample shows that no degradation has taken place.

The thin-layer chromatographic method and the accepted direct ultraviolet method (1) were applied to stable aqueous solutions of chloramphenicol currently available only on the foreign market. The results, shown in Table II, indicate considerably less active chloramphenicol present in samples tested by the thin-layer chromatographic method than by the direct spectrophotometric method.

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Despite the idealistic yearning of the medicinal chemist for theories and laws which will assure the introduction of the best possible member of an established class of drugs as well as of entirely novel drugs, such has not yet proved to be the case. Despite worthy efforts which one day may bear fruit, the exact architecture of not a single active site is known. Until the day of full enlightenment has arrived, superior drugs will arise through molecular modification with its components of intuition, industry, theory, technical ability, flash of genius, and fortuitous circumstance.

The introductory chapter by Dr. Max Tishler combines a scholarly with a practical approach in convincing fashion. Particularly impressive is the unpredictable fact of the presence of the sulfonamide group in a variety of sructures constituting agents for bacterial infections, inhibition of penicillin excretion, gout, cardiac edema, and diabetes, not to mention the old chloramine antiseptics. Professor John C. Sheehan describes how chemists modified the penicillin molecule so as to render it effective against resistant staphylococcal infections. In the chapter by Dr. Gerhard Zbinden, one can see evidence of how, despite the deterrent effect of early theories of action which implied that better sulfas would not be found, chemists, pharmacologists, and clinicians have, nevertheless, produced valuable new sulfas.

So the story goes through 18 well-documented chapters by experts in medicinal chemistry and allied fields. Discrete molecular modifications have brought forth exciting and valuable new antihypertensives, *e.g.*, methyldopa, and antidepressants, *e.g.*, imipramine. Modifications of the steroid hormones have produced dramatic increases in biological activity beyond that of the parent hormone, and also important uses have emerged, as in the case of the antifertility agents.

Every medicinal chemist should read this book. It will teach him not to adhere rigidly to theories of drug action as we presently know them. It may help him to create valuable new drugs.

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Molecular Modification in Drug Design. Advances in Chemistry Series, American Chemical Society, Washington, D. C., 1964. vii + 228 pp. 15.8 × 24 cm. Price $5.00.

A few years ago the Division of Medicinal Chemistry of the American Chemical Society held a well attended two-day symposium on the subject of Molecular Modification in Drug Design, which also has become the title of a book summarizing the lectures and discussion. The late Professor Fred W. Schueler, chairman of the symposium, succinctly stated in the preface the essence of the subject: "Molecular modification guided by deduction, induction, and serendipity *via* the prepared mind has produced in the last 25 years more potent and more useful drug agents in various areas of therapeutics than have been reported in all previous history."

Despite the idealistic yearning of the medicinal chemist for theories and laws which will assure the introduction of the best possible member of an established class of drugs as well as of entirely novel drugs, such has not yet proved to be the case. Despite worthy efforts which one day may bear fruit, the exact architecture of not a single active site is known. Until the day of full enlightenment has arrived, superior drugs will arise through molecular modification with its components of intuition, industry, theory, technical ability, flash of genius, and fortuitous circumstance.

The introductory chapter by Dr. Max Tishler combines a scholarly with a practical approach in convincing fashion. Particularly impressive is the unpredictable fact of the presence of the sulfonamide group in a variety of sructures constituting agents for bacterial infections, inhibition of penicillin excretion, gout, cardiac edema, and diabetes, not to mention the old chloramine antiseptics. Professor John C. Sheehan describes how chemists modified the penicillin molecule so as to render it effective against resistant staphylococcal infections. In the chapter by Dr. Gerhard Zbinden, one can see evidence of how, despite the deterrent effect of early theories of action which implied that better sulfas would not be found, chemists, pharmacologists, and clinicians have, nevertheless, produced valuable new sulfas.

So the story goes through 18 well-documented chapters by experts in medicinal chemistry and allied fields. Discrete molecular modifications have brought forth exciting and valuable new antihypertensives, *e.g.*, methyldopa, and antidepressants, *e.g.*, imipramine. Modifications of the steroid hormones have produced dramatic increases in biological activity beyond that of the parent hormone, and also important uses have emerged, as in the case of the antifertility agents.

Every medicinal chemist should read this book. It will teach him not to adhere rigidly to theories of drug action as we presently know them. It may help him to create valuable new drugs.

Reviewed by Joseph H. Burckhalter
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NOTICES

An Introduction to Pharmaceutical Formulation. By A. G. FISHBURN. Pergamon Press Inc., 44-01 21st St., Long Island City, New York, N. Y. 11101, 1965. vii + 192 pp. 13 × 19.5 cm. Price $4.50.

Diet and Live: A Guide to Corrective Eating. By JOSEPH I. GOODMAN, M.D. The World Publishing Co., 2231 W. 110th St., Cleveland, Ohio, 1966. x + 300 pp. 14 × 21 cm. Price $4.95.

Current Topics in Thyroid Research. Proceedings of the Fifth International Thyroid Conference, Rome, 1965. Edited by C. CASSANO and M. ANDREOLI. Academic Press Inc., 111 Fifth Ave., New York, N. Y. 10003, 1965. xxvii + 1219 pp. 15.5 × 23 cm.

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Boletim de Escola Superior de Farmacia de Lisboa. Vol. VII (1962–1964). By ANTONIO PEDRO DE GOIS LUPI NOGUEIRA. Escola Superior de Farmacia, Quinta da Torrinha, Avenida 28 de Maio, Lisbon, Portugal. 391 pp. Paperbound.

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Review Article

Distribution and Biological Effects of Substance P

By H. DIX CHRISTENSEN* and THOMAS J. HALEY

CONTENTS

EXTENSIVE INVESTIGATIONS in recent years of the physiological and pharmacological properties of endogenous biologically active substances have increased interest in the polypeptide substance P (SP). Although SP is one of the oldest known peptides, discovered by Euler and Gaddum (1) in 1931 while investigating acetylcholine distribution, its biological function is largely unknown. Many speculative functions have been proposed for SP; as an essential factor for the rhythmic motility of the intestine (2–5), both as an excitatory and inhibitory neurotransmitter (6–9), as a general hyperpolarizing modulator (10, 11) or physiological tranquilizer (12, 13), as a carrier of various transmitter substances (14–16), and as a capillary permeability increasing agent (17, 18), but conclusive evidence for any or several of these functions has not been obtained.

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Substance P exerts its pharmacological effects primarily on three organic systems: the central nervous, gastrointestinal, and circulatory systems. While the initial publication described SP as a smooth muscle stimulating substance and blood pressure depressor, it also affects the central nervous system. This review will summarize the knowledge of SP to the present, particularly on these three systems. Other reviews (19–27) cover additional details and aspects.

CHEMICAL CHARACTERISTICS

Substance P is a basic straight chain polypeptide, with molecular weight 1650 ± 350 (19, 28). Total hydrolysis gives the following 13 amino acids: lysine, arginine, aspartic acid, glutamic acid, proline, glycine, alanine, valine, leucine, isoleucine, phenylalanine, threonine, and serine (28–31). Arginine-proline-proline are the first three amino acids from the *N*-terminus, as in bradykinin, but the remainder of the chain is different (29, 32). Owing to the extreme instability of purified SP in solution, the specific sequence and synthesis have not been determined. A 10^{-6} dilution of the purified substance loses over half of its activity within 2 min., while a 10^{-3} dilution is stable for several hours (28). Cleugh and Gaddum (33) suggest that this instability of the pure SP is due to its adsorption properties.

Substance P is very soluble in water, methanol, ethanol, and acetic acid and is insoluble in ether and chloroform. It can be precipitated from

alcoholic solutions with acetone or picric acid, and from watery solutions with phosphotungstic acid, mercuric chloride, or by 70% saturation with ammonium sulfate. Substance P is thermostable from pH 1–7, but activity is rapidly lost in an alkaline state. It dialyzes rapidly through cellophane, parchment, and collodion membranes (2, 34).

Paper chromatography using different solvent systems: *n*-butanol–acetic acid–water (40:10:50), *n*-butanol–pyridine–acetic acid–water (30:20:6:24), and pyridine–acetic acid–water (30:50:15) gave R_f values for SP of 0.34–0.37, 0.46, 0.61, respectively (21, 31, 35). Zetler (35) found that SP migrated 116 mm. to the cathode during paper electrophoresis (pH 4.95, acetate buffer, duration 6 hr.). SP migrates to the cathode at pH below 10.5. Using the buffer system formic acid–acetic acid–water (15:10:75) and pH 1.9, bovine SP migrated at a velocity 0.8 times that of glutamic acid (28); horse SP, however, migrated 1.0 times that of glutamic acid (29). The isoelectric point was 8.6 ± 0.2 (28). In countercurrent distribution in the system *n*-butanol–pyridine–acetic acid–water (40:10:5:45) at 20°, SP gave a value of K = 0.61 (28).

No specific chemical reaction for substance P is known, so SP concentration is estimated using bioassays, comparing the response against a standard preparation. One unit of SP is contained in approximately 20–30 mg. of horse intestine (34). Purified SP contains 120,000–150,000 units of activity per milligram (unit/mg.) (28, 30).

The isolated guinea pig ileum provides the most specific assay available (36). The most commonly used assay (37) uses a segment from the distal ileum in a 2–5-ml. intestinal bath containing Tyrode's solution at 34–38°. Hyoscine (10^{-8} Gm./ml.), pyrilamine[1] (10^{-6} Gm./ml.), UML 491 (5×10^{-8} Gm./ml.), or other suitable blocking agents, which will antagonize acetylcholine, histamine, and serotonin, respectively, are added to the bath solution. Many other active substances which may be present in crude extracts can be eliminated by specific tachyphylaxis (36) or by enzymatic deamination (38, 39). The contraction curve for SP is very similar to acetylcholine, with the exceptions of a smaller maximum and a slightly delayed onset and maximum response time (28). Maximum contraction occurs usually within 1 min. and doses can be repeated at 3–4-min. intervals (37). In this bath 0.2 unit/ml. should give a good response and a 10% change can be detected. In a 0.05-ml. microbath the guinea pig ileum is sensitive to 0.001–0.01 unit of SP (40). Purified SP (50,000 units/mg.) in a dose of 10^{-9} Gm./ml. caused a submaximal contraction of the isolated guinea pig ileum, so SP on a molar basis is about 10 times more active than acetylcholine (28). This sample produced no tachyphylactic phenomenon, even with the time interval as short as 1 min., which is opposite to the results obtained using impure SP (41). Substance P stimulates the longitudinal but not the circular muscle (42). Below 20° contractions are inhibited (43) and highly variable results occur after storage (43, 44). Substance P is reported to increase in potency with increased pH (45) or to be unaffected (46).

Only two reasonably specific SP antagonists have been found: cystine-di-β-naphthylamide and trimethaphane-*d*-camphorsulfonate.[2] They have a slight antagonist effect against acetylcholine, histamine, and 5-hydroxytryptamine, but no effect on oxytocin, vasopressin, and bradykinin (47, 48).

The contraction of the isolated hen rectal caecum, rabbit jejunum, goldfish intestine, and fall in blood pressure of the atropinized rabbit are other bioassays which are sensitive to purified SP as well as crude preparations (36, 49–54).

Three extraction procedures are in common use. The standard extraction procedure consists of boiling the tissue at pH 4, precipitation of inactive protein by adding ethanol, and further concentrating SP by precipitation with ammonium sulfate (37, 55). Substance P is extracted from small quantities of tissue by an acid extraction of the acetone insoluble residue (37, 56, 57). Lembeck *et al.* (58) have recently developed a new chloroform–methanol extraction procedure. Crude extracts can be further purified by adsorption on an aluminum oxide column; SP is eluted with decreasing concentrations of methanol (37). Purified SP has been obtained by elaborate procedures involving repeated adsorption and ion exchange chromatography and countercurrent distribution (28–31).

Zetler (35, 59–64) in purifying SP extracts used carbon tetrachloride to remove salts and ammonium sulfate from the extract. Substance P from cattle brain treated in this way yields three biologically active polypeptides, while extracts from the horse intestinal tract gave ten. While some of the peptides may be artifacts from protein denaturation, they are probably

[1] Marketed as Neo-antergan by Merck & Co., Inc., Rahway, N. J.

[2] Marketed as Arfonad by Roche Laboratories, Nutley, N. J.

not all so. One of the active peptides was found in both the brain and intestine, which also had all of the properties of SP (62, 65). Zetler (62) concluded that the brain F_b and intestinal F_{b3} fractions are SP, which creates a slight problem, as Lembeck *et al.* (58) in their new extraction method, extract only fraction F_a from nervous tissue and found no SP activity remaining in the tissue after extraction.

Biological activity of highly purified SP is completely destroyed by chymotrypsin, but only partially by trypsin (32, 54). Impure SP is also inactivated by pepsin, cathepsin, papain, diamine oxidase, bacterial proteolytic enzymes and extracts from nervous tissue, intestinal tract, urinary bladder, ureter, and uterus (66–72). Eber and Lembeck (66) found kidney and spleen extracts most potent in inactivating SP, with no relationship between the tissue SP content and its enzymatic activity. Blood serum from gravid animals destroys SP, while normal serum has no or only slight inactivation ability (73, 74). In subcellular studies an enzyme which inactivates SP appears to be located in the cytoplasm–the supernatant fraction (75, 76). Substance P is very resistant to autolysis (66, 77, 78).

DISTRIBUTION

The gastrointestinal tract and nervous system of all investigated vertebrates contain SP (21, 79–86), but none of the noncordates (87, 88). The tunicate, *Ciona intestinalis*, is the most elementary species in which SP (0.02 unit/Gm.) has been found (87). Substance P distribution in five species is shown in Table I.

In the intestinal tract, the content is low in the esophagus and stomach, high in the duodenum and jejunum. The ileum, colon, and rectum contain moderate amounts (21). The amount of SP in the intestine varies with the species, in decreasing order: dog, monkey, horse, bovine, man, rat, guinea pig, sheep, cat, pig, ray, and dogfish (21, 87, 92). While SP is found in all layers of the gastric and intestinal walls, the muscularis mucosa contains the greatest concentration (21, 96).

Amphibians have the highest total brain concentration (200–820 units/Gm.) with moderate amounts in reptiles and smaller amounts in birds, mammals, and fish (79–81). Differences in forebrain and brain stem concentrations are less in primitive than more developed brains. With increased differentiation of the central nervous system, the concentration decreases in the whole brain (81) and in specific nuclei (90). The topographic distribution shows that while SP is present in all parts, the concentration varies over a wide range. Cell-rich phylogenetic older parts of the brain have the primary concentration with the cerebellum a notable exception.

In the cortex there is an uneven distribution, the anterior cingulate gyrus has a high concentration; the olfactory, somatomotor, and somatosensory cortices have moderate amounts, and the visual cortex has little. The cerebellar cortex contains small amounts of SP, but considered as a whole there is no more than in white matter.

Closely neighboring brain stem structures show neurohumoral differentiation (78), as the thalamus (12 units/Gm.), globus pallidus (112 units/Gm.), and putamen (64 units/Gm.); anterior (70 units/Gm.) and posterior (22 units/Gm.), hypothalamus, and mammillary body (34 units/Gm.); ala cinerea (248 units/Gm.), trigonum hypoglossi (37 units/Gm.), and area postrema (290 units/Gm.); substantia nigra (699 units/Gm.) and red nucleus (30 units/Gm.).

The concentration of SP in functional systems shows significant differences; for example, the visual system, other than the retina, has much lower concentrations than the auditory system.

In the spinal cord as in the brain, the gray matter shows a higher concentration than the white. There is more SP in the dorsal than the ventral halves of the spinal cord, except in the frog where they are the same (98). This high concentration of SP in the sensory pathway: the dorsal roots, fasciculi gracilis and cuneatus, and associated nuclei with very low concentrations of other well-known pharmacologically active substances, led to Lembeck's suggestion (6) that SP may be the transmitter of the sensory neurons.

All peripheral nerves contain SP, but in much smaller concentrations than in the central nervous system. The distribution indicates the autonomic nerves, in particular the sympathetic preganglionic, contain the largest amounts (21).

Differential and density gradient centrifugation studies of brain homogenates show that the intracellular distribution of SP is similar to acetylcholine (36, 76, 99–105). The distribution is bimodal, between the supernatant ($^1/_3$) and synaptic vesicles and the incompletely disrupted synaptosomes fractions ($^2/_3$). Substance P is found, as is acetylcholine, in the hollow or type 1 synaptic vesicle. Substance P in the vesicle-synaptosomes fractions is in a bound form and must be released before assay. Lembeck *et al.* (58) think it is bound to phosphatides. Substance P is released rapidly upon exposure to hypotonic solutions or extraction at pH 4.0.

TABLE I.—REGIONAL DISTRIBUTION OF SUBSTANCE P IN FIVE SPECIES (UNITS OF ACTIVITY/GM. OF TISSUE)

Species Ref.[a]	Human (18, 78, 89–92)	Bovine (21, 78, 90, 93–95)	Dog (21, 56, 90, 94, 96, 97)	Cat (21, 81, 95)	Ray (82, 83, 87)
G.I. Tract					
Esophagus	...	2.0	Low	1.3	...
Stomach	...	7.5	Moderate	2.5	...
Duodenum	...	15.0	64.9	8.0	3.0
Jejunum	19.0	22.5	53.6	6.0	
Ileum	...	...	...	...	
oral	...	16.2	40.1	4.0	...
middle	...	13.8	36.4	2.8	...
coecal	...	10.3	39.7	3.3	...
Colon	...	...	41.3	...	...
oral	8.4	15.3	...	5.3	...
rectal	9.4	15.8	...	5.0	...
Rectum	...	16.3	28.3	5.5	...
Nervous System					
Telencephalon	...	...	...	21	5
Pallium	34	...	...	...	...
precentral gyrus	43	7	21	...	...
postcentral gyrus	39	6	20	...	...
striate cortex	...	4	14	...	...
anterior cingulate	85	...	...	...	...
lateral olfactory gyrus	...	...	29	...	...
Corpus callosum	2	...	7	...	...
Basal nuclei	102	...	128	155	...
Caudate	85	80	46	...	...
Lenticular	...	45	...	...	...
putamen	64	...	...	...	...
globus pallidus	112	...	...	...	...
Hippocampus	...	25	15	...	...
Olfactory bulb	...	...	8	...	...

(continued on next page.)

The observed intracellular distribution is similar by using various methods of preparation, or different species (rat, sheep, guinea pig, rabbit) or region of the CNS (cortex, midbrain, cerebellum, and ventral and dorsal halves of the spinal cord) except that the total amount reflects the gross regional distribution (101, 104).

Euler (76, 106) found that the intracellular distribution of SP in peripheral nerves (vagus and splenic nerves of the cow and sciatic and brachial nerves of the dog) was primarily in granules as is the case in the central nervous system.

The subcellular and regional distribution indicates that SP is found within nerve cells rather than glial cells. This is supported also by Grabner and Lembeck's finding (107) that glial tumors contain no substance P activity, and that SP increases in the proximal and decreases in the distal part of cut peripheral nerves in degeneration experiments (98, 108–110). No change occurs in SP concentration in the spinal cord of the rat after dissection between the first and second lumbar vertebrae (111).

Substance P occurs in other organs besides the nervous system and the gastrointestinal tract, but only in minute amounts (1, 21). The ureter and uterus (5 units/Gm. of tissue in the dog) show the relatively highest amounts (21). Substance P is also found in minute amounts in blood (1) but not in urine (112).

ENDOGENOUS CHANGES IN CONCENTRATION

Besides the distribution, which indicates SP's physiological role is connected with function rather than structure, there are endogenous changes under different functional states. In an ontogenetic study on the bovine fetus (113), a brain weighing only 1 Gm. has a high content (21.5 units/Gm.) of SP. The brain stem, which is active during the development of the embryo, contains SP concentrations comparable to the adult, while the forebrain has none until after birth. The intestine contains increasing amounts of SP during the second half of embryonic development. Immature undifferentiated nervous tissues, neural tumors, do not contain SP (107).

In the gravid rat, there is an increase in SP content in the uterus and a marked decrease in the brain, particularly in the second half of pregnancy (74). Up to 4 times the concentration of SP is found in the retina of cattle, whose

TABLE I.—(*Continued.*)

Species Ref.[a]	Human (18, 78, 89–92)	Bovine (21, 78, 90, 93–95)	Dog (21, 56, 90, 94, 96, 97)	Cat (21, 81, 95)	Ray (82, 83, 87)
Diencephalon	...	...	...	...	
Thalamus	12	21	13	22	
lateral geniculate	5	...	...	...	
medial geniculate	37	...	...	...	
Red nucleus	30	...	...	...	
Substantia nigra	710	...	...	...	8
Hypothalamus	102	83	70	...	
mammillary bodies	34	...	...	...	
Mesencephalon	...	...	68	...	
Corpora quadrigemia	...	...	20	...	...
superior colliculus	55	92	...	55	...
inferior colliculus	141	122	...	44	...
Tegmentum	...	108	74	...	...
Cerebral peduncles	...	45	41	...	...
Substantia grisea centralis	119	149	...	...	...
Cerebellum	2	1	2	4	...
Medulla oblongata	...	...	35	...	39
Pons	3	...	21	52	...
Floor of fourth ventricle	...	34	45	...	...
ala cinerea	248	410	...	...	...
trigonum hypoglossi	37	34	...	...	...
area postrema	...	143	375	...	...
Spinal cord	...	...	34	...	23
Grey matter	...	...	68	...	...
White matter	...	...	9	...	...
Dorsal half	...	166	27	...	25
Ventral half	...	47	6	...	15
Nucleus gracilis	49	165	79	...	...
Nucleus cuneatus	25	125	55	...	...
Other tissue	...	...	...	...	...
Retina	...	5	24	...	...
Optic nerve	...	16	6	...	...
Dorsal roots	...	25	40	...	...
Ventral roots	...	3	6	...	...
Vagus nerve	...	5	22	...	...

[a] Kopera and Lazarini (95) and Douglas *et al.* (96) used different standards from the other authors to measure the biological activity.

eyes were closed for 2 hr. as compared to those illuminated for the same length of time. The extent of change from normal is about the same for each case. No change occurs in the SP content of an isolated retina *in vitro* to illumination or darkness (114). However, in the brain of the rat and the rabbit the content increases after illumination and decreases after darkness or blindness (115, 116). Stern (116) measured also changes in brain concentration of the rat when deprived of other sensory stimuli. Elimination of smell and hearing reduced SP content, while elimination of the tactile sense or the "radar apparatus" in the bat had the opposite effect. Vestibular stimulation also reduced the SP content. Milin (117) found that exogenous SP reduces the movement of earthworms to light and vibrational stimulation. The morphodynamic changes indicated that SP's action is to preserve the integrity of the photo- and tactoreceptor cells in the epidermis.

Walaszek *et al.* (118, 119) found less SP in the hypothalamus of rabbits, which had been previously injected with serum from schizophrenic patients. Serum from normal patients produced no change. While glial tumors do not normally contain SP, 13 units/Gm. was found in 1 multiform glioblastoma of a previously irradiated patient (107).

No SP release has been found in the mammalian brain on electrical stimulation (19, 52, 120), but stimulation is reported to release SP in the frog spinal cord (121) and stomach muscles (122–124). Cervical vagotomy increases the SP content of rabbits' small intestine from 2.8 to 7.2 units/Gm., while vagus stimulation significantly decreases the SP content with a return to control levels within 30 min. (125). This is opposite to earlier findings that vagus stimulation increased SP content in the intestine (126–128).

Pernow and Wallensten (92) found the SP content of human intestinal segments was higher when their motility was increased by intraluminar administration of hypertonic glucose. In Hirschsprung's disease the proximal hyperactive part was higher than controls, and the aganglionic and inactive segments of the colon had significantly less SP (91).

PHARMACOLOGICAL EFFECTS

Most studies on the pharmacological actions of SP have used very impure preparations containing many other active substances. The usual

so-called purified samples (250–1000 units/mg.) still contain less than 1% pure SP, while the highly purified SP (50,000–120,000 units/mg.) is extremely labile in aqueous solutions, so many of the reported pharmacological actions are not completely conclusive.

Substance P has a strong stimulatory effect on the mobility of the gastrointestinal tract of most vertebrates, both in isolated segments and *in situ* (1, 21, 50, 129, 130), with a positive correlation between SP content and sensitivity (21). The amplitude and frequency of the peristaltic waves are increased, besides a lower peristaltic threshold, when SP is applied intraluminally or in the bath of isolated intestines of rabbit or guinea pig (4, 131). Substance P restores fatigue or temperature blocked peristaltic activity (4, 132). This response is dependent upon an intact mucous membrane. Intravenous injections of SP markedly increase the segmental and peristaltic movement of the small intestine *in situ* of rabbits and man (129, 131). The increase is observed within a few minutes after start of infusion (50 units/min.) and lasts for 20 min. after ceasing the infusion. In the case of patients with paralytic ileus, SP induced both segmental and peristaltic activity only for the duration of the infusion. Intravenous administration of SP has been also observed to increase the spontaneous movement of the intestinal villi (133). Substance P (50 units/Kg.) injected intra-aortally increased the tonus of the ileocaecal valve of the cat (134). A strong peristaltic response occurs with rhythmic movement of the sphincter ani in the anesthetized dog (28). There was, however, no visible effect on the intestine of mice, with doses to 50,000 units of purified SP (28).

In the guinea pig, intravenous SP has a strong bronchoconstrictor effect (17, 28, 135, 136) but no effect on inhalation (137). No tachyphylaxis occurs (17). Substance P protects against protein induced anaphylactic shock in the guinea pig (138), while plasma levels of SP are independent of occurrence of anaphylaxis induced in dog (139). Euler and Pernow (140, 141) found intraventricular administration of SP (300 units/mg.) produced both tachypnea and hyperpnea in rabbits and cats. Haefely *et al.* (142, 143) found no effect with purified SP (200 or 24,000 units/mg.) on the respiration rate, but did with a crude preparation (20 units/mg.). Substance P given intravenously has no effect on respiration in the cat (142), but subcutaneously produced bradypnea in the guinea pig (8).

Intravenous or intra-arterial infusion of SP causes peripheral vasodilation with an accompanying brief fall in blood pressure (1, 21, 25, 112, 144–146). It is about 100 times more active than acetylcholine and 5 times more active than bradykinin as a vasodilator on a molar basis (28, 50, 147). Bilateral cervical vagotomy has no effect on the depressor response (50, 112). Substance P-evoked pressure increases in the recipient head were abolished by bilateral cervical vagotomy, but not the systemic depressor effect in cross circulation experiments in dogs (148). Intravenous injections of SP in man produced tachycardia and a slight fall in arterial blood pressure. Cardiac output was increased, but not stroke volume, intracardiac, and pulmonary blood pressures (147, 149). An increase in blood flow in skin and muscles occurs within 1 min. after the start of intravenous infusion of SP, and this is coupled with an intense burning sensation in the face and a throbbing head pain. The splanchnic blood flow is, however, not altered. Intra-arterial injections (1 unit/min.) tripled the blood flow in the forearm within seconds, with increased oxygen saturation of both deep and superficial venous blood (147, 150). A transient tachyphylactic effect occurs, so tremendously high doses have only slightly greater effects than a minimum effective dose in mammals. After 15–30 min., identical hemodynamic effects can be reinduced. In the chicken the response is directly related to the amount of SP injected (112). In the conscious dog high doses of SP (7500 units/Kg. i.v.) increase the duration of the depressor effect accompanied by a marked bradycardia which persists after the blood pressure has returned to normal (28). The minimum effective dose in the cat is about 10 times that of other species (21, 28, 50). Circulatory shock could not be induced in the cat, mouse, or man (28, 147). Intraventricular administration of purified SP (24,000 units/mg.) in the cat had no effect on blood pressure (143). No changes in frequency or contractility of the myocardium were found by direct application of SP (28). Substance P has a vasoconstrictor effect on the perfused frog leg (56).

Slow intravenous infusion of an impure SP killed decerebrated mice sooner than normal. The toxic dose is 12,000–18,000 units/Kg. in normal mice. With slow infusion most animals die in convulsions, while rapid injection produces paralysis at death. Physiological saline or inactivated SP controls never produced convulsions (116).

Capillary permeability is significantly increased by small doses of SP in the guinea pig but not in

the rabbit (17, 50, 151–153). Topical application of 0.5–2 ng. of pure SP on the mesenterium of the anesthetized rat shows a clear relaxation of the venule walls followed by a diffused efflux of leukocytes (50). Lewis (154) has also found that SP causes migration and accumulation of leukocytes. Fibrinolysis is inhibited by 20–80 units of SP (155). Cutaneous injections of 10–30 ng. of pure SP produce a burning pain and erythema after a latency of 10–20 sec., which lasts for 10–30 min. (50). A burning sensation occurs when it is applied to an exposed blister base (17, 156). Intra-arterial, but not intravenous, injections of SP induce visceral pain in dogs (157).

An antidiuretic and milk ejection effect are observed, but only in doses having a marked depressor action (17). The ACTH releasing ability of SP is due to an impurity (158, 159). It has no epinephrine releasing action on the adrenal medulla (160). Substance P inhibits oxytocin induced contractions of the rat uterus *in vivo* (161).

Ten minutes after an intraperitoneal dose (3000 units/Kg.), SP increases in concentration in the brain and spinal cord of the rat, reaching a maximum in 30–40 min., and after 60–120 min. returns to normal or slightly below (116). This time course for SP uptake could account partially for many observations on the effects of exogenous SP alone and with central acting drugs on the nervous system. Sedation, *i.e.*, a reduction in spontaneous and induced motor activity, is the most often reported behavioral effect of administered SP (8, 10, 12, 13, 141, 162–165). Oral automatisms were often provoked. All of these observations are somewhat in question, as Haefely *et al.* (143) found no observable behavioral effects after intraventricular injection of purified SP in the cat, but did observe sedation with crude extracts. They showed that ammonium sulfate in the concentrations contained in the crude extracts would produce sedation. This observation does not explain, however, the chymotrypsin inactivated SP controls used by other authors which produced no effect. Also the same investigators (19, 28) reported that 7500 units/Kg. of pure SP produced sedation in the unanesthetized dog. In mice no clear effect was observed with doses up to 1000 units per animal given intravenously or intracerebrally. With 5000 units, spontaneous, but not induced, activity was reduced (28).

Ganglionic transmission in the superior cervical ganglion of the cat was not affected by high doses of the purified SP (19), although a dual effect of potentiation and inhibition has been reported using impure extracts (140, 166, 167). Caspers (10, 168, 169) correlated behavioral with bioelectrical changes in the unanesthetized rat after administration of an impure SP extract. The EEG and d.c. potential patterns of the cortex were those of natural sleep following intraperitoneal and topical SP application. Dendritic potentials are increased in voltage and duration. The reactivity of the animals to acoustic stimulation was easily depressed, while tactile stimulation was more resistant. Conditioned responses were more resistant to SP than unconditioned ones. Besides a hyperpolarizing effect at the cortex, Caspers found SP reduced the unit discharge in the brain stem reticular formation, but had little effect at the thalamic relay nuclei. Schneiderman *et al.* (170), however, found SP (75 units/Kg. i.v.) decreases the threshold of the thalamocortical evoked potential in the rabbit, depressing the amplitude of the first surface-positive component and enhancing the second and third surface-negative components. Substance P increased cortical Δ activity and the number of spindles. The onset of changes occurred within 5 min. of administration, reaching a maximum within 15, and lasting about 1 hr. Cortical activation and hippocampal synchronization have been found with intracarotid injection (7) or topical application (171) of SP, but are probably a secondary effect produced by blood pressure depression (10).

Stern *et al.* (172) found an inhibitory influence of SP on polysynaptic, but not monosynaptic reflexes in the cat, while Kissel and Domino (173) found no effect on either. No effect was found on polysynaptic reflexes in the frog (121). Substance P had no influence on conditioned reflexes, discrimination tests, and maze performance of the rat (174).

Substance P produces no change in the dorsal root potential (175–177) or the amplitude variation in the electric knife fish (*G. eigenmannia*) (11). Substance P stimulates the sensory nerve endings in the rabbit ear (178).

DRUG INTERACTION

The failure of atropine to block the contracting action of SP on the intestine led to its discovery (1). Since then the interaction of many compounds with SP on isolated intestinal tissue has been determined, primarily to find a specific antagonist. Only two reasonably specific antagonists have been found: cystine-di-β-naphthylamide and trimethaphane-*d*-camphorsulfonate (47, 48). Drugs which exert no inhibitory or an unspecific effect on the SP action on the isolated guinea pig ileum are as follows: adenosine,

adermine, γ-aminobutyric acid, *p*-aminobenzoic acid, 1-AMP, 2-AMP, 5-AMP, antimit, antazoline phosphate, antipernicin, atropine, ATP, antihistamines, camylofine, azulen benzoquinone, biotin, 2-bromo-*d*-lysergic acid diethylamide, hyoscine butylbromide, bulbocapnine, barbital, bradykinin, caffeine, catechol, chlorpromazine, ε-amino-*n*-caproic acid, citric acid, cocaine, codeine, cysteine, cyanocobalamin, dehydrocholate, deoxyribonucleic acid, dihydroergotamine, dichloroisoproterenol, buphenine, eutison, glutathione, heparin, hexamethonium, histamine, hydrochloric acid, hydrazines, pentylenetetrazol riboflavin, *d*-lysergic acid diethylamide, morphine, iproniazid, kallidine, procaine hydrochloride, NaF, NaCN, mephenesin, meprobamate, 1,2-naphthoquinone, 1,4-naphthoquinone, methysergide, α-naphthylamine, β-naphthylamine, *n*-(naphthyl)-methylenediamine, narceine, dihydralazine, nicotine, oxytocin, patulin, phenacemide, phenoxybenzamine, polyethylene sulfate, protamine, pyridoxalphosphate, pyridoxine, quiloflex, reserpine, quinine, 8-hydroxyquinoline, 6-ethoxy-7-methoxy-1-(3′,4′-diethoxybenzyl)-3-methyl isoquinoline, renin, scopolamine, prenylamine, semicarbazide, sparteine, strychnine, tetraethylammonium bromide, thiamine, thioglycerin, thiosemicarbazide, trimethadione, tripelennamine, urea, vasopressin (1, 21, 40, 41, 47, 48, 50, 96, 179–184).

A few drugs, while not specific, inhibit the SP response, as 5-adenylic acid, noscapine, acetyl thiamine, adrenochrome, papaverine, trasentin-6H, D-tubocurarine, gallamine, trihexyphenidyl hydrochloride, ibogaine, harmoline (47, 50, 182–186).

While LSD potentiates the response of SP on the guinea pig ileum (187–189), BOL-148 has an inhibitory effect (183). Succinylcholine and psilocybin also potentiate the effect of SP (185, 190). Substance P potentiates the intestinal response to acetylcholine and nicotine with slight depression of serotonin and no effect on histamine or γ-aminobutyric acid responses (184, 191).

Hexamethonium, morphine, and morphine-like analgesics inhibit the stimulating action of SP on the peristaltic reflex (132, 192). Substance P is able to restore peristalsis when abolished by either external or internal application of serotonin or tubocurarine (4, 5, 193) but not bradykinin (194). Purified SP has a potentiating effect on epinephrine induced contractions of the nictitating membrane (19).

Morphine, physostigmine, or atropine injected 30 min. before sacrifice produced no change in the SP content of the small intestine of the rat (181). Physostigmine significantly decreases the SP content of the rabbit's intestine, while hexamethonium, chlorisondamine, atropine, reserpine, chlorpromazine, *p*-hydroxymercuribenzoate, and nicotine have no definite effect (125). Intraperitoneal injections of 100 units/Kg. of SP for 3 days significantly increases the serotonin content of the rat's ileum and stomach, but not of the spleen. Intraluminal application for 30 min. of SP (60–80 units) doubles the serotonin content of the isolated guinea pig ileum (132, 195).

Acetylsalicylate, mepyramine, atropine, or lysergic acid diethylamide do not inhibit the bronchoconstrictor response of SP in the guinea pig (135, 136).

Atropine, ganglionic blocking agents, antihistamines, or guanethidine have no effect on circulatory responses of SP in mammals (21, 144, 150, 196). Dihydroergotamine, caffeine, mecamylamine, chlorisondamine, hexamethonium, and methysergide diminish the SP arterial depressor response in the intact chicken, but have no vascular effect on the isolated chicken wing preparation or cat blood pressure. The response of these drugs is nonspecific. Atropine, *p*-bromdylamine, LSD, 1-benzyl-2-methyl-5-hydroxytryptamine (BAS-phenol), phenethylamine, harmaline, ibogaine, morphine, and reserpine have no or slight antagonistic effects in the intact chicken (112). Mepyramine reduces SP-induced increased capillary permeability (17).

The effect of drugs on SP concentration in the central nervous system has been investigated by several authors with no unanimity of results. Stern (116) found that strychnine (0.5 mg./Kg. i.p.) after 1–3 hr. reduced the amount of SP in the brain and spinal cord of the rat, while procaine (50 mg./Kg. i.p.) increased the amount. Mephenesin (150 mg./Kg. i.p.) had no effect at 1 hr. and caused only a slight reduction at 3 hr. Allergic encephalitis does not change the SP concentration. Haefely (197) found no change in content in the rat's brain after giving reserpine, barbiturates, chlorpromazine, amphetamine, and LSD. Stern and Kocic-Mitrovic (198), however, found a large increase in SP in the brain of the rat after reserpine treatment, but no change in concentration after phenobarbital, chlorpromazine, meprobamate, mephenesin, or syrosingopine. No change in the rat's brain SP content occurred after giving neostigmine, epinephrine, norepinephrine, serotonin, histamine, factor I, or γ-aminobutyric acid (199). Capsaicine, given subcutaneously, significantly reduces SP in the spinal cord of the rat, but not in the brain. *p*-Bromophenylacetyl urea had no

effect on the spinal cord concentration (111). Cocaine increases SP content in the dorsal roots proximally to site of topical application (200).

No change in SP brain concentration in mice was found after LSD, reserpine, morphine, chloroform, ether, chlorpromazine, barbital, insulin, diphenylhydantoin, pentylenetetrazol, or trihexyphenidyl hydrochloride treatment (182). Zetler and Ohnesorge (201) observed an increase in SP in the mouse brain after amphetamine and morphine and a decrease after chloroform, urethan, and phenobarbital, while LSD produced no change. While no change in total brain concentration was found after picrotoxin or LSD, if the "free" and "bound," *i.e.*, easy or difficult to extract portions were separately determined; picrotoxin increased the "free" and lowered the "bound" amounts of SP and LSD decreased the "bound" form.

Physostigmine decreased the concentration of SP in the rabbit brain, while reserpine, chlorpromazine, and *p*-hydroxymercuribenzoate had no effect (202).

In anesthetized dogs there was no change in the SP content in the hypothalamus and caudate nucleus after amphetamine, ephedrine, insulin, β-tetrahydronaphthylamine, caffeine, and reserpine (94). Reserpine has no effect on SP content in peripheral nerves of the dog (76).

The effect of exogenous SP on the action of central acting drugs is no more conclusive than the effect of these drugs on the endogenous concentration of SP. Zetler (8, 203, 204) found impure SP protected mice against strychnine and picrotoxin convulsions. It prevented the central effects of harmine and methamphetamine and prolonged hexobarbital sleeping time and bulbocapnine catelepsy. Substance P also antagonized morphine analgesia and caused hyperalgesia, potentiated caffeine convulsions, and lowered the threshold to painful stimuli. It had no effect on seizures induced by electroshock, ammonium acetate, nicotine, or pentylenetetrazole. These drugs were tested after 15–30 min. of SP treatment. However, Stern *et al.* (205, 206) found that a slightly purified SP sample potentiated strychnine convulsion, and shortened hexobarbital sleeping time—the opposite to the impure sample, but still antagonized morphine analgesia. Substance P acted as an antagonist to morphine analgesia in the mouse writhing tests (207), while Bonta *et al.* (208) found a strychnine antagonist in their impure SP preparation, but it had no effect on hexobarbital sleeping time or morphine analgesia. They found the strychnine antagonistic property also in other SP-free fractions. Pure SP had no strychnine antagonistic ability (50). Gaddum *et al.* (209) later separated an antistrychnine substance from the crude SP. Kapek (210) found that intracerebral injections of SP increased seizure thresholds to convulsions induced by pentylenetetrazol, strychnine, and electroshock. Stern *et al.* (172, 211, 212) found SP has an antagonistic effect against tetanus toxin, and iminodipropionitrile, and a synergistic effect with mephenesin and meprobamate. Substance P inhibits epileptic attacks induced by audiogenic stress in mice (213).

After observing the changes in concentration with time in the central nervous system after giving exogenous SP, Stern (116) re-examined several drugs to determine the influence of time of administration on the results, using an impure SP sample. Zetler's results were confirmed when SP was given 15 min. before administration of the other drugs. Strychnine, picrotoxin, caffeine, or morphine actions are, however, potentiated when administered 60 min. after SP and hexobarbital sleeping time is shortened. The action of chloral hydrate is lengthened when administered 15 or 60 min. after SP. Bulbocapnine catalepsy is lengthened at all times. Audiogenic stress is not inhibited after 60 min., but is at 15 min. Harmine tremor is antagonized by SP at all times tested. Pentylenetetrazole convulsions are only slightly influenced by SP after 60 min. Substance P not only antagonized morphine analgesia, but also suppressed the withdrawal symptoms in addicted rats. Impure SP given 60 min. prior shortened morphine, pethantine, and heptadon analgesia. It did not act against mephenesin paralysis after either 15 or 60 min. Substance P shows protection during the first 30 min. to strychnine convulsions and hexobarbital narcosis, then excitement for the next 1.5 hr., and by the second hour a return to the central behavior pattern.

Substance P had no influence on cocaine or procaine local anesthesia. The lethal dose of slow intravenous infusion of SP after LSD administration in the decorticated mouse is one-third to one-fourth of that in the nontreated normal animal (116). Both crude and pure SP (10,000 units/mg.) enhance the fourth dorsal root potential after LSD administration (175–177). The amplitude variations of the electric knife fish are significantly decreased following LSD and SP administration (11). Local application of γ-aminobutyric acid antagonizes the effect of SP on the d.c. potential in the cortex (168).

CONCLUSIONS

The chemistry, distribution, physiology, and pharmacology of SP has been reviewed. The presence of SP in all cordates, the characteristic

distribution in the gastrointestinal tract and nervous system, the changes in endogenous concentration with different functional states, and the high pharmacological activity of the pure substance indicate teleologically that SP has a definite physiological function. However, this function is still largely obscure, particularly within the nervous system. This makes SP an interesting and important physiological and pharmacological problem.

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Alkaloids of *Acronychia Baueri* Schott I

Isolation of the Alkaloids and a Study of the Antitumor and Other Biological Properties of Acronycine

By GORDON H. SVOBODA, GERALD A. POORE, PATRICK J. SIMPSON, and GEORGE B. BODER

Utilizing a differential extraction technique for the examination of the bark of the Australian plant *Acronychia Baueri* Schott (*Bauerella australiana* Borzi), has resulted in the isolation of the triterpene lupeol and the alkaloids melicopine, acronycine, and normelicopidine. The experimental antitumor activity associated with the crude alkaloidal mixture obtained from the ether extract has been shown to be attributable to acronycine. Experimental evidence is herein given, showing acronycine to have the broadest antitumor spectrum of any alkaloid isolated to date in these laboratories. By virtue of its being chemically unrelated to any of the presently utilized antitumor agents it represents a new lead in the search for agents effective in the chemotherapeutic management of human neoplasms.

THE GENUS *Acronychia*, family *Rutaceae*, consists of approximately 20 species of trees and shrubs native to Australia and tropical Asia. Of the Australian species, one is also found in New Caledonia, six others being endemic. The species are characterized by opposite or alternate leaves, sepals, petals, and other flower parts being in fours (1).

Acronychia Baueri Schott, commonly called the scrub ash or scrub yellowwood, grows 50 to 60 ft. in New South Wales and Queensland. It resembles the evergreen *A. laevis* but has rather longer leaves and a hard fruit. The wood is said to be excellent for mallet and chisel handles (2). No references to its medicinal use were found.

While this species is usually classified as follows: family, *Rutaceae*; subfamily, *Toddalioideae*; tribe, Toddalieae; subtribe, Toddaliinae; Engler (3) considers it as being *Bauerella australiana* Borzi. "Whether or not *Bauerella* is a genus distinct from *Acronychia* is basically one of evaluation of separating characters. In 1931, Engler kept them distinct, but the material in the herbaria at Harvard cannot be distinguished at sight and the characters used by Engler to separate the two concepts are trivial indeed: for *Bauerella*, he cited sepals imbricate, stamen filaments treadlike, style long; for *Acronychia*, sepals valvate, filaments short and broad, style short. None of these is biologically critical. The genus must be based upon a significant biological difference. I, therefore, cannot accept *Bauerella*. The plant described in 1836 as *Acronychia Baueri* Schott was redescribed in 1897 as *Bauerella australiana* Borzi. I believe that the proper name of this plant should be *Acronychia Baueri* Schott." [1]

The choice of plants selected in the authors' phytochemical screening program is determined

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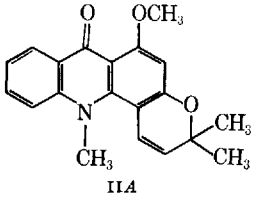
The drug used in this investigation was obtained from the Meer Corp., New York, N. Y.

Added in proof: Recently an alternative structure for acronycine has been proposed (IIA) (19):



IIA

Previous work was somewhat ambiguous in that either structure was possible, and the former structure had been selected on the basis of what could be considered as tenuous NMR data.

Oxidative degradation of acronycine has produced an acid which was identified and which could only have been derived from structure IIA.

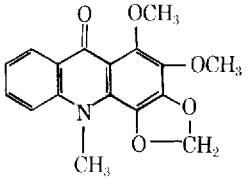
[1] Personal communication from Dr. Richard Evans Schultes, Curator, Botanical Museum, Harvard University, Cambridge, Mass.

by their reported folkloric usage and/or their reported alkaloidal content. Selection of *Acronychia Baueri* Schott falls into the second category, thereby being within the scope of the authors' original intent. Although the comprehensive work of Lahey *et al.* (4–10) had determined the alkaloidal content and the nature and structures of the various alkaloids, no biological activities were reported.

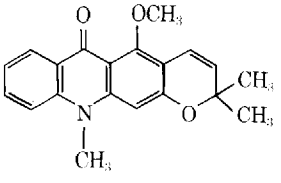
While the defatted ethanolic extract of the bark gave a negative response in the cancer screen *versus* the P-1534 leukemia, Mecca-lymphosarcoma, and adenocarcinoma 755, it elicited an interesting CNS depressant activity in the mouse behavior test (11). The observation of this latter activity was responsible for the ensuing phytochemical effort.

Inasmuch as the approach to the problem of isolation and purification involved a differential extraction technique, the results can be expected to be at variance with those of the total-extraction procedure with ethanol so frequently reported in the literature.

Defatting of the bark with *n*-hexane yielded an unexpectedly large amount of fat-soluble extractive, from which the triterpene lupeol was readily isolated. Extraction of the defatted drug with ether yielded an extract from which crystals readily deposited upon concentration. These crystals were eventually found to be a mixture of melicopine (I) and acronycine (II).



I



II

Although the original extract was inactive against the neoplasms used, it was found that the alkaloidal mixture was significantly effective *versus* the C-1498 leukemia, X-5563 myeloma, and AC-755. Fractional crystallization from methanol yielded the pure alkaloids. Melicopine was found to be ineffective against these experimental neoplasms, acronycine being responsible for the observed activity.

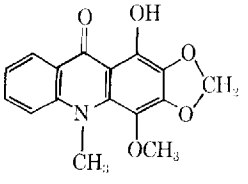
EXPERIMENTAL

A quantity of coarsely ground bark (15.0 Kg.) was extracted by stirring with two 35-L. portions of *n*-hexane. Concentration *in vacuo* yielded 253.0 Gm. of a nondrying oil. A copious quantity of fine needles deposited from the concentrate at room temperature. The oily mass was dissolved in 250 ml. of hot acetone, and the solution was allowed to cool spontaneously to room temperature, yielding 9.82 Gm. of fine colorless needles which exhibited parallel extinction and a high birefringence under polarized light, m.p. 210–212°. Its X-ray diffraction pattern was identical in all respects to that of the authentic triterpene lupeol.

The crude mother liquor (CML) elicited some activity against the C-1498 leukemia, and it was assumed that this activity was due to the acronycine content, as this alkaloid was shown to be present when examined by thin-layer chromatography (1:1 ethyl acetate–benzene on Silica Gel G).

Dissolving 128.5 Gm. of the *n*-hexane extract·CML in 2 L. of ether, with the subsequent addition of ethereal HCl to the chilled solution, produced 13.444 Gm. of a red-orange amorphous hydrochloride. Suspending the salt in H_2O and extracting with ethylene dichloride yielded 9.37 Gm. of amorphous free bases. Crystallization from acetone yielded three crops of crystals: first crop, 0.348 Gm. of what appeared to be essentially normelicopidine (III); second crop, 1.248 Gm. of acronycine contaminated with some normelicopidine; third crop, 0.644 Gm. of a mixture of apparently equal parts of acronycine and normelicopidine. A small amount of normelicopidine (0.012 Gm.) was obtained by crystallizing the mother liquor from methanol.

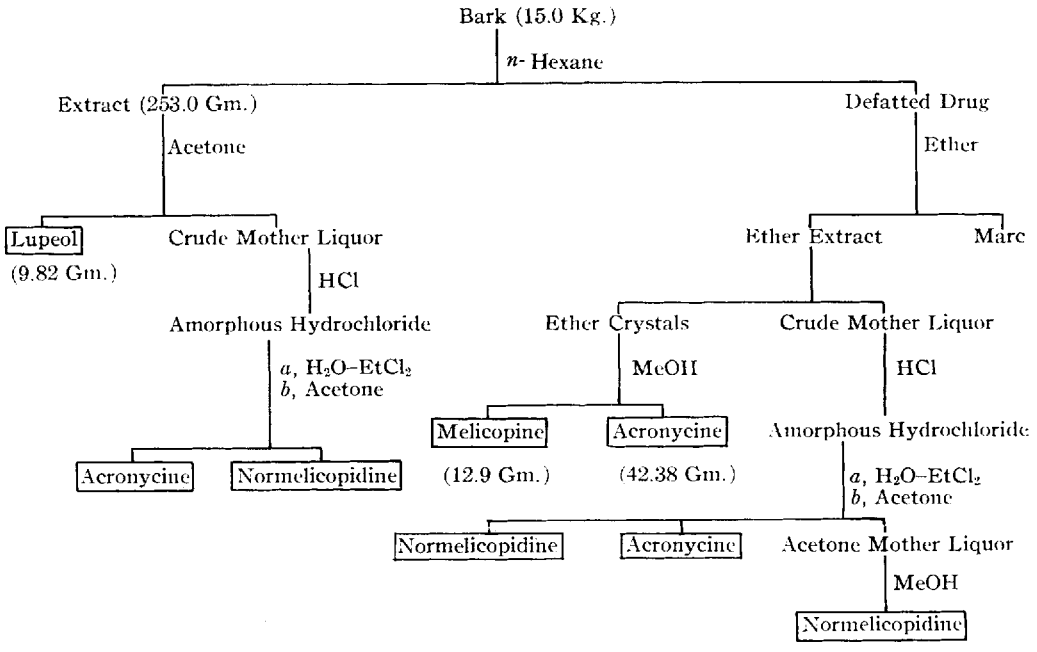
Recrystallization of the first crop from hot acetone yielded 0.298 Gm. of pure normelicopidine; recrystallization of the second crop from hot methanol yielded 0.028 Gm. of normelicopidine and 0.836 Gm. of acronycine; recrystallization of the third crop from hot methanol yielded 0.244 Gm. of normelicopidine and 0.236 Gm. of acronycine. The separation of normelicopidine from acronycine could be easily effected inasmuch as the former is quite insoluble in methanol.



III

The defatted bark was then extracted by stirring with three 40-L. portions of ether. Concentration of this extract to 10 L. produced 115.0 Gm. of yellow-gold crystals. Interpretation of physical data (X-ray, ultraviolet and infrared spectra, and TLC) indicated that this mixture was comprised of acronycine and melicopine, the former predominating.

Recrystallization of this mixture from methanol (10 ml./Gm.) afforded four crops of crystals: 12.9 Gm. of melicopine, 26.0 Gm. of slightly impure acronycine, 13.9 Gm. of a mixture of acronycine and melicopine, and 16.9 Gm. of slightly impure



Scheme I

acronycine, respectively. The second and fourth crops were combined (42.9 Gm.) and were recrystallized from methanol (8 ml./Gm.), yielding 41.4 Gm. of chromatographically pure acronycine. An additional 0.98 Gm. of pure alkaloid was obtained by crystallization of the finished mother liquor from acetone.

Treating an ether solution containing a 64.5-Gm. aliquot of the ether extract crude mother liquor[2] with ethereal HCl afforded 31.0 Gm. of a red-orange amorphous hydrochloride. The free bases were obtained (27.2 Gm.) by suspending the salt in H_2O and extracting with ethylene dichloride. Crystallization of normelicopidine (1.9 Gm.) was achieved with hot acetone (10 ml./Gm.). Concentration of the mother liquor to a low volume yielded 11.8 Gm. of essentially pure acronycine. (Chromatographic purity could be achieved by recrystallization from methanol as above.) An additional amount of normelicopidine (0.38 Gm.) was obtained by crystallization of the acetone mother liquor from methanol.

A flow diagram for the extraction scheme is presented in Scheme I.

Authentic samples of the alkaloids were not available for direct comparison, and it was necessary to resort to data published in the literature cited. All physical data[3] were in good agreement with those in the original references. As an additional aid to their identification, the infrared spectra of the individual alkaloids are presented in Fig. 1. NMR examination indicated that the integrated intensities, chemical shifts, and splitting patterns were consistent with the structures shown.

[2] Total weight = 169.0 Gm., but 100 Gm. reserved for chromatographic examination.

[3] Melting points were determined on a Kofler microstage. Ultraviolet absorption spectra were obtained using a Cary model 14 spectrophotometer; infrared spectra with a Perkin-Elmer model 21 double beam recording infrared spectrophotometer; NMR spectra with a Varian Associates 60-Mc. spectrometer. A standard Norelco powder camera, 114.6 mm. in diameter, was used in the X-ray examination.

Melicopine.—The base crystallizes from methanol as yellow orthorhombic blades elongated on the *c* crystallographic axis. Crystals show 100 and 010 faces and occasionally the 110 face crystals show pale yellow-dark yellow pleochroism, m.p. 179–181°.

Anal.—Calcd. for $C_{17}H_{15}NO_5$: C, 65.17; H, 4.82; N, 4.47; O, 25.53. Found: C, 64.62; H, 4.96; N, 4.67; O, 25.66.

$\lambda_{max.}^{EtOH}$ 270 mμ (log ε 4.74), 301 mμ (log ε 4.13).

Acronycine.—The base crystallizes from methanol as opaque, yellow, porous particles of which neither the extinction positions nor crystal shape

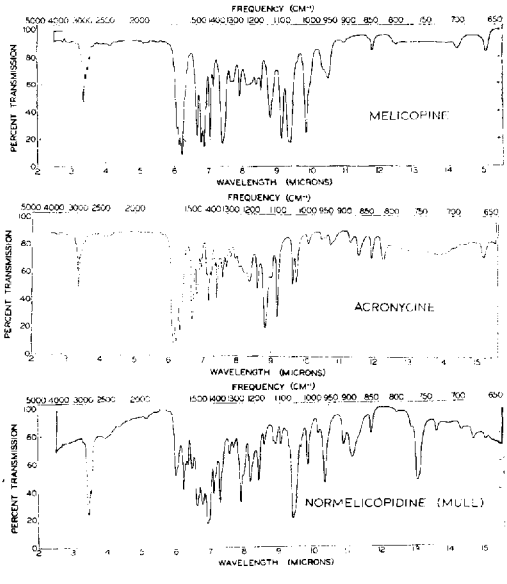


Fig. 1.—Infrared spectra of the alkaloids.

TABLE I.—EXPERIMENTAL TUMOR SPECTRUM OF ACRONYCINE

Tumor	Host, 10 Animals	i.p. Dosage, mg./Kg./Day	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm. T/C	Av. Life, T/C	% No Takes, T/C[a]	% Activity
		75 × 1 × 3					
B-82 leukemia	C58B1/6	37.5 × 1 × 7	−1.4/+0.4	...	23.7/14.6	...	61
C-1498 leukemia	C57B1/6	28 × 1 × 10	−1.5/+0.8	...	31.5/17.6	...	79 (7)
P-1534 leukemia	DBA/2	30 × 1 × 10	−0.2/−0.7	...	16.5/18.2	...	0
L-5178Y leukemia	DBA/2	28 × 1 × 10	+2.2/+3.2	...	24.2/15.0	...	62
AKR leukemia	AKR	28 × 1 × 10	+0.1/+0.8	...	38.3/21.5	...	78 (5)
Ehrlich ascites	Cox std.	30 × 1 × 10	+5.6/+7.8	...	21.8/18.4	...	0 (1)
Freund ascites	CAF[1]	48 × 1 × 10	+2.6/+6.5	...	9.8/14.5	...	0
S-180 ascites	CAF[1]	30 × 1 × 10	+3.0/+6.2	...	20.0/22.1	...	0
Taper hepatoma, ascites	SPF·ND4	48 × 1 × 10	+3.4/+8.6	...	17.7/14.8	...	20
Sarcoma 180	CAF[1]	30 × 1 × 10	+3.2/+6.0	7.1/11.9	...	0/0	40 (9)
Mecca-lymphosarcoma	AKR[c]	30 × 1 × 7	−0.4/+2.5	6.2/16.9	...	42/14	63 (7)
Ridgeway osteogenic sarcoma	AKR	48 × 1 × 9	−0.6/+3.4	0/9.6	...	100/0	100 (10)
X-5563 myeloma	C3H	30 × 1 × 8	+0.1/+0.3	0/9.1	...	100/13	100 (8)
Adenocarcinoma 755	C57B1/6	30 × 1 × 10	−0.5/+1.9	11.9/19.7	...	0/0	40 (10)
Shionogi carcinoma 115	dd/s (male)	36 × 1 × 9	+1.4/+1.4	0/15.3	...	100/5	100 (7)
High malignancy clone	C3H[d]	37.5 × 1 × 9	+1.6/+6.5	0/22.6	.	100/0	100 (5)
S-91 melanoma	DBA/1	36 × 1 × 9	−1.4/+0.1	0/14.1	...	100/0	100 (4)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors on solid tumors, indefinite survivors at 45 days on leukemia or ascitic tests. [c] Seven animals. [d] Five animals.

could be determined, from ethanol as yellow clear needles in fan-shaped clumps, the long crystals of which exhibited extinction values of approximately 18° from the elongation axis. Also in evidence were some short, thick crystals (almost cubic) that had parallel extinction to one side of the crystal, from acetone as monoclinic rods, having an extinction angle of approximately 22°. There were also a number of crystals twinned on the *a* and *c* axes, m.p. 174–176°.

Anal.—Calcd. for $C_{20}H_{19}NO_3$: C, 74.74; H, 5.96; N, 4.36; O, 14.94. Found: C, 74.76; H, 6.19; N, 4.29; O, 15.01.

$\lambda_{max.}^{EtOH}$ 280 mμ (log ε 4.60), 291 mμ (log ε 4.54), 304 mμ (log ε 4.28), 392 mμ (log ε 3.84).

Normelicopidine.—The base crystallizes from either methanol or acetone as red, equant monoclinic prisms in which occasional elongated forms are seen. The crystals show pronounced yellow-red to dark red pleochroism, m.p. 210–211°.

Anal.—Calcd. for $C_{16}H_{13}NO_5$: C, 64.21; H, 4.37; N, 4.68; O, 26.73. Found: C, 64.29; H, 4.55; N, 4.74; O, 26.46.

$\lambda_{max.}^{EtOH}$ 218 mμ (log ε 4.175), 282 mμ (log ε 4.53); shoulders at 251 mμ (log ε 4.35), 304 mμ (log ε 4.088); in alkaline solution a typical phenolic shift was seen: $\lambda_{max.}^{EtOH}$ 250 mμ (log ε 4.358), 284 mμ (log ε 4.448), 312 mμ (log ε 3.968).

BIOLOGICAL PROPERTIES

Acronycine possesses the broadest antitumor spectrum (Table I) of any alkaloid isolated to date in these laboratories. The two systems of choice for assaying its activity are the C-1498 myelogenous leukemia and the X-5563 plasma cell myeloma.

Both of these experimental neoplasms are unique in some characteristics: the C-1498 leukemia does not respond to any of the known clinically active chemotherapeutic agents presently available, while the X-5563 myeloma presents most of the same features that are associated with multiple myeloma in man. Also of special interest is the activity against the Shionogi carcinoma 115, an androgen-dependent tumor which might be of value in predicting clinical activity against prostatic cancer.

Methodology.—Procedures for animal tumor testing in these laboratories have previously been described (12) but consist essentially of subcutaneous trocar implantation of solid tumors in the axilliary region and intraperitoneal inoculation of ascitic and leukemic cells with standard cell inocula. Treatment is usually initiated 24 hr. later, exceptions being made with the X-5563 myeloma, Shionogi carcinoma 115, and the S-91 melanoma. In the case of the X-5563, treatment was initiated 3 days after transplantation, while with the other two it was begun 5 days after transplantation. Activity of a compound is defined in terms of per cent inhibition, using treated *versus* controls (T/C) when testing against the solid tumors and in terms of per cent prolongation of life, using treated *versus* controls (T/C) when testing against the leukemias and ascites systems.

Preparation of Acronycine for Testing.—The relative insolubility of this alkaloid has obviously not precluded its being tested. A suitable suspension was prepared by grinding the compound with small volumes of a nonionic dispersant[4] to obtain a uniform suspension and diluting with this diluent to the desired volume. (The dispersant is a polyoxyethylated fatty acid that is H_2O-miscible and nontoxic when diluted to the proper concentration of 1:10 with either sterile distilled H_2O or sterile physiological saline solution.) Acronycine was neither soluble in, nor were satisfactory suspensions obtained with carboxymethylcellulose, saline, sesame oil, olive oil, or acacia solution.

ANTITUMOR ACTIVITY OF ACRONYCINE

In this laboratory acronycine has been shown to be a potent antitumor agent against a multiplicity of mouse neoplasms, significant activity having been demonstrated against 12 of 17 tumors tested with a wide range of dose levels. Not only is it broad

[4] Marketed as Emulphor by General Aniline and Film Corp., Melrose Park, Ill.

Table II.—Activity of Acronycine *Versus* C-1498 Leukemia *Via* Various Routes of Administration

Route	Dosage, mg./Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Life, T/C	% Prolongation	Indefinite Survivors
Intraperitoneal	15	−0.8/+0.8	25.9/17.6	47	..
	20	−1.1/+0.8	29.0/17.6	65	6
	24	−1.9/+0.8	31.6/17.6	80	3
	28	−1.5/+0.8	31.5/17.6	79	7
	30	−1.2/+0.8	31.0/17.6	76	3
	75	−2.8/−0.2	23.0/13.3	72	..
	80	−3.1/+0.1	24.0/16.1	49	5
	90	−2.7/+0.1	29.2/16.1	81	2
	100	−2.0/+0.1	19.6/16.1	21	7
	125	−2.4/−0.2	14.6/13.3	0	..
	150	−2.5/−0.2	13.0/13.3	0	..
	175	−1.0/−0.2	12.4/13.3	0	..
Oral	30	−1.1/+0.4	23.6/17.9	31	..
	45	−1.4/+0.4	31.4/17.9	76	5
	60	−1.8/+0.4	31.3/17.9	75	7
	75	−1.4/+0.4	24.4/17.9	37	5
	80	−2.1/+0.4	18.7/17.9	0	6
Subcutaneous	15	−0.8/+0.4	19.9/18.5	0	..
	20	−1.5/+0.4	20.4/18.5	0	..
	24	−1.1/+0.4	22.8/18.5	23	..
	28	−1.8/+0.4	27.8/18.5	57	..
	30	−1.8/+0.4	27.3/18.5	48	1

Table III.—Activity of Acronycine *Versus* C-1498 Leukemia with 3-Day Delayed Treatment

Route	Dosage, mg./Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Life, T/C	% Prolongation
Intraperitoneal	15	−0.1/+1.6	23.3/16.2	43
	20	−0.3/+1.6	25.1/16.2	55
	24	−1.2/+1.6	26.0/16.2	61
	28	−1.0/+1.6	26.1/16.2	61
	30	−0.8/+1.6	25.6/16.2	58
Oral	30	−1.4/+0.4	21.4/16.6	29
	45	−1.6/+0.4	27.1/16.6	64
	60	−2.3/+0.4	26.8/16.6	61
	75	−0.8/+0.4	30.6/16.6	85
	80	−2.0/+0.4	28.8/16.6	73
Subcutaneous	15	+0.8/+1.1	18.7/16.8	0
	20	+0.5/+1.1	19.1/16.8	0
	24	−0.5/+1.1	20.3/16.8	21
	28	−0.2/+1.1	19.7/16.8	0
	30	+0.9/+1.1	19.7/16.8	0
Intravenous	3.75	+0.6/+0.7	17.8/15.6	0
	7.5	−0.4/+0.7	18.4/15.6	0
	10	−1.6/+0.7	20.2/15.6	29

Table IV.—Activity of Acronycine *Versus* C-1498 Leukemia with 6-Day Delayed Treatment

Route	Dosage, mg./Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Life, T/C	% Prolongation
Intraperitoneal	15	−1.7/−2.1	19.0/15.8	20
	20	−3.0/−2.1	21.8/15.8	34
	24	−3.0/−2.1	20.6/15.8	27
	28	−2.3/−2.1	24.0/15.8	51
	30	−2.8/−2.1	23.0/15.8	45
Oral	30	−2.0/0	18.7/16.2	0
	45	−2.6/0	19.6/16.2	21
	60	−3.4/0	19.9/16.2	23
	75	−3.3/0	26.7/16.2	65
	80	−3.0/0	22.7/16.2	33
Subcutaneous	15	−1.0/+0.6	17.4/16.2	0
	20	−1.1/+0.6	18.2/16.2	0
	24	−0.9/+0.6	17.9/16.2	0
	28	−1.8/+0.6	18.5/16.2	0
	30	−1.4/+0.6	18.7/16.2	0
Intravenous	3.75	−0.4/0	17.8/16.4	0
	7.5	−0.4/0	18.2/16.4	0
	10	−1.0/0	19.0/16.4	0

TABLE V.—ACTIVITY OF ACRONYCINE *Versus* X-5563 MYELOMA

Route	Dosage, mg./ Kg. × 1 × 8	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	24	−0.6/+0.3	0/9.1	100/13	100 (8)
	28	+1.3/+0.3	0/9.1	100/13	100 (6)
	30	+0.1/+0.3	0/9.1	100/13	100 (8)
	36	+2.5/+0.3	0/9.1	100/13	100 (3)
	45	−1.4/+0.3	0/9.1	100/13	100 (2)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors.

TABLE VI.—ACTIVITY OF ACRONYCINE *Versus* X-5563 MYELOMA WITH 3-DAY DELAYED TREATMENT

Route	Dosage, mg./ Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Tumor Size mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	15	−3.8/−0.6	1.9/10.8	55/6	82 (8)
	20	−1.4/−0.6	0.7/10.8	55/6	94 (6)
	24	+0.1/−0.6	0/10.8	100/6	100 (4)
	28	−1.2/−0.6	0/10.8	100/6	100 (6)
	30	−2.5/−0.6	0/10.8	100/6	100 (2)
Oral	30	−3.5/+0.9	1.3/13.3	67/0	90 (8)
	45	−/+0.9	−/13.3	−/0	N.S.[c]
	60	−/+0.9	−/13.3	−/0	N.S.
	75	−/+0.9	−/13.3	−/0	N.S.
	80	−1.1/+0.9	0/13.3	100/0	100 (1)
Subcutaneous	15	−2.9/−0.5	7.9/14.6	11/0	46 (8)
	20	−1.6/−0.5	4.9/14.6	22/0	67 (9)
	24	−3.5/−0.5	5.5/14.6	0/0	62 (8)
	28	−2.4/−0.5	6.5/14.6	11/0	56 (9)
	30	−3.0/−0.5	7.0/14.6	11/0	52 (9)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

TABLE VII.—ACTIVITY OF ACRONYCINE *Versus* X-5563 MYELOMA WITH 6-DAY DELAYED TREATMENT

Route	Dosage, mg./ Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Tumor Size mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	15	+0.6/+3.9	13.4/18.3	0/7	28 (7)
	20	−0.8/+3.9	10.3/18.3	14/7	43 (3)
	24	+0.9/+3.9	13.7/18.3	0/7	25 (4)
	28	+2.6/+3.9	16.5/18.3	0/7	0
	30	−1.8/+3.9	8.2/18.3	0/7	55 (2)
Oral	30	−2.1/+3.5	10.2/15.7	14/7	35 (5)
	45	−1.2/+3.5	16.0/15.7	0/7	0
	60	−2.7/+3.5	7.0/15.7	0/7	56 (1)
	75	−/+3.5	−/15.7	−/7	N.S.[c]
	80	−3.0/+3.5	9.0/15.7	0/7	43 (1)
Subcutaneous	15	+0.7/+5.4	14.0/18.4	14/7	0
	20	+0.7/+5.4	16.9/18.4	0/7	0
	24	+0.2/+5.4	15.0/18.4	0/7	0
	28	−3.5/+5.4	13.2/18.4	14/7	28 (6)
	30	−1.8/+5.4	14.1/18.4	0/7	0
Intravenous	3.75	+2.8/+5.2	16.9/24.4	0/0	30 (7)
	5	+1.0/+5.2	15.1/24.4	14/0	38 (7)
	7.5	+1.9/+5.2	19.3/24.4	0/0	0

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

spectrum in character, but it is also effective by various routes of administration. The oral and subcutaneous activities are of special interest since most of the clinically proved oncolytic agents presently used are ineffective orally and elicit intolerable side effects when administered subcutaneously.

When tested against the adenocarcinoma 755, C-1498 leukemia, and the X-5563 myeloma, acronycine also displayed significant activity by both the oral and subcutaneous routes. Furthermore, there was no evidence of skin irritation or alopecia when administered subcutaneously.

Only minimum activity was observed when the alkaloid was administered intravenously. In all probability this is attributable to its insolubility, sufficiently high blood levels not being attained. In several instances blockage of the circulatory system was evidenced, autopsy revealing an actual aortic block by the compound itself.

In determining the efficacy of a potential clinically useful compound it is always of interest to determine its effect under conditions which simulate, to the best of one's ability, the human situation, *i.e.*, its effectiveness against a well-established neoplasm. This condition can be achieved experimentally by implanting the mice with the tumors and withhold-

TABLE VIII.—ELECTROPHORETIC STUDIES ON X-5563 TUMOR-BEARING, ACRONYCINE-TREATED MICE

Dosage, 4 Animals, mg./Kg. × 1 × 10	Serum Fraction	% on Day of Sampling 0	7	12	19
20	γ-like	18	24	17	21
	β	23	24	19	19
	α	11	13	13	7
	Albumin	50	39	50	53
	Protein[a]	130	98	87	64
28	γ-like	14	23	18	18
	β	21	21	21	22
	α	12	13	14	7
	Albumin	53	43	47	53
	Protein[a]	130	96	84	58
36	γ-like	18	24	20	15[b]
	β	21	21	15	17[b]
	α	11	15	14	0[b]
	Albumin	50	60	51	68[b]
	Protein[a]	119	100	70	53[b]
Saline controls	γ-like	18	22	21	32
	β	23	20	18	17
	α	11	13	11	9
	Albumin	48	45	50	42
	Protein[a]	148	89	85	84

[a] Total count of Analytrol integrator teeth under each protein fraction peak. The number of teeth would be directly proportional to the density of the dyed protein band on the actual electrophoretic strip. [b] One animal only.

ing treatment until the neoplasm is ravaging its host. It should be noted that animals inoculated with the C-1498 leukemia at a known cell concentration normally live for a period of 14–18 days. Therefore, a delay of 6 days after inoculation before initiating therapy has without question allowed the tumor to become well established.

Acronycine also has the demonstrable ability to produce "cures" or indefinite survivors of animals inoculated with the C-1498 leukemia. An indefinite survivor is defined as one which survives 45 days or longer after inoculation, having undergone the usual 9- or 10-day regimen of daily treatment. Furthermore, it has demonstrated the ability to inhibit the growth of several neoplasms. These phenomena are detailed in the tables and summaries which follow.

Activity Against the C-1498 Leukemia.—As stated earlier, this neoplasm is singularly nonresponsive to chemotherapeutic agents. Table II summarizes the dose-response relationships for the various routes of administration, while Tables III and IV give the results of 3- and 6-day delayed therapy *via* various routes. It is readily seen that acronycine is effective when administered by any of several routes and even when treatment is delayed, the neoplasm having become well established.

Activity Against the X-5563 Plasma Cell Myeloma (Tables V, VI, and VII).—This neoplasm is intended to serve as a model for human multiple myeloma. In both the animal and human situations an abnormal protein is evidenced in the γ-globulin area of the blood serum.[5] The presence of this abnormal protein seems to increase with the progressive growth of the tumor in both hosts. The depression of this protein has been observed in patients responding to active chemotherapeutic agents, and this has also been seen in the serum of these experimental animals (Table VIII, Figs. 2 and 3).

[5] Beckman model R paper electrophoresis system, using Spinco procedure A, a method for serum proteins utilizing bromphenol blue dye in aqueous solution. The procedure is described in "Technical Bulletin 6095A," Spinco Division, Beckman Instruments, Inc., Stanford Industrial Park, Palo Alto, Calif.

It should be noted, however, that in man it has been possible to witness a regression of the tumor mass without a depression of this protein. In mice bearing the X-5563 neoplasm and responding to treatment with acronycine, regression of the tumor has always been accompanied by a drop in this abnormal protein. This technique, therefore, provides one with an interesting correlative tool in working with a compound active against this particular tumor.

Activity Against the Shionogi Carcinoma 115.—Of special interest in the tumor spectrum is the activity against this neoplasm. This is an androgen-dependent system which may be of value in serving as a model for activity against prostatic cancer in man. While this tumor originally arose in females, it will not grow in female mice, castrated males, or males treated with progesterone, estradiol, or other female sex hormones. Dose-response relationships are presented in Table IX.

Activity Against the Adenocarcinoma 755.—The activity of acronycine against this neoplasm by various routes of administration (Table X) is another feature which distinguishes it from the alkaloids of *Catharanthus roseus* G. Don (*Vinca rosea* Linn.).

OTHER BIOLOGICAL PROPERTIES

Electrophoretic Patterns and Leukopenia.—The methodology involved in these determinations is herein described. Sixteen C3H mice[6] were randomly set up in small hanging cages with four animals in each cage and one group serving as saline controls. The first bleeding was done on day zero, by the orbital technique, to establish both the normal white blood cell counts and the normal electrophoretic patterns for each group. After the blood samples were taken, all of the animals were implanted subcutaneously with the X-5563 tumor. Food and water were supplied *ad libitum* for the

[6] Supplied by Microbiological Associates Laboratory, Bethesda, Md.

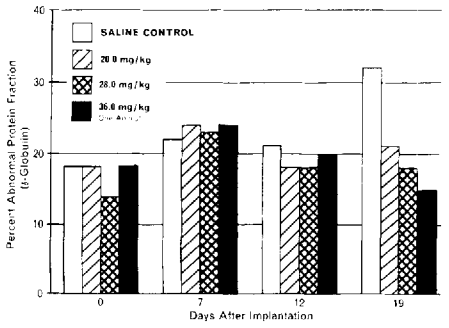


Fig. 2.—Electrophoretic patterns of X-5563 tumor-bearing, acronycine-treated mice.

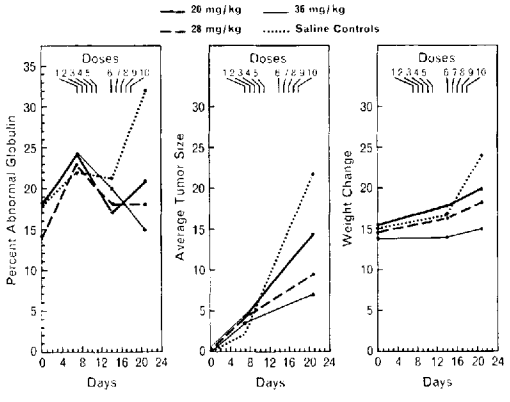


Fig. 3.—Additional parameters of electrophoresis WBC experiment.

next 6 days. On day 7 another bleeding was done, tumor measurements were made, and all animals were weighed. Intraperitoneal treatment was then initiated, three groups of mice receiving acronycine, one group receiving only saline for a total of 10 injections. Similar bleedings were done on day 12, after five injections, and on day 19, the day after the tenth and final injection. On day 19 all animals were weighed, tumor measurements were made, and the experiment was terminated (Table XI).

The electrophoresis was carried out in the following manner: 10 μl. of mouse plasma was used on each strip with Beckman B2 barbital[7] buffer, pH 8.6, having a 0.075 *M* strength. A 16-hr. run at room temperature was made, employing a constant current of 5 ma./cell. At the end of this period, the strips were stained by the Beckman method, scanned, and evaluated with a Beckman Analytrol.

Some leukopenia was evidenced at all dose levels. The method used to determine the white count was as follows: 20 μl. of whole blood was taken from each of the treated and control mice by the orbital technique. The fresh whole blood was then diluted with 10 ml. of particle-free physiological saline. Hemolysis of the red cells was achieved by adding a small amount of 2% saponin solution, allowing all of the intact white cells to be enumerated. Counts were made using a Coulter counter, the instrument being set to correlate with counts done by the hemocytometer method.

It should be noted that mice are somewhat limited in the amount of stress that can be tolerated, and that those on the electrophoresis-white blood cell count experiment were subjected to a great deal of stress because of a combination of factors—treatment with the alkaloid, several bleedings, the amount of blood withdrawn, and daily handling. The drug-treated animals were apparently unable to replenish the protein removed by the blood samplings under the conditions of daily therapy. This factor could be partly responsible for some deaths occurring during the experiments.

Mitotic Studies.—Acronycine was compared with vincaleukoblastine sulfate in several tissue culture cell lines for its ability to exert metaphase arrest. The procedures and methods have been described in detail elsewhere (13) but may be briefly summarized.

Two different cell lines were used, a diploid Chinese hamster lung and the polyploid HeLa cell. The alkaloid was dissolved in undenatured ethanol, sterile-filtered, and diluted in the appropriate tissue culture medium to a maximum concentration of 25 mcg./ml. in 0.25% ethanol. Log-phase cultures were exposed to the compounds for 4 and 24 hr. The cells were prepared according to two different methods—a hematoxylin-eosin stain (H and E) and a chromosome preparation according to the method of Puck (13).

No differences in effect were seen in the two cell lines and there was no apparent mitotic arrest associated with acronycine. At concentrations of 25 mcg./ml., acronycine exerted a very rapid cytotoxicity to cells which was produced in less than 3 hr.; yet at 10 mcg./ml. there was apparently no cytological effect. The TCD_{50} had previously been established at 13 mcg./ml. The results of a typical experiment are shown in Table XII.

Adrenal Apoplexy.—Acronycine exhibited significant activity in blocking the apoplexy effect of 7,12-dimethylbenz(a)anthracene (7,12-DMBA) when administered orally at 40 and 80 mg./rat in a single feeding 24 hr. prior to the oral administration of the 7,12-DMBA. Huggins *et al.* (14) orally administered 20 mg. of 7,12-DMBA to 50-day-old virgin female Sprague-Dawley rats and on necropsy found that the adrenals were completely engorged with blood and greatly hypertrophied. The same investigators further found that this effect could be blocked by certain other polycyclic hydrocarbons such as 3-methyl cholanthrene.

The strain, age, and sex of the animal are important factors in inducing this phenomenon. There is apparently some interrelationship between the female hormones being produced at this age, the role of the pituitary and that of the adrenal cortex, but it is not yet completely understood.

The same effects have been seen in these laboratories and the assay was performed in the following manner. Sprague-Dawley virgin female rats were received at 43 days of age. They were given food and water *ad libitum* until day 49. On this day they were weighed and dosed orally with the test compound, the controls receiving only saline. Twenty-four hours later they received the 7,12-DMBA, along with food and water *ad libitum*. On day 53 all of the animals were sacrificed by decapita-

[7] Marketed as Veronal by Winthrop Laboratories, Inc., New York, N. Y.

TABLE IX.—ACTIVITY OF ACRONYCINE *Versus* SHIONOGI CARCINOMA 115

Route	Dosage, mg./ Kg. × 1 × 9	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	36	+1.4/+1.4	0/15.3	100/5	100 (7)
	45	−0.8/+1.4	0/15.3	100/5	100 (4)
	50	−0.4/+1.4	0/15.3	100/5	100 (1)
	60	−/+1.4	−/15.3	−/5	N.S.[c]

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

TABLE X.—ACTIVITY OF ACRONYCINE *Versus* AC-755 *Via* VARIOUS ROUTES OF ADMINISTRATION

Route	Dosage, mg./ Kg. × 1 × 10	Av. Wt. Change, Gm., T/C	Av. Tumor Size, mm., T/C	% No Takes, T/C[a]	% Inhibition[b]
Intraperitoneal	15	+0.3/+1.9	15.7/19.7	0/0	0
	20	+0.2/+1.9	14.4/19.7	0/0	27 (10)
	24	−1.2/+1.9	12.6/19.7	0/0	36 (10)
	28	−0.3/+1.9	12.3/19.7	0/0	38 (10)
	30	−0.5/+1.9	11.9/19.7	0/0	40 (10)
	75	−0.1/+2.0	13.3/15.6	0/0	0
	80	−2.3/+2.0	13.8/24.1	10/0	42 (10)
	90	−2.2/+2.0	11.1/24.1	10/0	54 (10)
	100	+0.6/+2.0	14.0/24.1	10/0	42 (10)
	125	+0.3/+2.0	8.0/15.6	10/0	49 (3)
	150	−/+2.0	−/15.6	−/0	N.S.[c]
	175	−/+2.0	−/15.6	−/0	N.S.
Oral	30	−1.6/+0.9	11.1/20.4	0/0	46 (10)
	45	−2.5/+0.9	10.6/20.4	0/0	48 (10)
	60	−3.9/+0.9	9.7/20.4	0/0	52 (9)
	75	−5.1/+0.9	7.6/20.4	0/0	63 (10)
	80	−4.2/+0.9	6.5/20.4	0/0	68 (10)
Subcutaneous	15	+1.0/+2.7	13.9/20.9	0/0	34 (10)
	20	−0.1/+2.7	13.0/20.9	0/0	38 (10)
	24	−0.3/+2.7	11.7/20.9	0/0	44 (10)
	28	−0.8/+2.7	10.5/20.9	0/0	50 (7)
	30	−0.8/+2.7	13.0/20.9	0/0	38 (10)

[a] Zeros or "no takes" are also indicative of activity, as no "no takes" in the control animals and 100% in the test animals are interpreted as 100% inhibition. [b] The number in parentheses indicates survivors. [c] No survivors.

tion and exsanguinated. The adrenals were then removed and weighed.

Determinations of hemoglobin were not done, the adrenals instead having been observed macroscopically and rated according to the degree of apoplexy present. With an active blocking agent the adrenals appeared yellowish in color as in normal untreated rats and weighed less than those of the controls. Per cent activity of a compound in preventing apoplexy was carried by the following arbitrary protocol:

4+ = total apoplexy (inactive compound)
3+ = 30% absence of apoplexy
2+ = 60% absence of apoplexy
1+ = 80% absence of apoplexy
0 = 100% absence of apoplexy

In assaying acronycine in the preceding manner 72 and 92% blocking effects were observed at 40 and 80-mg. doses per rat, respectively.

Effect of Topical Application.—Acronycine was suspended in the nonionic dispersant[4] as previously described and a suspension of 3 mg./ml. was applied daily to the skin of normal SPF-ND4 mice for a total of 22 applications. There was no evidence of skin irritation, epilation, or hyperplasia.

Algae, Protozoa, and Mammalian Cell Studies.—A dose response of acronycine was run against six algal and protozoan agar-diffusion type assays by the method of Johnson *et al.* (15). The systems involved were *Chlorella vulgaris*, *Scenedesmus basiliensis*, *Tetrahymena pyriformis*, *Ochromonas malhamensis*, *O. danica*, and *Euglena gracilis*. Trace activities were seen against the *S. basiliensis* and *T. pyriformis*, while significant measurable zones of inhibition were observed against both of the *Ochromonas* sp. from paper disks dipped into an ethanol solution containing 500 mcg./ml. of the alkaloid.

Acronycine was further found to be inactive against the bacterial agar diffusion systems of *Clostridium feseri* and *Lactobacillus casei*. However, significant activity was observed in the tissue culture bioautograph systems employing the HeLa human epidermoid cancer cells and the AV_2 human amniotic cells. While several dose levels were used, the lowest dose level employed to give significant zones of activity was 500 mcg./ml.

These data are summarized in Table XIII.

Tetrahymena Motility.—*Tetrahymena pyriformis* is an actively motile protozoan cell. The ciliate's powerful swimming ability allows it to remain in suspension for long periods despite its almost macroscopic size. Any compound exerting a gross cellular toxicity in general or a neurotoxic effect in particular would disrupt the ciliary apparatus causing sedimentation of the immobilized cell. The effect of acronycine upon *Tetrahymena* motility was studied by comparing the sedimentation rate of alkaloid-treated cell suspensions with untreated controls. Significant biological response in this system would suggest a rapid and economical method for comparison of structural modifications

TABLE XI.—W.B.C. AT VARIOUS TIME INTERVALS DURING TREATMENT OF X-5563 TUMOR-BEARING MICE WITH ACRONYCINE

Dosage, 4 Animals, mg./Kg. × 1 × 10	Count/mm.,[a] 0 (Normal)	Day of Sampling 7	12	19	Av. Wt. of Animals, Day of Sampling 0	7	12	19	Mean Tumor Diam., mm., Day of Measurement 7	19	% Inhibition
20	4900	6000	8200	2300	15.6	16.9	19.3	20.0	4.1	14.5	34
28	4700	6200	6000	2500	14.7	16.3	17.5	18.2	4.1	9.5	57
36	4800	6200	6300	2300	13.7	14.0	15.0	15.0	2.3	7.0	68
Saline controls	5500	6100	9400	14800	15.0	16.6	19.9	23.9	2.2	21.9	...

TABLE XII.—EFFECT OF ACRONYCINE ON HELA CELLS, 24-hr. EXPOSURE–CHROMOSOME PREPARATION

Compd.	Concn., mcg./ml.	No. Mitosis	Total No. Cells Counted	% Mitoses
Acronycine	25	Too cytotoxic	...	...
	10	28	712	3.9
	5	24	650	3.7
	1	14	496	2.8
	0.5	27	725	3.7
EtOH control	0.25%	16	422	3.8
VLB sulfate	0.5	120	725	16.6
Control	...	30	830	3.6

TABLE XIII.—AGAR-DIFFUSION PAPER-DISK TYPE ASSAYS OF ACRONYCINE ON ALGAE, PROTOZOA, AND MAMMALIAN CELLS

Concn., mcg./ml.[a]	System *C.v.*[b]	*S.b.*	*T.p.*	*O.m.*	*O.d.*	*E.g.*	*C.f.*	*L.c.*	HeLa	AV_2
2000	...	H, Tr	Tr	H, 20	H, 22	...	...	...	21	16
1000	...	H, Tr	Tr	H, 18	H, 21	...	...	...	18	15
500	...	...	...	H, 17	H, Tr	...	...	...	12	14
250	...	...	...	H, 15	H, Tr	...	...	...	10	Tr

[a] Figures represent mm. inhibitory zone diameter; ..., inactive; Tr, zone less than 10 mm.; H, hazy zone. [b] *C.v., Chlorella vulgaris; S.b., Scenedesmus basiliensis; T.p., Tetrahymena pyriformis; O.m., Ochromonas malhamensis; O.d., O. danica; E.g., Euglena gracilis; C.f., Clostridium feseri; L.c., Lactobacillus casei;* HeLa, human epidermoid cancer cells; AV_2, human amniotic cells.

of the compound and as an additional tool for future mechanism of action studies. Other workers have utilized *Tetrahymena* to study the effect of the phenothiazine tranquilizers (16) and the neuromuscular blocking action of some methonium compounds (17). This is not to say that these protozoa will detect all neurologically active agents—curare and leurocristine sulfate show no toxic effect upon motility of the organism. These motility studies represent a relatively simple method of observing the biological effect of a compound. The import of these observations depends upon possible correlation to the biological effects seen in drug-treated animals.

Dilute suspensions of viable *Tetrahymena* cells were prepared in particle-free saline. Acronycine was freshly dissolved in undenatured ethanol and added to the test suspension at zero time. The final drug concentration in the cell suspension was 50 mcg./ml., the final ethanol concentration being 1% (v/v). A similar volume of ethanol was added to the cell control. The sedimentation rate of the protozoa was measured by carefully inserting the counting stylus of the Coulter counter, an electronic particle counter, into the upper third of the suspension and counting the swimming cells at timed intervals. Rate of sedimentation would be reflected by the decreasing cell counts with time. Cell counts were obtained by utilizing three different threshold settings on the instrument to give a size distribution of the viable population at each counting interval. The protozoa were separated into three size groups: small, large, and very large, and the relative sedimentation rates were obtained for each group.

The line graph (Fig. 4) shows the profound toxic effect of acronycine upon the motility of the total cell population. The bar graphs (Fig. 5) illustrate the relative sedimentation rates of the sized cells. It is interesting to note that the rate and degree of cell immobilization is less with the very large cells than in the large and small groups. No firm explanation can be given for this phenomenon at this time.

SUMMARY

Acronycine has been shown to be a very potent alkaloid against a multiplicity of mouse neoplasms.

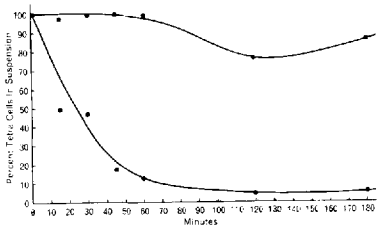


Fig. 4.—Tetrahymena motility studies. Key: upper curve with control c 1% EtOH; lower curve, acronycine, 50 mcg./ml.

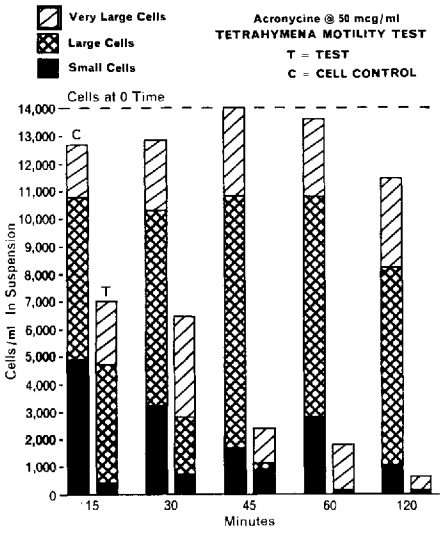


Fig. 5.—Tetrahymena motility test.

It possesses the broadest antitumor spectrum of any alkaloid isolated to date in these laboratories, being significantly active against 12 of 17 experimental neoplasms. Furthermore, it is not only effective when administered by a variety of routes, but it has demonstrated significant activity in delayed therapy experiments.

Unlike the alkaloids derived from *Catharanthus roseus* G. Don (*Vinca rosea* Linn.) (18), whose most striking experimental feature is their activity against the P-1534 leukemia in DBA/2 mice, acronycine is inactive against this neoplasm. Those systems of special interest which are responsive to this alkaloid are: the X-5563 plasma cell myeloma, a model system of multiple myeloma in man; the Shionogi carcinoma 115, an androgen-dependent tumor, potentially a model system for prostatic cancer; the C-1498 myelogenous leukemia which is nonresponsive to any of the clinically useful chemotherapeutic agents.

Other biological parameters include activity against certain protozoa and tissue culture activity against human epidermoid cancer cells and human amniotic cells. Acronycine has been shown to be an effective agent in blocking DMBA-induced adrenal apoplexy. Although some degree of leukopenia has been seen in certain experiments, the correlative weight loss associated with leukopenia was not in evidence.

No apparent mitotic arrest was seen in tissue culture studies utilizing either diploid Chinese hamster lung cells or the polyploid HeLa cells. This observation can be construed as lending credence to the theory that metaphase arrest is not a necessary function of an active oncolytic agent.

Acronycine, being an *N*-methyl acridone, possesses structural features unrelated to the *Catharanthus roseus* alkaloids or to any of the other presently known antitumor agents. Therefore, it represents a new lead toward chemotherapeutic management of a variety of neoplasms.

Studies are continuing in an attempt to ascertain as thoroughly as possible the character of the isolatable ingredients of this plant. The preparation of various derivatives and the determination of their biological activities, in an attempt to establish structure-activity relationships, are currently under way, and the results of these efforts will be reported at a later date.

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Biphasic Elimination of Noscapine

By MILO GIBALDI* and NORMAN D. WEINER

Published data concerning the elimination of noscapine from various species were critically re-examined. It would appear that noscapine is distributed into a third compartment prior to exponential elimination. An analogy between the pharmacodynamics of noscapine and the barbiturate, thiopental, is considered.

NOSCAPINE, an isoquinoline alkaloid of opium, is an antitussive agent which depresses the medullary centers and suppresses the cough reflex. Pharmacological and clinical investigations have confirmed the value of noscapine as a potent nonaddictive cough suppressant (1).

Despite the antiquity of this opium derivative, little is known concerning its absorption, distribution, and elimination in the body. In 1961, Vedsö (2) studied the absorption and excretion of noscapine in man. More recently, Nayak *et al.* (3) investigated the rate of metabolism, urinary excretion, and organ distribution of noscapine in laboratory animals.

Vedsö concluded that the rapid disappearance of noscapine from the blood is attributable to rapid and cumulative tissue uptake. Conversely, Nayak and co-workers claimed that cumulation of the drug in the various tissues does not occur. These investigators indicated that the rapid disappearance of noscapine from the plasma and tissues is due to a rapid, first-order biotransformation. A further consideration of the data of both Vedsö (2) and Nayak *et al.* (3) strongly suggests that the elimination of noscapine involves a more complex mechanism than those postulated in either study.

DISCUSSION

Representative log tissue concentration *versus* time plots of noscapine, after intravenous administration of drug to rats, are shown in Fig. 1. These plots were constructed by utilizing literature data (3). As may be observed in Fig. 1, the blood, kidney, and skeletal muscle data demonstrate linearity after a lag of about 8 min. It is, therefore, apparent that after 8 min., the drug is eliminated from both the tissues and blood in a first-order fashion. In accord with Teorell's model for drug distribution and elimination (4), the rate of elimination of noscapine from the blood is in good agreement with the elimination rate found in the tissues. The half-lives for the elimination of noscapine from the blood, kidney, and muscle were calculated by the method of least squares and were found to be 12, 13, and 15 min., respectively.

Rapid first-order elimination of noscapine has been observed in a number of species. Nayak *et al.* (3) report that the first-order disappearance of noscapine from the blood of both mice and rabbits has a half-life of about 9 min. Vedsö (2), who contended that biotransformation was not involved in the elimination of noscapine, did not attempt to quantify the elimination rate in man. However, his data provide sufficient evidence to indicate that the plasma concentration-time curve contains a postabsorptive semilogarithmic phase. Representative semilog plots are presented in Fig. 2. Contrary to Vedsö's conclusions, the elimination of a significant portion of the noscapine from human plasma follows first-order kinetics with a half-life, according to our calculations, of about 40 min.

Further examination of the plots presented in Fig. 1 reveals an interesting phenomenon. Each of the plots is biphasic, consisting of a very rapid initial elimination component and a significantly slower elimination component; the latter phase is observable about 8 min. after administration. Biphasic elimination of noscapine also appears to occur in the rat brain and liver (3). To interpret the significance of these phenomena it is relevant to consider the pharmacokinetics of distribution after intravenous administration.

Theoretical Considerations of Drug Distribution.—Numerous studies have shown that a semilog plot of plasma concentration *versus* time, after rapid intravenous injections of various substances, demonstrates linearity only after a certain time has elapsed (5). The initial rapid fall in blood concentration is generally attributable to the diffusion of drug from the blood to other body fluids and tissues. The start of the linear portion of the plot is considered to coincide with the steady state of distribution where the apparent volume of distribution has reached a maximum and, thereafter, remains constant. The duration of this initial phase of elimination from the plasma is a function of the drug properties. A highly water-soluble substance, restricted to the extracellular fluid, will rapidly attain steady-state conditions. Drugs which can penetrate to deeper tissues will generally show a more prolonged initial phase prior to exponential elimination.

After intravenous administration, in a two-compartment model system, tissue concentration increases continually to a maximum and, thereafter, declines in a first-order fashion at a rate equivalent to the rate of decline of drug in the plasma. According to the steady-state approximation, the time required for maximum tissue concentration coincides with the time lag before the initiation of exponential elimination of drug from the plasma (5, 6). Furthermore, in the idealized two-compartment system, the fraction of drug in each compartment is independent of amount of drug in the body. Thus, plasma concentration is directly proportional to the amount of drug in the body. For compounds which adhere to

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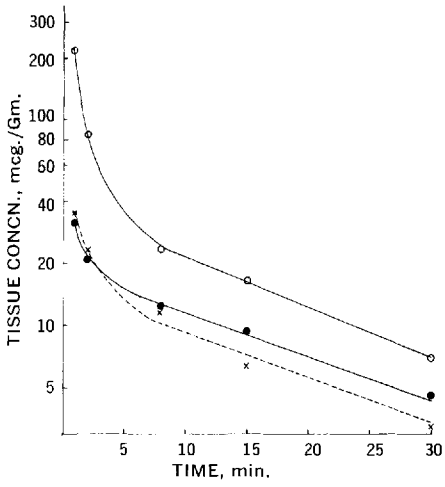

Fig. 1.—Semilog plots for the disappearance of noscapine from various tissues after intravenous administration of 25 mg./Kg. in rats. Plots were constructed with data taken from *Reference 3*. Key: O, kidney; ●, muscle; ×, blood.

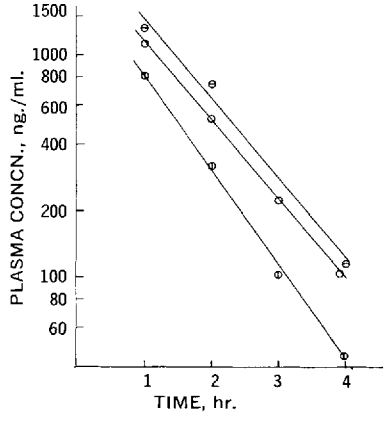

Fig. 2.—Semilog plots for the disappearance of noscapine from the plasma of three humans after oral administration of 250–300 mg. of noscapine chloride. Plots were constructed with data taken from *Reference 2*.

Teorell's model for distribution and elimination, a plot of plasma level, at time t, after intravenous administration *versus* dose, will be rectilinear (5).

Pharmacokinetic Interpretation of Noscapine Data.—Nayak *et al.* (3) state that there is a rapid disappearance of noscapine from the blood concomitant with a rapid uptake by organs perfused by a rich vascular supply. This is evidenced by the fact that drug concentration is maximal, within 1 min. after administration, in the brain, kidneys, lungs, liver, and skeletal muscle. However, as shown in Fig. 1, plasma concentration continues to decline rapidly until about 8 min. after administration. If uptake, by the tissues studied, was responsible for the very rapid initial elimination of noscapine from the plasma, drug concentration in each organ should be maximal at about 8 min. This is obviously not the case.

Although the various tissues studied constitute a compartment separate from the plasma, the rate of distribution between compartments is apparently so rapid that the defined system is kinetically indistinguishable from a one-compartment system.

Despite the fact that noscapine is very quickly distributed into a relatively large volume, it is evident from the data in Fig. 1 that, at the time of maximal tissue level, noscapine has not yet achieved its ultimate apparent volume of distribution. If the initial rapid loss of noscapine from the plasma is simply due to rapid uptake by the tissues studied, the steady-state approximation could be applied at the first minute, and each of the drug concentration-time plots in Fig. 1 could be described by a simple exponential expression. Quite to the contrary, each set of data in Fig. 1 was found to be fitted by an equation which is the sum of two exponentials. Using the method of least squares, the following equations were calculated:

$$C_B = 42e^{-0.80t} + 17e^{-0.057t} \quad \text{(Eq. 1)}$$

$$C_M = 50e^{-1.22t} + 18e^{-0.045t} \quad \text{(Eq. 2)}$$

$$C_K = 700e^{-1.33t} + 37e^{-0.055t} \quad \text{(Eq. 3)}$$

where C_B, C_M, and C_K refer to the concentrations of noscapine, at time t, in the blood, skeletal muscle, and kidneys, respectively.

At this point, it is reasonable to consider why such data are unusual. It is quite possible that many drugs really occupy a three-compartment model and distribution from the plasma into each compartment occurs at a somewhat different rate. Furthermore, sampling may be so spaced that the initial phase or phases of elimination from the plasma is not observed. For example, if Nayak *et al.* (3) had sampled at 10-min. intervals, only a single exponential elimination would be manifest. This interpretation of the noscapine data is in accord with Teorell's model but still leaves two unanswered questions.

First, since the pharmacokinetic evaluation has suggested the existence of a third compartment (real or hypothetical) what is the nature of this compartment? Second, as noted above, since a three-compartment model is not at variance with Teorell's model, one would expect a rectilinear relationship between plasma level and dose. However, as reported by Vedsö (2), noscapine plasma concentration, 2 hr. after oral administration of the drug, increased in a hyperbolic fashion as a function of dose (Fig. 3).

An explicit explanation for the pharmacokinetic behavior of noscapine is not possible without more extensive distribution and metabolism studies. However, the pharmacodynamic properties manifested by the barbiturate thiopental (7) are sufficiently analogous to consider in explaining the noscapine data.

Thiopental, like noscapine, is rapidly eliminated from the plasma and reaches maximal tissue levels in a number of organs, including the brain, liver, and muscle, almost immediately after administration. Nevertheless, plasma concentration continues to decline rapidly for a prolonged period of time after maximal tissue levels are achieved. Although drug concentration is maximal in a number of organs a few minutes after administration of thiopental, steady-state kinetics are not observed for a significant time thereafter (7). After a single 400-mg. dose of the

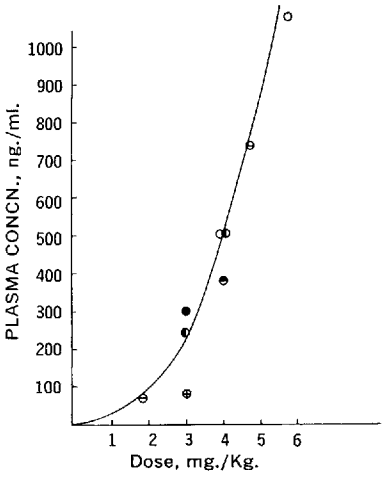

Fig. 3.—Relationship between dose of noscapine chloride and plasma concentration in various individuals 2 hr. after administration. (Taken from *Reference 2.*)

barbiturate to man, plasma levels fall abruptly and subjects awaken in about 10 min. Subsequently, the plasma level declines slowly, 10–15%/hr. (8). This rate reflects the true rate of metabolism of thiopental.

Studies designed to determine the movement of thiopental between plasma and tissue served to clarify this pharmacodynamic phenomenon. After intravenous administration, most tissue, including brain, rapidly take up considerable amounts of thiopental; the tissue levels then decline in parallel with the plasma level. In contrast, the level in fat, initially quite low, rises slowly, and approaches a peak in about 3 hr., when it is then 10 times higher than the plasma level.

The application of the thiopental distribution model to the noscapine data provides a near-perfect explanation of the results, but must remain speculative in the absence of experimental data of noscapine concentrations in adipose tissue. The organs selected by Nayak *et al.* did not include the adrenals or carcass, organs which would provide insight to drug deposition into a fat compartment.

Nevertheless, other analogies existing between noscapine and thiopental provide further support for the proposed hypothesis. The importance of lipid solubility in defining the pharmacological action of thiobarbiturates is emphasized by a comparison of thiopental and pentobarbital, its oxygen analog. Both drugs are distributed to almost the same extent in most tissues, but the concentration of pentobarbital is much less than that of thiopental in adipose tissue because of the decreased lipid solubility of the oxygen analog (8).

Noscapine, like thiopental, is a highly lipid soluble drug (9). An excellent indication of the comparable lipid characteristics of these drugs is provided by a consideration of transport across the blood-brain boundary, a process which is quite dependent on the lipid solubility of the permeating molecule. On the basis of distribution studies, both thiopental and noscapine show rate constants for entry into the central nervous system of about 0.8–1.0 min.$^{-1}$, significantly higher than rate constants observed for such drugs as pentobarbital (about 0.2 min.$^{-1}$), barbital (about 0.05 min.$^{-1}$), and salicylic acid (0.006 min.$^{-1}$) (3, 10). In view of the similar properties of noscapine and thiopental, it is not surprising that both drugs act in the central nervous system, and both show extremely prompt, but very fleeting, therapeutic activity.

Unfortunately, one piece of data does not fit well into the over-all scheme. Vedsö (2) has claimed the existence of a hyperbolic relationship between plasma level of noscapine and dose. Indirect evidence based on repetitive dosing leads one to believe that the hyperbolic relationship does not exist in the case of thiopental. For example, a dog can be anesthetized with a given dose of thiopental, then be allowed to recover, and this procedure can be repeated a number of times without having to reduce the dose to obtain the same depth of anesthesia. Each time, the recovery of the animal is due almost entirely to localization in fat, which acts as a seemingly infinite reservoir (7).

The third compartment proposed for noscapine, be it a fat compartment or not, does not share the ability of the fat compartment for thiopental, to function as a sink. Whether this difference is qualitative or simply a quantitative distinction remains to be determined.

The proposed model, based on fat distribution, is by no means the only possible way to explain the pharmacodynamics of noscapine. There are a number of other models which may be proposed to account for the data (11), including other compartments and complex biotransformation processes. Although these other models are mathematically correct, they do not appear to be as reasonable from physiologic and pharmacologic viewpoints.

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Theoretical Analysis of Comparative Studies of Complex Formation

Solubility, Spectral, and Kinetic Techniques

By KENNETH A. CONNORS and JOSEPH A. MOLLICA, JR.

The stoichiometric ratios and the equilibrium constants describing the extent of formation of complexes are the basic information required in studies of complex formation between two species. Some of this information can be obtained by measuring the apparent solubility, the absorption spectrum, or the reactivity of one species (the substrate) as a function of the concentration of the other species (the ligand). The stability constant evaluated by these methods, assuming that a single complex of 1 : 1 substrate–ligand ratio is present, can be related to the actual constants of the system. Analyses of some of the complex systems most likely to be encountered show that the three experimental approaches may not always yield the same numerical result and that comparative studies with several techniques may yield valuable information concerning the natures of the complexes. The solubility, spectral, and kinetic methods for studying complexes are, in general, subject to about the same degree of nonselectivity in their responses to multiple complexes and interactions.

THE CONCEPT of complex formation has been adopted as a simple hypothesis that can account for nonadditive behavior in the physical and chemical properties of solutions of two or more species. With this hypothesis it becomes possible to utilize quantitative measures of these properties to describe the extent of interaction between the species and to investigate the nature of the interaction product, or complex. Many definitions of "a complex" have been proposed, but for the purpose of this paper it will not be necessary to limit sharply the chemical nature of the species, and the techniques to be discussed may be applicable to the study of reactions that may not be accepted as complexation reactions. Throughout this paper complex formation is considered to be a reversible chemical reaction in which the rate of attainment of equilibrium is much greater than any rates involved in the measuring processes. The system is, therefore, considered to be at equilibrium.

The basic purpose of studies of complex formation is to provide a comprehension of the properties of complexes, including their structure and reactivity. Since the reversibility of complex formation is the fundamental aspect relating all of these processes, the general reaction may be written

$$mS + nL \rightleftharpoons S_mL_n$$

where S represents the substrate and L is the ligand. (The substrate is the compound whose apparent properties are measured.) Given adequate evidence that a complex is present in a system, the first information to be sought is its stoichiometry, *i.e.*, the values of m and n. It is probable that in many (perhaps most) systems more than one complex is formed, and the stoichiometries of all species are desired. Note also that it is entirely conceivable that two or more complexes may co-exist with the same stoichiometry but different structures (1). (A single complex species will possess a unique average molecular and electronic configuration.)

The strength or stability of a complex is specified in terms of its stability (association, formation) constant. The over-all stability constant K_{mn} for the complex formation reaction is written

$$K_{mn} = \frac{[S_mL_n]}{[S]^m[L^n]} \qquad \text{(Eq. 1)}$$

where brackets signify molar concentrations, and K_{mn} is the constant applicable to the solvent system and temperature employed. The standard state of the solute is taken to be the infinitely dilute solution in the experimental solvent. Often the concentrations of S and L are sufficiently low that they do not affect the value of the stability constant. An alternative description of the stability of S_mL_n is available in the step stability constant; the assumption is that S_mL_n is formed from $S_mL_{(n-1)}$ by reaction with one L, or from $S_{(m-1)}L_n$ by reaction with one S. Rossotti and Rossotti (2) review methods for the determination of stability constants.

Most of the molecules of pharmaceutical and biological interest are of complicated structure and contain numerous functional groupings. Such molecules will, therefore, possess multiple interaction sites for complex formation. A given system of substrate, ligand, and solvent can be described as a member of one of the following two classes.

(*a*) Only one complex species is formed. This

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complex may be a resultant of multiple interactions. The possibility is admitted that the complex contains only substrate or only ligand molecules.

(*b*) Two or more complex species are formed. The several complexes may be formed by means of different types of interaction forces or by the same interactions differently oriented.

In the studies of complexation equilibria and their relation to enzyme specificity behavior from these laboratories, solubility measurements, absorption spectroscopy, and rate measurements for the determination of complex stability have been employed. These are all well-known techniques, but few investigators have systematically applied more than one of them to a complexation system (3–5). The three methods do not always yield the same numerical result (taking into account the expected experimental uncertainty), and the authors' analysis of these differences may be of value to others.

The usual procedure is to assume that a single complex of one-to-one stoichiometry is responsible for the observed effects. If the data suggest that this simple assumption is untenable, another will of course be made in its place, but the observations are not always susceptible to such an interpretation. The problem, therefore, is to find the relationship between the apparent 1:1 stability constant (as evaluated by each of the experimental methods) and the actual parameters of the system. This analysis will be carried out for each technique as applied to the systems most likely to be encountered. A comparison of the three methods will then be given.

ONE COMPLEX PRESENT

Solubility Method.—*1:1 Complex.*—The theory and practice of the solubility method have recently been reviewed in detail (6), and only a brief outline will be given here. The experimental operation entails the addition of an equal weight (in excess of its normal solubility) of the slightly soluble substrate into each of several vials. A constant amount of solvent is added to each container, then successively increasing portions of the relatively soluble ligand are added to these vessels, which are closed and brought to solubility equilibrium at constant temperature. The solution phases are analyzed for their total concentration of S, no matter what its molecular state may be. A phase diagram is constructed by plotting, on the vertical axis, the total molar concentration of S found in the solution, S_t, against the total molar concentration of L added, L_t. Here only the formation of soluble complexes is considered; these produce a phase diagram consisting of a smooth curve with a positive slope. In general, if the solution contains but one complex, S_mL_n, the concentrations at any point on the curve can be expressed (see *Appendix* for an explanation of the symbols):

$$\begin{aligned} [S] &= S_0 \\ [S_mL_n] &= (S_t - S_0)/m \\ [L] &= L_t - n[S_mL_n] \end{aligned} \qquad \text{(Eq. 2)}$$

since the concentration of free S is maintained constant by the presence of solid substrate. Consider the case in which $m = n = 1$. Then Eq. 1 for K_{11} is combined with Eq. 2 to give

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0} + S_0 \qquad \text{(Eq. 3)}$$

showing that the plot of S_t *versus* L_t is a straight line with intercept S_0 on the vertical axis; the slope of this line is $K_{11}S_0/(1 + K_{11}S_0)$, leading to

$$K_{11} = \frac{\text{slope}}{S_0(1 - \text{slope})}$$

In general, the apparent 1:1 stability constant is evaluated from solubility measurements by means of Eq. 4; if a single 1:1 complex is present, the apparent K_{11} (symbolized K'_{11}) determined in this manner is equal[1] to the actual K_{11}.

$$K_{11}' = \frac{\text{slope}}{\text{intercept}\,(1 - \text{slope})} \qquad \text{(Eq. 4)}$$

2:1 Complex.—If, more generally, $n = 1$ but m assumes any value, Eqs. 1 and 2 give

$$S_t = \frac{mK_{m1}S_0{}^mL_t}{1 + K_{m1}S_0{}^m} + S_0 \qquad \text{(Eq. 5)}$$

The phase diagram is linear as long as the complex contains only 1 molecule of L. If the slope is greater than unity, then at least one species must be present in which m is greater than 1, for it is clearly impossible for 1 mole of L to take more than 1 mole of S into solution if the complex is of the 1:1 type. On the other hand, a slope smaller than 1 does not necessarily mean that a 1:1 complex is formed, though this assumption is usually made. More definite statements concerning the order with respect to S cannot usually be made since the presence of solid substrate is responsible for maintaining a constant activity of S in the system.

If a single 2:1 complex is present, the slope is given by Eq. 6.

$$\text{slope} = \frac{2K_{21}S_0{}^2}{1 + K_{21}S_0{}^2} \qquad \text{(Eq. 6)}$$

If this quantity is less than 1, the system will be interpreted as a probable 1:1 complex, and an apparent K'_{11} will be calculated from Eq. 4. The actual nature of this quantity is found by combining Eqs. 4 and 6,

$$K_{11}' = \frac{2K_{21}S_0}{1 - K_{21}S_0{}^2} \qquad \text{(Eq. 7)}$$

where K_{11}' is the apparent 1:1 stability constant. (If the true stoichiometry were known, it would be a simple matter to evaluate the correct constant, K_{21}, but this information will seldom be available.)

This discussion has ignored the route of formation of S_2L. This can conceivably occur in three ways: $2S + L = S_2L$; $S_2 + L = S_2L$; $SL + S = S_2L$. The first of these has been employed in the preceding discussion. The other possibilities require the presence of another complex, and can be treated with methods developed later for these more complicated systems.

1:2 Complex.—When a complex is present that is second-order in L, the solubility diagram will not be linear but will show a positive curvature (6). Such a curvature would be recognized and would prevent evaluation of an apparent K_{11}'. If, however, the system contains both 1:1 and 1:2 com-

[1] This is not exactly true, of course, for the general solvent effect of S and L on the constant has been neglected; these relatively minor effects may be responsible for small differences, but can be ignored as long as S_t and L_t remain fairly small. The "statistical" or "contact" complexes formed as a result of random distribution of the molecules have also been neglected (7, 8).

plexes, the deviation from linearity may be unnoticed and misinterpretation may result. This system will be analyzed in a later section.

Substrate Dimer.—Suppose S undergoes reaction to form the dimer S_2 with dimerization constant K_{SS}. Then the total concentration $S_t = [S] + 2[S_2]$, or $S_t = S_0 + 2K_{SS}S_0^2$. Obviously the apparent complexation constant evaluated from the phase diagram will be zero, but the intercept will give $S_0 + 2K_{SS}S_0^2$ rather than S_0. No solubility experiment will reveal this anomaly, however.

Spectral Method.—*1:1 Complex.*—If the molar absorptivities of the complex and the substrate differ at the same wavelength, it may be possible to determine the stability constant spectrophotometrically. It is assumed that Beer's law is followed by all species. Then at a concentration S_t of substrate, in the absence of ligand, the solution absorbance is

$$A_0 = a_S b S_t \qquad \text{(Eq. 8)}$$

In the presence of ligand at total concentration L_t, the absorbance of the solution containing the same total substrate concentration is

$$A_L = a_S b[S] + a_L b[L] + a_{11} b[SL]$$

which, combined with the material balance on S, gives

$$A_L = a_S b S_t + a_L b L_t + \Delta a b[SL]$$

where $\Delta a = a_{11} - a_S - a_L$. By measuring the solution absorbance against a reference containing ligand at concentration L_t, the measured absorbance becomes

$$A_L' = a_S b S_t + \Delta\, ab[SL] \qquad \text{(Eq. 9)}$$

Combining Eqs. 8 and 9 with the stability constant definition leads to

$$\Delta A/b = K_{11}\Delta a[S][L]$$

where $\Delta A = A_L' - A_0$. Utilizing the expression $[S] = S_t/(1 + K_{11}[L])$, this becomes

$$\Delta A/b = \frac{K_{11}S_t\Delta a[L]}{1 + K_{11}[L]} \qquad \text{(Eq. 10)}$$

This equation can be put into several linear forms, one of these being Eq. 11, which is similar to the equation used by Benesi and Hildebrand (9, 10) to determine stability constants spectrophotometrically.

$$b/\Delta A = 1/K_{11}S_t\Delta a[L] + 1/S_t\Delta a \qquad \text{(Eq. 11)}$$

If [L] can be approximated by L_t, a plot of $b/\Delta A$ *versus* $1/L_t$ will be linear. The stability constant K_{11} is taken as the ratio *intercept/slope* of this plot. This is the operational definition of the spectrally measured 1:1 stability constant. Note that the types of interaction or their distribution in the complex are irrelevant, the only requirement in the application of the method being that Δa is not equal to zero.

No approximations have been introduced in the derivation of Eq. 11, but it is necessary to assume $[L] = L_t$ in its application. This assumption is equivalent to supposing that $1 + K_{11}[S] = 1$, because of the relationship $L_t = [L](1 + K_{11}[S])$. The assumed equality $[L] = L_t$ is, therefore, sensitive to the magnitude of the stability constant and to the substrate concentration. If K_{11} is quite large, it is essential to hold S_t to a small value if Eq. 11 is to be applied.

As noted above, Eq. 11 is exact, but its use requires an approximation. It is possible to introduce the approximation during the derivation (2), leading to the equation

$$bL_t/\Delta A = (S_t + L_t)/\Delta a S_t + 1/\Delta a K_{11}S_t$$

It can be shown that the application of this equation requires conditions that are similar to those adopted in the use of Eq. 11. Throughout this paper the spectral method will be discussed in terms of Eq. 11.

2:1 Complex.—In this system the equation corresponding to Eq. 9 is written

$$A_L' = a_S b S_t + \Delta ab[S_2L]$$

where $\Delta a = a_{21} - 2a_S - a_L$. The concentration of free substrate is given by

$$[S] = \frac{S_t}{1 + 2K_{21}[S][L]}$$

The resulting equation in its reciprocal form is

$$b/\Delta A = 1/K_{21}S_t\Delta a[S][L] + 2/S_t\Delta a \qquad \text{(Eq. 12)}$$

so the apparent spectral constant is

$$K_{11}' = 2K_{21}[S] \qquad \text{(Eq. 13)}$$

Note, however, that the plot should not theoretically be linear, since the slope is a function of [S]. This complication will be treated in more detail in later sections dealing with multiple complexes.

Substrate Dimer.—If S dimerizes with a change in spectrum, the quantity ΔA will be independent of L_t. The apparent stability constant will be zero. Beer's law will not be followed by the substrate if the dimer's molar absorptivity is not twice that of the monomer.

Kinetic Method.—*1:1 Complex.*—The kinetic method, as it has been most frequently applied, utilizes a reduction in rate of a reaction of S when L is present to obtain information about the nature of the complex; the basic assumption is that the decreased reactivity is the result of complexation, the complexed S being less reactive than free S. The kinetic scheme can be represented

$$S + L \overset{K_{11}}{\rightleftharpoons} SL$$

$$S + R \overset{k_S}{\rightarrow} \text{products}$$

$$SL + R \overset{k_{11}}{\rightarrow} \text{products}$$

If, as is usually the case, a reagent R is involved in the reaction, k_S is the second-order rate constant (often determined under pseudo first-order conditions with reagent in excess). It is assumed that R does not form complexes with S or L. The theoretical rate equation is

$$v = k_S[S][R] + k_{11}[SL][R] \qquad \text{(Eq. 14)}$$

and the experimental rate equation is

$$v = k_{obs.}S_t \qquad \text{(Eq. 15)}$$

where $k_{obs.}$ is the pseudo first-order rate constant. Setting Eqs. 14 and 15 equal and dividing through by [R] and S_t,

$$k_S' = k_S f_S + k_{11} f_{11} \qquad \text{(Eq. 16)}$$

where k_S' is the apparent second-order rate constant, f_S is the fraction of S in the uncomplexed form, and f_{11} is the fraction present as SL. The stability constant K_{11} is combined with the definitions of these fractions, giving Eq. 17.

$$f_S = \frac{1}{1 + K_{11}[L]} \qquad f_{11} = \frac{K_{11}[L]}{1 + K_{11}[L]} \qquad \text{(Eq. 17)}$$

In the special case that $k_{11} = 0$, Eq. 16 can be expressed in the forms Eqs. 18 and 19.

$$k_S' = k_S f_S \qquad \text{(Eq. 18)}$$

$$k'_S/k'_S = K_{11}[L] + 1 \qquad \text{(Eq. 19)}$$

According to Eq. 18, a plot of k_S' *versus* f_S is linear, passing through the origin, with slope k_S. Prior knowledge of K_{11} is required to calculate f_S. Equation 19, however, can be plotted without this knowledge if the equality $[L] = L_t$ may be made. Then the slope of the plot of k_S/k_S' *versus* L_t gives K_{11}.

If $k_{11} \neq 0$, the general Eq. 16 must be used. Since $f_S + f_{11} = 1$, this can be written

$$k_S - k_S' = f_{11}(k_S - k_{11}) \qquad \text{(Eq. 20)}$$

Introducing the definitions $r_{11} = k_{11}/k_S$ and $q_{11} = 1 - r_{11}$ permits Eq. 20 to be transformed to Eq. 21.

$$k_S - k_S' = q_{11} k_S f_{11} \qquad \text{(Eq. 21)}$$

or

$$k_S - k_S' = \frac{q_{11} k_S K_{11}[L]}{1 + K_{11}[L]} \qquad \text{(Eq. 22)}$$

Equation 22 can be placed in the following three forms amenable to linear graphing:

$$\frac{1}{k_S - k_S'} = \frac{1}{q_{11} k_S K_{11}[L]} + \frac{1}{q_{11} k_S} \qquad \text{(Eq. 23)}$$

$$\frac{[L]}{k_S - k_S'} = \frac{[L]}{q_{11} k_S} + \frac{1}{q_{11} k_S K_{11}} \qquad \text{(Eq. 24)}$$

$$\frac{k_S - k_S'}{[L]} = -K_{11}(k_S - k_S') + q_{11} k_S K_{11} \qquad \text{(Eq. 25)}$$

Throughout this paper the kinetic method will be treated in terms of Eq. 23, which predicts that a plot of $1/(k_S - k_S')$, or of $k_S/(k_S - k_S')$, *versus* $1/[L]$ should be linear. The kinetically determined 1:1 stability constant is then defined as the ratio *intercept/slope* of this plot. From the intercept value the quantity q_{11}, and ultimately k_{11}, can be evaluated.

Equation 19 has frequently been employed for the estimation of stability constants from rate measurements. This procedure is not recommended, however, for the reason made evident in Fig. 1. In this figure Eq. 19 is plotted for three hypothetical systems having q_{11} values of 0.5, 0.9, and 1.0; in each instance the true $K_{11} = 25.0\ M^{-1}$. Only the topmost line should be straight, since Eq. 19 is valid only when $q_{11} = 1.0$ and in fact the other lines do exhibit a slight negative curvature. But if these were experimental points based on ordinary rate data, rather than calculated theoretical points, it is probable that these curves would be interpreted as straight lines.[2] The slopes of these lines, which are

[2] As K_{11} is made larger, the curvature in these plots becomes more evident.

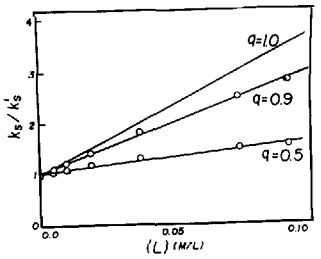

Fig. 1.—Plots of Eq. 19 for systems containing a single 1:1 complex with stability constant $K_{11} = 25.0\ M^{-1}$ and the q values shown.

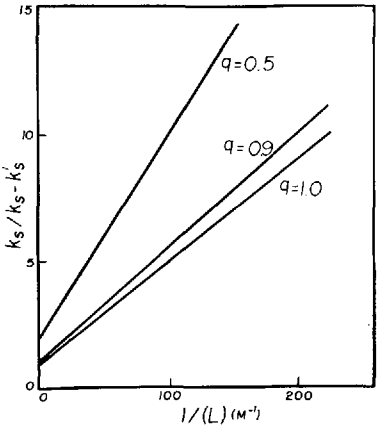

Fig. 2.—Plots of the data shown in Fig. 1 according to Eq. 23.

the apparent 1:1 stability constants according to Eq. 19, are 25 (for $q_{11} = 1.0$), 18 (for $q_{11} = 0.9$), and 5.4 (for $q_{11} = 0.5$). It is suggested that Eqs. 23, 24, or 25 be used in analyzing kinetic data. The same data plotted in Fig. 1 have been replotted in Fig. 2 according to Eq. 23; the apparent 1:1 stability constant is 25 M^{-1} in each case.

2:1 Complex.—In dealing with this system it becomes necessary to take into account various possible fates of the complex S_2L. It may undergo reaction with R to give products from one S molecule, releasing the other unreacted, or both S molecules may react, or 2 molecules of R may be required, etc. The simplest assumption will be adopted, that $k_{21} = 0$, recognizing that this places a limit on the applicability of the result. Then the basic equation, corresponding to Eq. 16, is

$$k_S' = k_S f_S \qquad \text{(Eq. 26)}$$

The fraction $f_S = [S]/S_t$, while $S_t = [S] + 2[S_2L]$. Combining these equations with the definition of K_{21} gives

$$f_S = 1/(1 + 2K_{21}[S][L])$$

which, with Eq. 26, leads to

$$k_S' = k_S/(1 + 2K_{21}[S][L]) \qquad \text{(Eq. 27)}$$

Equation 27 shows that k_S', at a given ligand concentration, is a function of substrate concentration; in other words, the apparent rate constant will vary with time as the reaction proceeds. If measurements

are made for only a small portion of the total reaction time, it is quite possible (taking into account ordinary experimental uncertainties) to overlook the variability of k_S' and to interpret the system as belonging to the 1:1 class. Equation 27 can be converted to the usual plotting form:

$$1/(k_S - k_S') = 1/2k_S K_{21}[S][L] + 1/k_S \quad \text{(Eq. 28)}$$

The kinetically evaluated K_{11}' is equal to the ratio *intercept/slope*, or

$$K_{11}' = 2K_{21}[S] \quad \text{(Eq. 29)}$$

Substrate Dimer.—If S dimerizes, and the dimeric form is essentially unreactive, the apparent constant k_S' will be related to the substrate concentration by the equation $k_S' = k_S(1 + 2K_{SS}[S])$. The apparent constant will, therefore, vary with time during a reaction. Since k_S' is not dependent upon the ligand concentration, however, the apparent 1:1 stability constant will be zero.

TWO COMPLEXES PRESENT

Solubility Method.—*Two 1:1 Complexes.*—It is possible that two complexes of 1:1 stoichiometry but different structures may co-exist. If one distinguishes between these by representing them as SL and LS, the solubility conservation equations may be written

$$S_0 = [S]$$

$$S_t = [S] + [SL] + [LS]$$

$$L_t = [L] + [SL] + [LS]$$

These are combined with the stability constants to give

$$S_t = \frac{(K_{SL} + K_{LS})S_0L_t}{1 + (K_{SL} + L_{LS})S_0} + S_0 \quad \text{(Eq. 30)}$$

which has the same form as Eq. 3 for a single 1:1 complex. Applying Eq. 4 shows that

$$K_{11}' = K_{SL} + K_{LS} \quad \text{(Eq. 31)}$$

Thus the apparent 1:1 stability constant evaluated by solubility measurements gives the sum of the individual constants. This can be generalized to any number of 1:1 complexes. Note that the slope of the phase diagram cannot exceed unity as long as only 1:1 complexes are present.

1:1 and 2:1 Complexes.—The step stability constants are defined as

$$K_{11} = [SL]/[S][L]$$

$$K_{(21)} = [S_2L]/[S][SL]$$

The development follows the lines already indicated. The equation of the phase diagram is

$$S_t = \left[\frac{K_{11}S_0 + 2K_{11}K_{(21)}S_0^2}{1 + K_{11}S_0 + K_{11}K_{(21)}S_0^2}\right] L_t + S_0 \quad \text{(Eq. 32)}$$

Combining the slope of this plot with Eq. 4:

$$K_{11}' = \frac{K_{11} + 2K_{11}K_{(21)}S_0}{1 - K_{11}K_{(21)}S_0^2} \quad \text{(Eq. 33)}$$

That a mathematically equivalent expression would be obtained if the over-all stability constant had been employed can be seen from the equality $K_{21} = K_{11}K_{(21)}$.

1:1 and 1:2 Complexes.—Combining the step constants K_{11} and $K_{(12)}$ with the material balance equations gives

$$S_t = \left[\frac{K_{11}S_0 + K_{11}K_{(12)}S_0[L]}{1 + K_{11}S_0 + 2K_{11}K_{(12)}S_0[L]}\right] L_t + S_0 \quad \text{(Eq. 34)}$$

which, with Eq. 4, leads to

$$K_{11}' = \frac{K_{11} + K_{11}K_{(12)}[L]}{1 + K_{11}K_{(12)}S_0[L]} \quad \text{(Eq. 35)}$$

According to Eq. 34 the phase diagram should show a positive curvature; but if the 1:1 complexing is much more extensive than the 1:2 type, this nonlinearity may not be noticed. Methods are available to analyze this system, when it is recognized, to obtain the individual stability constants (6).

1:1 Complex and Ligand Dimer.—The equation of the phase diagram is easily developed as before:

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0 + 2K_{LL}[L]} + S_0 \quad \text{(Eq. 36)}$$

where $K_{LL} = [L_2]/[L]^2$. The phase diagram will exhibit a negative curvature, but if the curve is mistaken for a straight line the apparent 1:1 stability constant that will be evaluated is given by Eq. 37.

$$K_{11}' = \frac{K_{11}}{1 + 2K_{LL}[L]} \quad \text{(Eq. 37)}$$

1:1 Complex and Substrate Dimer.—In this case the equation of the phase solubility diagram is

$$S_t = \frac{K_{11}S_0L_t}{1 + K_{11}S_0} + S_0 + 2K_{SS}S_0^2$$

The slope of the straight line is the same as that which would be observed in the absence of dimer formation, but the intercept is different. The apparent constant is

$$K_{11}' = \frac{K_{11}}{1 + 2K_{SS}S_0} \quad \text{(Eq. 38)}$$

Spectral Method.—*Two 1:1 Complexes.*—If the two complexes SL and LS are formed and at least one of them possesses a molar absorptivity different from free S, a spectral change will be observed. The analysis follows that given for a single 1:1 complex. The concentration of free substrate is related to the other system variables by Eq. 39.

$$[S] = \frac{S_t}{1 + (K_{SL} + K_{LS})[L]} \quad \text{(Eq. 39)}$$

The reciprocal form of the equation for this system is

$$b/\Delta A = \frac{1}{S_t(K_{SL}\Delta a_{SL} + K_{LS}\Delta a_{LS})[L]} + \frac{K_{SL} + K_{LS}}{S_t(K_{SL}\Delta a_{SL} + K_{LS}\Delta a_{LS})} \quad \text{(Eq. 40)}$$

(where $\Delta a_{SL} = a_{SL} - a_S - a_L$ and $\Delta a_{LS} = a_{LS} - a_S - a_L$), showing that the apparent 1:1 stability constant is given by

$$K_{11}' = K_{SL} + K_{LS} \quad \text{(Eq. 41)}$$

This result has been pointed out by several authors (1). Even if one of the complexes has an absorption spectrum identical with that of the free substrate,

K_{11}' will be given by Eq. 41. This may be intuitively pictured as the result of a depletion of free S by formation of this second complex, even though it is not spectrally distinctive.

1:1 and 2:1 Complexes.—This system should not, theoretically, yield a linear reciprocal plot; yet, as Johnson and Bowen have shown, the experimental plots may well appear to be linear (11). The analysis may be conducted as in earlier examples, leading to

$$\Delta A/b = K_{11}[S][L](\Delta a + \Delta a' K_{(21)}[S])$$

where $\Delta a = a_{11} - a_S - a_L$ and $\Delta a' = a_{21} - 2a_S - a_L$. For the present purpose the substrate concentration is written as

$$[S] = \frac{S_t}{1 + K_{11}[L](1 + 2K_{(21)}[S])}$$

These equations are combined to give

$$b/\Delta A = \frac{1}{K_{11}S_t(\Delta a + \Delta a' K_{(21)}[S])[L]} + \frac{1 + 2K_{(21)}[S]}{S_t(\Delta a + \Delta a' K_{(21)}[S])} \quad \text{(Eq. 42)}$$

from which it is seen that the apparent spectrally measured constant is

$$K_{11}' = K_{11} + 2K_{11}K_{(21)}[S] \quad \text{(Eq. 43)}$$

This conclusion is not always valid, however; a fuller discussion is given under the next system.

1:1 and 1:2 Complexes.—Again it is evident that the plot should be nonlinear, but Johnson and Bowen (11) have found that the curvature may be overlooked. The development of the appropriate equation is similar to that in the preceding example; the equation is

$$b/\Delta A = \frac{1}{K_{11}S_t(\Delta a_{11} + \Delta a_{12}K_{(12)}[L])[L]} + \frac{1 + K_{(12)}[L]}{S_t(\Delta a_{11} + \Delta a_{12}K_{(12)}[L])} \quad \text{(Eq. 44)}$$

where $\Delta a_{11} = a_{11} - a_S - a_L$ and $\Delta a_{12} = a_{12} - a_S - a_L$. The apparent stability constant is

$$K_{11}' = K_{11} + K_{11}K_{(12)}[L] \quad \text{(Eq. 45)}$$

That Eq. 44 is not the equation of a straight line must be kept in mind, however, and it may be expected that the range of ligand concentration over which the system is studied may affect the results. Suppose the ligand concentration is made very large, so that $\Delta a_{12}K_{(12)}[L] \gg \Delta a_{11}$; then Eq. 44 becomes

$$b/\Delta A = 1/\Delta a_{12}K_{11}K_{(12)}[L]^2 + 1/\Delta a_{12}S_t$$

and the apparent constant is

$$K_{11}' = K_{11}K_{(12)}[L] \quad \text{(Eq. 46)}$$

The necessary condition for Eq. 46 to be approached is a function not only of [L], but also of the quantities Δa_{11}, Δa_{12}, and $K_{(12)}$. This conclusion agrees with the calculations of Johnson and Bowen (11), who designed hypothetical systems to demonstrate these effects. (These remarks apply also to the system described by Eq. 42, which is, however, not as sensitive to these effects because [S] is usually much smaller than [L].) Thus, the apparent stability constant may vary with the wavelength at which the absorbance measurements are made.

1:1 Complex and Ligand Dimer.—This situation is made rather complicated because the assumption that $L_t = [L]$, made hitherto in the spectral analysis, is not valid; a very appreciable fraction of the uncomplexed ligand may exist as the dimer. This system does not appear to be amenable to a useful treatment according to the manner of the earlier examples.

1:1 Complex and Substrate Dimer.—This system does not lead to a useful analysis. The extent of dimerization will depend upon the ligand concentration (unlike the case, discussed earlier, where S_2 was the only complex present) because the free substrate concentration is a function of ligand concentration.

Kinetic Method.—*Two 1:1 Complexes.*—Proceeding as for a single complex, this basic equation is obtained

$$k_S' = k_S f_S + k_{SL} f_{SL} + k_{LS} f_{LS} \quad \text{(Eq. 47)}$$

which, since $f_S + f_{SL} + f_{LS} = 1$, leads to

$$k_S - k_S' = q_{SL}k_S f_{SL} + q_{LS}k_S f_{LS} \quad \text{(Eq. 48)}$$

where the q's are defined as before. Expressions for the fractional compositions are found by combination of the material balance and stability constant equations,

$$f_{SL} = \frac{K_{SL}[L]}{1 + K[L]} \qquad f_{LS} = \frac{K_{LS}[L]}{1 + K[L]}$$

where $K = K_{SL} + K_{LS}$. Substituting these into Eq. 48 and rearranging gives the linear form

$$\frac{1}{k_S - k_S'} = \frac{1}{(q_{SL}K_{SL} + q_{LS}K_{LS})[L]} + \frac{K_{SL} + K_{LS}}{(q_{SL}K_{SL} + q_{LS}K_{LS})} \quad \text{(Eq. 49)}$$

showing that K_{11}' is given by Eq. 50.

$$K_{11}' = K_{SL} + K_{LS} \quad \text{(Eq. 50)}$$

This result will be obtained even if one of the complexes has a reactivity equal to that of the free substrate (*i.e.*, if one of the q's equals zero).

1:1 and 2:1 Complexes.—Suppose that the S_2L complex is unreactive. Then the basic equation of the system is

$$k_S' = k_S f_S + k_{11} f_{11}$$

But the fractions f_S and f_{11} are functions of [S], so k_S' will vary during the course of the reaction, as pointed out in connection with Eq. 27. If this variability should not be evident, the usual kinetic treatment will be made. The above equation is transformed into

$$k_S - k_S' = k_S(q_{11}f_{11} + f_{21})$$

where $f_{11} = [SL]/S_t$ and $f_{21} = 2[S_2L]/S_t$. This leads finally to Eq. 51.

$$\frac{1}{k_S - k_S'} = \frac{1}{k_S K_{11}[L](q_{11} + 2K_{(21)}[S])} + \frac{1 + 2K_{(21)}[S]}{(q_{11} + 2K_{(21)}[S])k_S} \quad \text{(Eq. 51)}$$

This is not the equation of a straight line, but, if

k_S' appears to be constant during a reaction (perhaps because only a few per cent of total substrate is allowed to react during the observation period), then Eq. 51 will be essentially linear. The apparent stability constant will be

$$K_{11}' = K_{11} + 2K_{11}K_{(21)}[S] \qquad \text{(Eq. 52)}$$

If, however, $2K_{(21)}[S] \gg 1$, the apparent constant will be $K_{11}' = 2K_{11}K_{(21)}[S]$.

1:1 and 1:2 Complexes.—The basic equation is

$$k_S' = k_S f_S + k_{11} f_{11} + k_{12} f_{12} \qquad \text{(Eq. 53)}$$

which can be transformed to

$$k_S - k_S' = q_{11} k_S f_{11} + q_{12} k_S f_{12}$$

By means of the stability constant definitions and the material balance on S_t, this is converted to

$$\frac{1}{k_S - k_S'} = \frac{1}{k_S K_{11}[L](q_{11} + q_{12}K_{(12)}[L])} + \frac{1 + K_{(12)}[L]}{(q_{11} + q_{12}K_{(12)}[L])k_S} \qquad \text{(Eq. 54)}$$

This is not the equation of a straight line, but under many circumstances it will probably yield an essentially linear plot. Equation 54 has the same form as Eq. 44 for the spectral treatment of this system, and the earlier comments apply. The apparent stability constant can range from

$$K_{11}' = K_{11} + K_{11}K_{(12)}[L] \qquad \text{(Eq. 55)}$$

to

$$K_{11}' = K_{11}K_{(12)}[L] \qquad \text{(Eq. 56)}$$

depending on the relative magnitudes of q_{11} and $q_{12}K_{(12)}[L]$; thus, the value of K_{11}' may be dependent upon the quantities q_{11} and q_{12}.

1:1 Complex and Ligand Dimer.—As in the spectral method, this system does not give a simple analytical solution. If such a system is detected, perhaps the best way to treat it would be to determine by an independent method the ligand dimerization constant, then to calculate [L] as a function of L_t, and finally to treat the system as containing the single 1:1 complex (thus using Eq. 23), with the calculated monomer concentration taking the place of total concentration in constructing the graph. This procedure uses the approximation $L_t = [L] + 2[L_2]$; *i.e.*, consumption of ligand by formation of SL is ignored.

1:1 Complex and Substrate Dimer.—The experimental rate constant is a function of [S] and, therefore, varies during the reaction. This system cannot be conveniently analyzed.

DISCUSSION

Criteria for System Classification.—The operational definitions of the apparent 1:1 stability constants may be summarized as follows:

Solubility.—Plot S_t *versus* L_t; then

$$K_{11}' = \frac{\text{slope}}{\text{intercept } (1 - \text{slope})}$$

Spectral.—Plot $b/\Delta A$ *versus* $1/L_t$; then

$$K'_{11} = \textit{intercept/slope}$$

Kinetic.—Plot $1/(k_S - k_S')$ *versus* $1/L_t$; then

$$K_{11}' = \textit{intercept/slope}$$

The results of the preceding analyses, giving K_{11}' in terms of stability constants and concentrations, are gathered in Table I. The earlier discussion should be consulted for details concerning assumptions, approximations, and limits of applicability of these relationships. With their aid, it would be appropriate to consider how the comparative study of complexation systems with several techniques may yield information inaccessible with a single probe.

The usual order of investigation of a complex system will be (*a*) the determination of the stoichiometrics of all complexes present in significant concentrations or proportions, (*b*) the evaluation of stability constants for these complexes, (*c*) ultimately the determination of the structure and chemical and physical properties of each complex. Several criteria can be suggested to help in establishing stoichiometries and stability constants.

TABLE I.—THEORETICAL EQUIVALENTS OF APPARENT STABILITY CONSTANTS DETERMINED ASSUMING 1:1 COMPLEXATION

Complexes Present	K_{11}' as Found from— Solubility	Spectra	Kinetics
None	0	0	0
L_2	0	0	0
S_2	0	0	0
SL	K_{11}	K_{11}	K_{11}
S_2L	$\frac{2K_{21}S_0}{1 - K_{21}S_0^2}$	$2K_{21}[S]$	$2K_{21}[S]$
SL + LS	$K_{SL} + K_{LS}$	$K_{SL} + K_{LS}$	$K_{SL} + K_{LS}$
$SL + S_2L$	$\frac{K_{11} + 2K_{11}K_{(21)}S_0}{1 - K_{11}K_{(21)}S_0^2}$	[a]	[a]
$SL + SL_2$	$\frac{K_{11} + K_{11}K_{(12)}[L]}{1 + K_{11}K_{(12)}S_0[L]}$	[a]	[a]
$SL + L_2$	$\frac{K_{11}}{1 + 2K_{LL}[L]}$	[b]	[b]
$SL + S_2$	$\frac{K_{11}}{1 + 2K_{SS}S_0}$	[b]	[b]

[a] Variable; see text for discussion. [b] See text.

Relative Values of K_{11}' by the Solubility, Spectral, and Kinetic Techniques.—Table I shows the rationale for this criterion. If a finite value of K_{11}' is obtained (concerning this point see the later discussion), its relative value by the three methods may allow a partial assignment of stoichiometric types. Thus, if all three methods yield the same numerical value, the system probably contains only 1:1 complexes. The possibility exists, however, that identical values can be observed with two methods by a coincidental combination of constant and concentrations. This can easily be detected as pointed out below.

Dependence of K_{11}' on Initial Total Substrate Concentration by the Spectral and Kinetic Techniques.—When a complex S_mL_n is present for which m is greater than 1, K_{11}' by the spectral and kinetic methods will be a function of substrate concentration. K_{11}' should be determined with at least two appreciably different initial substrate concentrations. A significant dependence of K_{11}' on substrate concentration means that at least one complex is present with m larger than 1. The functional form of this dependence may yield further information. Because of this dependence, the substrate concentration should be specified when complex stability constants are reported.

Dependence of K_{11}' on Ligand Concentration by the Solubility Technique.—In each of the three techniques the ligand concentration is the independent variable. As noted earlier, linear spectral plots may be observed even though a curve is theoretically to be expected, and similar results will apply in the kinetic method. The solubility method offers the best chance to detect a dependence of K_{11}' on ligand concentration. If a positive curvature is noted in the phase diagram at least one complex is present of the form SL_n, where n is greater than 1. Negative curvature may indicate dimerization (or higher aggregate formation) of the ligand, as in the system $SL + L_2$. A linear phase diagram does not prove that there are no complexes of these types, for certain combinations, as, for example, the system $SL + SL_2 + L_2$, may give rise to an essentially linear curve over wide ranges of ligand concentration (12).

Dependence of k_S' on Time.—When a complex is present with two or more S molecules per complex molecule, the apparent rate constant should vary with time. In order to detect this variation it may be necessary to follow the reaction for at least two half-lives. If variability of k_S' is not observed the conclusion cannot be positive that all complexes contain only one S molecule, because of the assumptions made concerning the fate of the higher order complex, but this is a reasonable tentative inference.

Dependence of K_{11}' on Wavelength in the Spectral Technique.—This criterion has been emphasized by Johnson and Bowen (11). If K_{11}' varies with the wavelength, at least one higher order complex is present. The theoretical reason for this dependence was pointed out in connection with Eq. 46.

Independent Evidence Relating to Stoichiometry and Stability.—Some of these additional sources are: estimate of stoichiometry from isolable complexes or from the solubility phase diagram (6), Beer's law behavior of pure substrate and ligand, liquid–liquid partition studies of substrate and ligand to detect and measure the extent of self-aggregation processes, and spectral studies leading to stoichiometric ratios (*e.g.*, the method of continuous variations).

These criteria will obviously not be capable of defining the nature of all complexation systems, but they should help considerably in this problem. The possibility that systems may be encountered that are more complicated than those in Table I is very real and must be kept in mind.

It is most important to realize that when the spectral K_{11}' is smaller than either the solubility or the kinetic constant this does not constitute evidence that only the charge-transfer portion of the complex interactions is being measured. If only 1:1 complexes are present, the three methods will yield the same apparent stability constant no matter what the distribution of forces responsible for maintaining the complexes. As long as one of these complexes possesses a changed absorption spectrum this will be true, even if the other complexes cause no spectral change. The same kind of argument applies to the kinetically determined K_{11}', if only 1:1 complexes exist, and at least one of these has an altered reactivity, the apparent K_{11}' will be equal to the sum of all the true 1:1 constants. The general statement may be made that if reliable K_{11}' values for a system differ when determined by the three methods, some complexes are present other than 1:1 combinations of substrate and ligand.

The reliability of stability constants evaluated spectrophotometrically as evidence for the existence of complexes has been explored by Person (13), who suggests that as a practical guide a 1:1 stability constant must be equal to or greater than $0.1/L_{max.}'$ where $L_{max.}'$ is the highest ligand concentration used in the study, in order for the constant to be considered significantly different from zero. Suppose, for example, that the upper limit of ligand concentration in a spectral study is 0.2 M; then the borderline value of K_{11}' is 0.5 M^{-1}. A value smaller than this cannot be taken as evidence for complexing. Similar guides could be formulated for other techniques. Throughout this paper the authors have supposed that nonzero values of stability constants can be demonstrated.

Capabilities of the Solubility, Spectral, and Kinetic Methods.—The solubility method is considered by many to possess the disadvantage of nonselectivity in that it measures the results of all types of interactions. But the foregoing analysis shows that the spectral and kinetic methods are also subject to this type of nonselectivity, and, in the mathematical terms of the analysis as represented in Table I, it may be held that the solubility method is actually more selective than the other techniques. The solubility method possesses two real drawbacks: it is primarily limited to slightly soluble solid substrates, and the substrate concentration cannot be varied. In those systems where the ligand is not too soluble the second disadvantage may be eliminated by reversing the system, treating S as L and vice versa. It is of course not possible to extrapolate a solublity K_{11}' to zero concentration of substrate.

Solubility studies are carried out at constant [S], and spectral studies are at constant S_t. Part of the difficulty in analyzing spectral data follows from this difference, but the capability of varying S_t when desired is an advantage of the spectral method. (It is possible to perform some spectral complexation studies at solubility equilibrium, thus setting [S]

$= S_0$ and letting S_t vary throughout the run; this may simplify some analyses.) The great disadvantage of the spectrophotometric method is of course that a spectral change must occur upon complexation, but when a change is observed the method is very convenient, especially since it provides wavelength as an additional variable. In a general sense, the spectral technique is neither more nor less selective, when applicable, than are other methods. The mathematics developed for spectral studies can be applied to any other physical property that is directly proportional to a species concentration; examples are refractive index (14), optical rotation (15), and fluorescence intensity.

The kinetic method is carried out with neither [S] nor S_t held constant (though if initial rates were measured S_t could be considered the constant factor). Mathematically it is similar to the spectral method; but it possesses the advantage that it is applicable even if no spectral change occurs, and the disadvantage that it does not include a convenient variable corresponding to wavelength. (The parallel to wavelength is complex reactivity, but this cannot easily be altered without changing the system.) Throughout this paper the inhibition of rates by complex formation has been taken as the basis for the analysis, but the complex may in some systems exhibit an enhanced reactivity, and this phenomenon also can serve for study of the complex equilibrium (16, 17). The outstanding potential advantage of the kinetic technique is its capability for providing information about the reactivity, and thus the structure, of the complex. This capability has not yet been exploited, although some attempts have been made to utilize it (5), and further studies in the chemistry of organic complexes may find its application valuable.

Conclusions.—The application of more than one experimental technique is advisable in the study of complexation systems. By comparing the apparent stability constants evaluated by the several methods on the basis of an assumed 1:1 stoichiometry between substrate and ligand, it may be possible to establish, in part, the stoichiometries of the complexes present. If the solubility, spectral, and kinetic techniques yield essentially identical values for the apparent 1:1 stability constant (and if certain other criteria suggested in this paper are satisfied), it may be concluded that only 1:1 complexes between substrate and ligand are present. If the three techniques do not give the same value, the manner in which they differ and their dependence on variables of the system may permit a further classification. On the basis of the mathematical analysis it is concluded that the solubility, spectral, and kinetic methods for studying complex formation are about equally nonselective in their responses to multiple complexes, with the solubility method perhaps possessing a slight advantage in specificity.

APPENDIX

S, substrate molecule
L, ligand molecule
S_mL_n, general formula for complex
S_t, total (formal) concentration of S
L_t, total (formal) concentration of L
S_0, equilibrium molar solubility of substrate monomer in absence of L
$[i]$, molar concentration of species i
$f_s = [S]/S_t$, fraction of S in uncomplexed form
$f_{mn} = m[S_mL_n]/S_t$, fraction of S in form of S_mL_n
$K_{mn} = [S_mL_n]/[S]^m[L]^n$, over-all stability constant for the complex S_mL_n
$K_{(mn)} = [S_mL_n]/[S][S_{m-1}L_n]$ or $[S_mL_n]/[S_mL_{n-1}][L]$, step stability constant for S_mL_n
K'_{11}, apparent stability constant assuming 1:1 stoichiometry
k_S, specific rate constant for a reaction of S
k'_S, apparent rate constant for S in presence of L
k_{mn}, specific rate constant for a reaction of S_mL_n
$r_{mn} = k_{mn}/k_S$, relative reactivity of S_mL_n
$q_{mn} = 1 - r_{mn}$
b, cell path length
a_S, molar absorptivity of S
a_{mn}, molar absorptivity of S_mL_n
A, absorbance

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Determination of Acetylsalicylic Acid and Barbiturate Combinations by Differentiating Nonaqueous Titration

By SONG-LING LIN* and MARTIN I. BLAKE

Acetylsalicylic acid and barbiturate mixtures are determined by differentiating nonaqueous titration. The titration solvent is methyl isobutyl ketone, the titrant is sodium methoxide in benzene–methanol, and the electrode system consists of the glass-calomel or antimony-calomel electrode pair. Satisfactory end points were realized when the acetylsalicylic acid-to-barbiturate ratio was as high as 14 to 1. Tetrabutylammonium hydroxide proved unsuitable as a differentiating titrant. Differentiation was not possible when dimethylformamide was the titration solvent. The barbiturates studied included phenobarbital, amobarbital, barbital, allylisobutylbarbituric acid, pentobarbital, and secobarbital.

A VARIETY of techniques have been proposed for the analysis of acetylsalicylic acid and barbiturates when used individually. These methods involve titrimetry, chromatography, colorimetry, and spectrophotometry. They have been reviewed by Zimmer and Huyck (1) and Connors (2). Titrimetric procedures in aqueous and nonaqueous media have been reviewed by Ashworth (3).

Since acetylsalicylic acid and the barbiturates are weakly acidic and since they differ significantly in their ionization constants, it seems appropriate that a suitable differentiating titration procedure can be developed by the proper selection of titration solvent, titrant, and electrode system. It was the purpose of this study to develop such a procedure.

A number of solvents have been employed in differentiating acids and bases. These have included acetone (4, 5), methyl ethyl ketone (6–8), methyl isobutyl ketone (9, 10), acetonitrile (11), isopropanol (8), *tert*-butyl alcohol (12, 13), nitrobenzene (14), dimethylformamide (10, 13), *N,N*-dimethyl fatty amides (15), pyridine (5, 13, 16), and ethylenediamine (17).

In the present report combinations of acetylsalicylic acid with a variety of barbiturates are determined by differentiating nonaqueous titration. The titration solvent is methyl isobutyl ketone and the titrant is sodium methoxide in benzene–methanol. Titration is effected potentiometrically using a glass-calomel or antimony-calomel electrode system.

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EXPERIMENTAL

Apparatus.—All titrations were performed potentiometrically with a Fisher titrimeter, model 35. The following electrodes were employed: glass electrode (Beckman No. 40495), sleeve-type calomel electrode (Beckman No. 41240), antimony electrode (Beckman No. 39027), and silver-silver chloride electrode (Beckman No. 41236). The calomel electrode was used as such unless otherwise indicated.

Reagents and Solutions.—Acetylsalicylic acid U.S.P. (Mallinckrodt) was dried at 60° for 4 hr. Analysis by U.S.P. assay indicated a purity of better than 99.5%. Phenobarbital U.S.P. (Mallinckrodt) was recrystallized from diluted ethanol and dried at 100° for 2 hr., m.p. 176–178°. Analysis by U.S.P. assay indicated a purity of better than 99.0%. Amobarbital was recrystallized from diluted alcohol and dried at 105° for 4 hr., m.p. 156–158°. Analysis by U.S.P. assay indicated a purity of better than 99.0%. Allylisobutylbarbituric acid was dried at 105° for 2 hr., m.p. 138°. Analysis by N.F. X method indicated a purity of better than 99.5%. Other chemicals and all solvents used in this study were reagent grade and were employed without further purification.

Tenth normal sodium methoxide in benzene–methanol (10:1) was prepared and standardized as described by Fritz and Lisicki (18).

Differentiating Titration of Acetylsalicylic Acid and Phenobarbital.—About 0.70 meq. of acetylsalicylic acid and 0.70 meq. phenobarbital, accurately weighed, were dissolved in 70 ml. of solvent in a 150-ml. beaker. The solution, magnetically stirred, was titrated potentiometrically with 0.1 *N* sodium methoxide solution, using a sleeve-type calomel and glass electrode system. A blank titration was performed. Titration curves were obtained by plotting potential reading (mv.) *versus* volume (ml.) of titrant. The exact end point was determined by plotting $\Delta E/\Delta v$ *versus* ml. The titration solvents in this study included dimethylformamide, acetonitrile, acetone, methyl ethyl ketone, methyl isobutyl ketone, isopropanol, and *tert*-butyl alcohol.

TABLE I.—DIFFERENTIATING TITRATION OF ACETYLSALICYLIC ACID AND PHENOBARBITAL IN VARIOUS SOLVENTS

Curve Ref.[a]	Solvent	Recovery, % Acetylsalicylic Acid	Recovery, % Phenobarbital
1	Dimethylformamide	One End Point[b]	
2	Acetonitrile	99.25 ± 0.56[c]	100.49 ± 0.95
3	Acetone	100.82 ± 0.91	99.32 ± 0.82
4	Methyl ethyl ketone	101.15 ± 0.70	98.78 ± 0.62
5	Methyl isobutyl ketone	100.35 ± 0.54	99.89 ± 0.69
6	Isopropanol	100.64 ± 0.92	100.58 ± 0.62
7	*tert*-Butyl alcohol	101.72 ± 0.48	99.03 ± 0.90

[a] Numbers correspond to curves in Fig. 1. [b] Corresponds to total acetylsalicylic acid and phenobarbital. [c] Standard deviation based on at least 4 determinations.

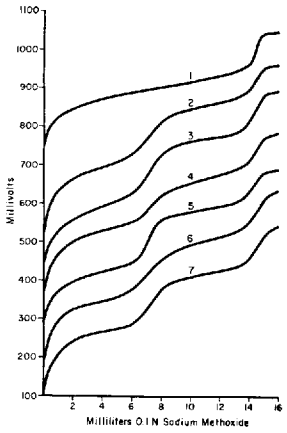

Fig. 1.—Typical curves for differentiating titration of acetylsalicylic acid and phenobarbital in various solvents. The titrant was 0.1 *N* sodium methoxide in benzene–methanol (10:1). The numbers above the curves correspond to those in Table I.

Electrode Systems.—About 0.60 meq. of acetylsalicylic acid and 0.40 meq. of phenobarbital, accurately weighed, were dissolved in 50 ml. of methyl isobutyl ketone. Three electrode pairs were employed, glass-calomel, antimony-calomel, and glass-silver-silver chloride. The sleeve-type calomel electrode was used as such or was modified by replacing the aqueous saturated potassium chloride solution in the bridge with a saturated solution of potassium chloride in methanol or with a saturated solution of potassium chloride in methyl isobutyl ketone.

Variation of Acetylsalicylic Acid-to-Phenobarbital Ratio.—A series of differentiating titrations was performed in which the milliequivalent ratio of acetylsalicylic acid to phenobarbital was varied from 1 to 1 to greater than 30 to 1. Calculated amounts of acetylsalicylic acid and phenobarbital to give the desired milliequivalent ratio of components were accurately weighed and dissolved in 70 ml. of methyl isobutyl ketone. The solution was titrated potentiometrically with 0.1 *N* sodium methoxide using the glass-calomel or antimony-calomel electrode system. The calomel electrode was employed without modification.

Analysis of Acetylsalicylic Acid Combinations with Various Barbiturates.—Accurately weighed quantities of acetylsalicylic acid and one of a number of barbiturates were dissolved in 50 ml. of methyl isobutyl ketone in a 150-ml. beaker. The solution was titrated potentiometrically with 0.1 *N* sodium methoxide using a glass-calomel or antimony-calomel electrode system. The calomel electrode was used without modification.

RESULTS AND DISCUSSION

Although acetylsalicylic acid and phenobarbital, as individual components, can be readily determined by titration in nonaqueous media, the titrimetric analysis of mixtures of these compounds has not been reported. Since the pKa of acetylsalicylic acid is 3.49 and the pKa of phenobarbital is 7.54, the differentiating titration of mixtures in a number of solvents is readily accomplished. The solvents examined in this investigation are listed in Table I, and typical titration curves are shown in Fig. 1. Titrations were effected potentiometrically with 0.1 *N* sodium methoxide in benzene–methanol using a glass-calomel electrode system. Basic solvents such as dimethylformamide, while excellent for the individual components, do not permit differentiation in the case of mixtures. Figure 1, curve 1, indicates a single end point corresponding to the total acid present when titration is performed in dimethylformamide. In preliminary studies similar results were obtained with ethylenediamine, butylamine, and pyridine.

TABLE II.—EFFECT OF ELECTRODE COMBINATION ON DIFFERENTIATING TITRATION OF ACETYLSALICYLIC ACID AND PHENOBARBITAL

Curve Ref.[a]	Electrode Combination	Electrolyte Bridge in Calomel Electrode	Recovery, % Acetylsalicylic Acid	Recovery, % Phenobarbital
1	Antimony-calomel	Sat. KCl in water	100.32[c]	101.09
2	Antimony-calomel	Sat. KCl in methanol	98.97	100.29
3	Antimony-calomel	Sat. KCl in MIK[b]	100.42	98.32
4	Glass-calomel	Sat. KCl in water	99.83	98.69
5	Glass-calomel	Sat. KCl in methanol	99.78	100.81
6	Glass-calomel	Sat. KCl in MIK	100.35	100.46
7	Glass-silver-silver chloride		101.70	99.77

[a] Numbers correspond to curves in Fig. 2. [b] Methyl isobutyl ketone. [c] Average of at least 3 determinations.

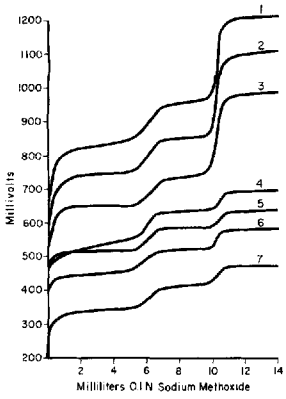

Fig. 2.—Effect of electrode combination on sensitivity of differentiating titration of acetylsalicylic acid and phenobarbital dissolved in methyl isobutyl ketone. The numbers above the curves correspond to those in Table II.

It is apparent that acetylsalicylic acid and phenobarbital are leveled to the same strength in these solvents and this precludes their differentiation. Acetonitrile and the ketones and alcohols listed in Table I did permit satisfactory differentiation. The curves in Fig. 1 show two distinct inflections, the first corresponding to the acetylsalicylic acid content and the second representing the phenobarbital end point. Methyl isobutyl ketone consistently produced the greater potential breaks for both end points when comparison is made with the other solvents tested. (See Fig. 1, curve 5.) Bruss and Wyld (9) found methyl isobutyl ketone an excellent solvent for differentiating acids or bases because of the large potential range which it affords.

Methyl isobutyl ketone was used as the titration solvent for subsequent studies reported in this investigation. The effect of electrode combination on the resolution of acetylsalicylic acid and phenobarbital mixtures was explored. The electrode systems and their modifications are listed in Table II. Typical titration curves are shown in Fig. 2. The most satisfactory results were realized with the antimony–calomel electrode pair. A comparison of the titration curves indicates that for acetylsalicylic acid the potential break was doubled and for phenobarbital the potential break was increased about ninefold when the antimony-calomel electrode pair replaced the glass-calomel electrode system. Little or no effect was noted when the supporting electrolyte in the calomel electrode was modified. In preliminary studies a number of other electrode systems were tested. The platinum-calomel and glass-antimony pairs gave poorly defined and unpredictable potential breaks corresponding to the first end point, although the second end point

Table III.—Effect of Acetylsalicylic Acid-to-Phenobarbital Ratio on Sensitivity of Differentiating Titration

Curve Ref.[a]	Amt. Weighed, meq. Acetylsalicylic Acid	Amt. Weighed, meq. Phenobarbital	Recovery, % Glass-Calomel Electrodes: Acetylsalicylic Acid	Glass-Calomel Electrodes: Phenobarbital	Antimony-Calomel Electrodes: Acetylsalicylic Acid	Antimony-Calomel Electrodes: Phenobarbital
1	1.00	1.00	100.46 ± 0.63[b]	100.06 + 0.88	100.35 ± 0.54	99.89 ± 0.69
2	1.00	0.75	99.42 ± 0.78	99.98 ± 0.39	100.14 ± 0.63	98.99 ± 0.46
3	1.00	0.40	99.18 ± 0.47	101.23 ± 0.49	100.97 ± 0.49	101.02 ± 0.66
4	1.00	0.30	99.04 ± 0.81	99.51 ± 0.62	99.77 ± 0.28	100.80 ± 0.34
5	1.00	0.25	100.93 ± 0.94	98.38 ± 0.48	98.62 ± 0.21	101.06 ± 0.29
6	1.00	0.20	98.75 ± 0.72	99.83 ± 0.79	101.78 ± 0.42	99.79 ± 0.50
7	1.00	0.15	101.82 ± 0.66	99.89 ± 0.96	99.97 ± 0.28	98.90 ± 0.63
8	1.00	0.10	One end point[c]		98.96 ± 0.48	101.85 ± 0.31
9	1.00	0.07	One end point		101.25 ± 0.67	99.06 ± 0.97
10	1.00	0.03	One end point		One end point	

[a] Numbers correspond to curves in Figs. 3 and 4. [b] Standard deviation based on at least 4 determinations. [c] Corresponds to acetylsalicylic acid plus phenobarbital.

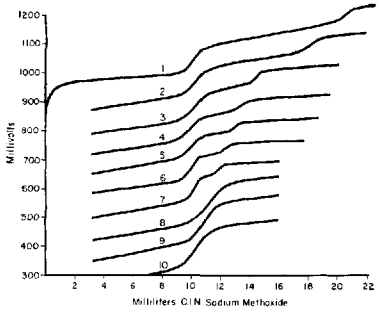

Fig. 3.—Effect of acetylsalicylic acid-to-phenobarbital ratio on sensitivity of differentiating titration of acetylsalicylic acid and phenobarbital dissolved in methyl isobutyl ketone. The glass-calomel electrode system was employed. The numbers above the curves correspond to those in Table III.

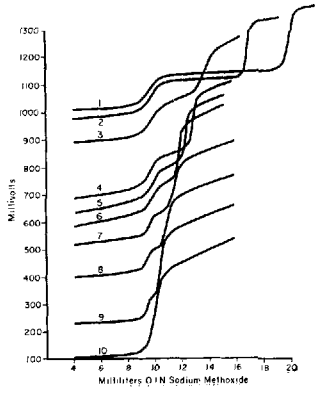

Fig. 4.—Effect of acetylsalicylic acid-to-phenobarbital ratio on sensitivity of differentiating titration of acetylsalicylic acid and phenobarbital dissolved in methyl isobutyl ketone. The antimony-calomel electrode system was employed. The numbers above the curves correspond to those in Table III.

TABLE IV.—DIFFERENTIATING TITRATION OF ACETYLSALICYLIC ACID AND BARBITURATE COMBINATIONS

Mixture	pKa	Amt. Weighed, meq.	Glass-Calomel Electrodes $\left(\frac{\Delta E}{\Delta v}\right)_{max.}$	Glass-Calomel Electrodes Recovery, %	Antimony-Calomel Electrodes $\left(\frac{\Delta E}{\Delta v}\right)_{max.}$	Antimony-Calomel Electrodes Recovery, %
Acetylsalicylic acid	3.49(21)[a]	0.50	80	99.81 ± 0.63 (*10*)[b]	80	99.72 ± 0.57 (*10*)
Allylisobutylbarbituric acid	7.68(22)	0.25	140	100.14 ± 0.55 (*10*)	250	100.28 ± 0.68 (*10*)
Acetylsalicylic acid	...	0.50	60	99.81 ± 0.62 (*9*)	65	100.32 ± 0.75 (*9*)
Amobarbital	8.02(23)	0.33	75	100.13 ± 0.56 (*9*)	150	98.90 ± 0.48 (*9*)
Acetylsalicylic acid	...	0.40	80	99.40 ± 0.44 (*4*)	80	99.32 ± 0.48 (*4*)
Barbital	8.06(23)	0.40	50	100.48 ± 0.60 (*4*)	340	98.84 ± 0.62 (*4*)
Acetylsalicylic acid	...	0.60	85	99.38 ± 0.49 (*5*)	95	100.40 ± 0.37 (*5*)
Pentobarbital	8.17(23)	0.30	60	98.97 ± 0.50 (*5*)	300	99.86 ± 0.62 (*5*)
Acetylsalicylic acid	...	0.50	60	99.82 ± 0.47 (*10*)	90	100.91 ± 0.56 (*10*)
Phenobarbital	7.54(23)	0.33	55	100.09 ± 0.73 (*10*)	520	100.34 ± 0.71 (*10*)
Acetylsalicylic acid	...	0.50	70	98.93 ± 0.53 (*4*)	85	99.88 ± 0.49 (*5*)
Secobarbital	8.08(22)	0.40	60	101.08 ± 0.62 (*4*)	110	100.09 ± 0.82 (*5*)

[a] Literature reference. [b] Standard deviation based on the number of determinations indicated in parenthesis.

representing total acid was satisfactory. Discernible end points were not obtained with the glass-platinum or antimony-platinum electrode systems.

Since in dosage forms there is usually a considerably greater amount of acetylsalicylic acid than phenobarbital, the effect of varying the ratio of concentrations of the components on the sensitivity of the differentiating titration was studied. The data for a series of titrations in which the milliequivalent ratio of acetylsalicylic acid to phenobarbital was varied from 1 to 1 to about 30 to 1 are reported in Table III. Typical titration curves are shown in Figs. 3 and 4. In one series of titrations (Fig. 3) the glass-calomel electrode pair was used, while in a second series of titrations (Fig. 4) the antimony-calomel electrode system was employed. With the glass-calomel electrodes two inflections in the titration curve were obtained when the milliequivalent ratio of acetylsalicylic acid to phenobarbital was not greater than about 7 to 1. When the ratio was greater than this, only one end point corresponding to the total acid was realized. The family of curves in Fig. 3 demonstrates clearly the effect of the ratio on the resolution of the two end points. With the antimony-calomel electrode pair the acetylsalicylic acid end point is well defined when the ratio of components was as high as 14 to 1. In Fig. 4, curve 9, two end points are clearly defined. However, in curve 10, only one end point is discernible. In general, both inflections in the curves shown in Fig. 4 are more satisfactory than those in Fig. 3.

Tetrabutylammonium hydroxide has been shown (4, 8, 9, 19, 20) to be extremely useful as a titrant for determining weak acids. This titrant in benzene–methanol (10:1) was prepared and standardized as described by Cundiff and Markunas (19). Some preliminary studies were conducted to evaluate this titrant in differentiating mixtures of acetylsalicylic acid and phenobarbital. Methyl isobutyl ketone was used as the titration solvent. In all cases only a single end point was obtained corresponding to the total acid present. When the individual components were titrated excellent results were obtained with distinct and reproducible end points. The unsuccessful differentiation with this titrant cannot be explained at this time.

The proposed procedures were applied to combinations of acetylsalicylic acid with a variety of barbiturates. The data are reported in Table IV. All titrations were performed with sodium methoxide as the titrant and methyl isobutyl ketone as the titration solvent. Both the glass-calomel and the antimony-calomel electrode pairs were employed. The maximum potential change per unit volume of titrant added is shown for each combination. While excellent results were obtained for all combinations, the antimony-calomel electrode system produced greater potential changes for the barbiturate end point than did the glass-calomel electrode pair.

The proposed procedures make possible the simple and accurate determination of combinations of acetylsalicylic acid and a variety of barbiturates without preliminary extraction of the components. The technique is applicable even when there is a disproportionate concentration of the acetylsalicylic acid which is the usual situation when these combinations are used therapeutically.

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Adsorption of Phenothiazine Derivatives by Solid Adsorbents

By DONALD L. SORBY, ELMER M. PLEIN*, and JOSEPH D. BENMAMAN

In simple aqueous media, the extent of adsorption of various phenothiazine derivatives by kaolin, talc, and activated charcoal is significant. Adsorption by talc and kaolin is dependent upon pH of the medium, while adsorption by activated charcoal is less affected by pH. Adsorption of promazine hydrochloride by all three adsorbents is sensitive to the electrolyte concentration of the medium. A variety of evidence suggests that adsorption of the phenothiazine derivatives by activated charcoal is a result of physical forces related to the tendency of the solute to accumulate at the air–water interface. Adsorption of these compounds by talc and kaolin occurs through more complex mechanisms which cannot be completely elucidated from knowledge obtained in this experiment. The effects of pH and electrolyte concentration may be important to the previously observed action of adsorbents in modifying absorption of promazine from the gastrointestinal tract.

A PREVIOUS publication (1) presented a partial report of results obtained in this experiment. Various medicinally active phenothiazine derivatives were found to be adsorbed to a significant extent by kaolin, talc, and activated charcoal. The purpose of this report is to describe various aspects of the adsorption process in further detail and to present results of studies concerned with attempts to elucidate the mechanisms of the adsorption interaction. In addition, there is interest in adsorption as a potential means of altering drug absorption from the gastrointestinal tract (2, 3). Certain factors have been studied for their ability to produce release of the absorbed material from the surface of the adsorbate complex. *In vivo* experiments have involved the use of promazine hydrochloride. Thus, several experiments reported here feature this compound. Results obtained for promazine hydrochloride should be qualitatively similar for other phenothiazine derivatives.

EXPERIMENTAL

Preparation of Compounds for Study.—The phenothiazine derivatives used in this study were obtained from various sources of supply.[1] Each compound was recrystallized from an appropriate solvent until the melting point agreed with the accepted value.[2] The 15 compounds studied during various phases of the experiment are listed in Table I.

All adsorption studies were performed using compounds in the form of their hydrochloride salts. Compounds not available as the hydrochloride salt, *viz.*, methoxypromazine maleate, acepromazine maleate, trimeprazine tartrate, and prochlorperazine ethanedisulfonate, were converted to the hydrochloride form by passing 1.1×10^{-2} *M* solutions through an ion exchange column charged with a strongly basic polystyrene quaternary amine type ion-exchange resin[3] in the chloride cycle. The nonaqueous titration procedure described below was used to determine the exact concentration of the solution recovered from the ion-exchange column. The effluent solutions were tested for completeness of anion exchange by a paper chromatographic procedure (4). Ethanedisulfonate could not be detected by this method. For other compounds mentioned, no evidence of the original acid anion could be found in the effluent from the ion exchange columns. Conversion to the hydrochloride salt form was considered to be complete in all cases.

Preparation of Adsorbents for Study.—Adsorbent materials were procured from commercial sources. Activated charcoal[4] was obtained from the American Norit Co., Jacksonville, Fla.

Average screen analysis data, supplied by the manufacturer, showed that 98% is passed through a 100-mesh U. S. standard screen, 90% through a 200-mesh, and 85% through a 300-mesh screen. Talc was U.S.P. grade, 99.5% passes a 100-mesh screen. Kaolin was N.F. grade, 99.5% passes a 325-mesh screen.

The total content of commercial packages of each adsorbent was blended in a twin shell blender, dried at 120° for 5 hr., and stored in air-tight bottles until used. The total amount of each adsorbent used in this study came from one single production lot of material.

Aqueous extracts of the kaolin and talc adsorbents

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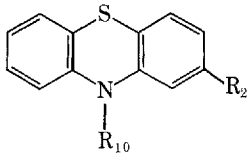
[1] The authors are grateful to Laboratories Clin-Comar, Lederle Laboratories, Sandoz Pharmaceuticals, G. D. Searle and Co., Smith Kline & French Laboratories, E. R. Squibb and Sons, The Upjohn Co., Warner-Chilcott Laboratories, White Laboratories, Inc., and Wyeth Laboratories, Inc., for their generosity in supplying the compounds used in this research.

[2] Melting point data were furnished by the supplier of each compound. Isopropanol was used as the recrystallizing solvent for all compounds except acepromazine maleate and prochlorperazine ethanedisulfonate. Ethyl acetate was used for the former and water–ethanol for the latter. Ethopropazine hydrochloride and thioridazine dihydrochloride were not recrystallized.

[3] Marketed as Amberlite IRA-400 by Rohm & Haas Co. Philadelphia, Pa.

[4] Marketed as Norit A.

TABLE I.—PHENOTHIAZINE DERIVATIVES INCLUDED IN THIS RESEARCH



Compd.	No. 2 Substituent (R_2)	No. 10 Substituent (R_{10})
Promazine	Hydrogen	3-Dimethylaminopropyl
Mepazine	Hydrogen	(*N*-Methyl-piperidyl)-methyl
Trimeprazine	Hydrogen	3-Dimethylamino-2-methylpropyl
Promethazine	Hydrogen	2-Dimethylamino-2-methylethyl
Ethopropazine	Hydrogen	2-Diethylamino-2-methylethyl
Pyrathiazine	Hydrogen	2-(1-Pyrrolidyl)-ethyl
Chlorpromazine	Chlorine	3-Dimethylaminopropyl
Methoxypromazine	Methoxy	3-Dimethylaminopropyl
Triflupromazine	Trifluoromethyl	3-Dimethylaminopropyl
Acepromazine	Acetyl	3-Dimethylaminopropyl
Prochlorperazine	Chlorine	3-(1-Methyl-4-piperazinyl)-propyl
Thiopropazate	Chlorine	3-[1-(2-Acetoxyethyl)-4-piperazinyl]-propyl
Trifluoperazine	Trifluoromethyl	3-(1-Methyl-4-piperazinyl)-propyl
Fluphenazine	Trifluoromethyl	3-(2-Hydroxyethyl-4-piperazinyl)-propyl
Thioridazine	Methylmercapto	2-(1-Methyl-2-piperidyl)ethyl

contained small amounts of ultraviolet absorbing impurities. The magnitude of this absorption was small, however, and was nearly constant in the range of the ultraviolet absorption spectrum where the phenothiazine derivatives show absorption maxima. The aqueous extracts showed a negative potassium ferrocyanide test for ferric ion.

Analytical Methods.—Two analytical methods were employed in this research.

Nonaqueous Titration Procedure.—The concentration of all stock solutions of phenothiazine derivatives was confirmed by a nonaqueous titration procedure adapted from the method of Milne *et al.* (5, 6). Aqueous solutions containing a particular phenothiazine derivative were made strongly basic with sodium hydroxide and then were extracted[5] with four 20-ml. portions of *n*-hexane. Sufficient acetone was added to the extract to make a solvent containing a 2:1 ratio of hexane to acetone. This solution was titrated with perchloric acid, 0.05 *N* in dioxane. The end point was determined potentiometrically using a Leeds & Northrup pH indicator equipped with glass indicator and calomel reference electrodes. The end point was taken as the point of maximum inflection of a curve representing observed E.M.F. plotted against volume of titrant added. A blank correction was made for a similar system containing no phenothiazine derivative. The method was used for all phenothiazine derivatives listed in Table I except ethopropazine. Accuracy of the method was within a range of ±3% of theoretical at the 1×10^{-2} *M* concentration level. Quantitative recovery of thiopropazate could not be obtained using this procedure.

Spectrophotometric Procedure.—The nonaqueous titration procedure lacked sufficient sensitivity to be used at the concentration ranges employed in experiments which measure adsorption isotherms. In such cases, an ultraviolet spectrophotometric procedure was used to measure equilibrium concentrations of the phenothiazine derivatives. The method was adapted from a background cancellation technique proposed by Flanagan *et al.* (7) for chlorpromazine. A Beckman DU spectrophotometer was used to measure absorbance of the samples at three different wavelengths. Absorbance at the wavelength of maximum absorbance ($\lambda_{max.}$) was always measured. The other two absorbance measurements were made at wavelengths where the sample absorbed less strongly. One measurement was always made at a shorter wavelength (λ_{short}) than $\lambda_{max.}$ and one at a longer wavelength (λ_{long}). Corrections for background absorbance were made in the fashion described by Flanagan *et al.* (7). However, they were calculated algebraically.

The following expressions illustrate this method of correcting for "background" interference in samples.

$$\text{Background correction (B.C.)} = As\ \lambda_{high} + \left[(As\ \lambda_{short} - As\ \lambda_{long}) \times \frac{\lambda_{max.} - \lambda_{long}}{\lambda_{short} - \lambda_{long}}\right]$$

$$\text{Corrected absorbance } (As^*) = \text{measured } As \text{ at } \lambda_{max.} - \text{B.C.}$$

A "corrected" molar absorption coefficient was calculated for each compound from the "corrected" absorbance of samples of known concentrations measured in the absence of adsorbent. The concentration of solute in test samples was calculated from the relationship

$$C = \frac{As^*}{(l)\ (e^*)}$$

where, As^* is the corrected absorbance of the sample at $\lambda_{max.}$, l is the path length of the solution, and e^* is the "corrected" molar absorption coefficient for the compound. Table II lists the wavelengths used in determining As^* and the values of e^* for compounds studied in this experiment. The range of usefulness of this method of assay is 5×10^{-6} *M* to 4×10^{-5} *M*.

[5] A benzene–dinitromethane solvent was recommended (5) for extracting dibasic compounds. Personal communication with the author indicated that the hexane–acetone mixture also worked well with dibasic compounds. Results obtained during this research have confirmed the hexane–acetone solvent as being suitable for extracting both monobasic and dibasic compounds.

TABLE II.—WAVELENGTHS USED FOR ANALYSIS AND THE CORRECTED MOLAR ABSORPTION COEFFICIENTS FOR VARIOUS PHENOTHIAZINE DERIVATIVES

Compd.[a]	Wavelengths for Absorption Measurements λ_{low}, mμ	$\lambda_{max.}$, mμ	λ_{high}, mμ	"Corrected" molar Absorption Coefficient × 10^{-5}
Methoxypromazine	232	251	270	0.155
Pyrathiazine	230	250	260	0.174
Triflupromazine	230	256	270	0.209
Acepromazine	230	242	290	0.099
Trifluoperazine	237	257	267	0.179
Trimeprazine	226	251	262	0.179
Thiopropazate	230	254	266	0.214
Chlorpromazine	236	255	265	0.196
Promethazine	225	250	260	0.188
Promazine	230	248	262	0.182
Prochlorperazine	234	255	266	0.213
Fluphenazine	234	256	268	0.200
Mepazine	234	253	262	0.188
Thioridazine	246	262	278	0.232
Ethopropazine	230	249	258	0.156

[a] All compounds were studied as the hydrochloride salt. All solutions also contained 0.01% sodium bisulfite.

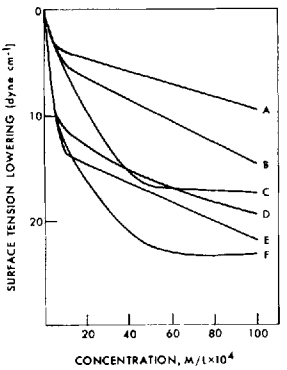


Fig. 1.—Surface tension lowering-concentration relationships. Key: A, promazine hydrochloride; B, mepazine hydrochloride; C, thioridazine hydrochloride; D, trifluoperazine; E, thiopropazate dihydrochloride; F, triflupromazine hydrochloride.

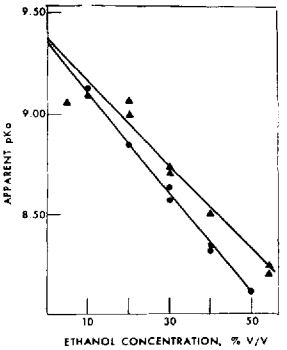


Fig. 2.—Apparent pKa as a function of ethanol concentration. Key: ▲, promazine hydrochloride; ●, pyrathiazine hydrochloride.

Preparation of Solutions.—Stock solutions of each compound were prepared on the day the adsorption isotherm was determined. Each solution was prepared to contain a 1×10^{-2} *M* concentration of the particular compound under study. Each solution also contained 0.01% sodium bisulfite.

Determination of Adsorption Isotherms.—The procedure for determining adsorption isotherms was reported elsewhere (1). Except where specified, all adsorption experiments were made at 20.0°.

Surface Tension Lowering Measurements.—The change in surface tension with concentration was measured for six phenothiazine derivatives. Dilutions of each compound in distilled water, ranging between 5×10^{-4} *M* and 1×10^{-2} *M*, were prepared. Surface tensions of the solutions were measured at 20 ± 1° with a Cenco-DuNoüy interfacial tensiometer, precision direct reading model. Fresh surfaces were prepared by flooding a small glass dish with solution, and two readings of the surface tension were immediately taken. This procedure was repeated until a total of six readings had been obtained at each concentration tested. The mean value was calculated from the six readings. A value for the surface tension of water was measured in a similar fashion. Figure 1 shows the variation of surface tension lowering with concentration for the compounds tested.

Determination of Apparent pKa.—Apparent pKa values for several compounds were measured by a titrimetric procedure similar to one employed by Marshall (8). Hydroalcohol solutions of the hydrochloride form of the appropriate phenothiazine derivative were prepared at a concentration of 1×10^{-3} *M*. These solutions were titrated with standard sodium hydroxide solution, 0.0500 *N*, at 20.0° under a nitrogen atmosphere. After each addition of titrant, the pH was measured with the instrument mentioned under *Nonaqueous Titration Procedure*. The apparent pKa was calculated at each pH reading on the titration curve using the relationship

$$\text{pKa} - \text{pH} - \log \frac{C_{R_3N}}{C_{R_3NH^+}}$$

where C_{R_3N} is the concentration of amine base, and $C_{R_3NH^+}$ is the concentration of the protonated species present at the particular pH value. Values for C_{R_3N} and $C_{R_3NH^+}$ were calculated from the amount of amine salt initially present and the amount of titrant added. An average of the several pKa values calculated in this fashion was taken as the apparent pKa for the compound in the particular hydroalcohol solvent. Titrations and apparent pKa calculations were made in duplicate for each compound at each alcohol strength, usually 10, 20, 30, 40, and 50% by volume ethanol.

The apparent pKa values measured for each compound in the various hydroalcohol solutions were plotted against alcohol concentration. The zero alcohol concentration intercept, calculated from the linear portion of the curve by the method of least squares, yielded the apparent pKa of the compound in water. Figure 2 shows a plot of apparent pKa *versus* alcohol concentration for pyrathiazine hydrochloride and chlorpromazine hydrochloride and is typical of the over-all results.

In certain cases, the amine base began to precipitate from the hydroalcohol system before a complete titration curve could be obtained. In such cases, the apparent pKa was calculated by assuming the concentration of the amine base actually in solution to be constant after the point where precipitation first occurred. The concentration of free base present at the point of precipitation was estimated from the amount theoretically present at the cloud point when a sample of identical concentration with respect to both alcohol and drug was titrated with a more dilute titrant solution. Using this method, apparent pKa values could be determined for most

TABLE III.—APPARENT pKa VALUES FOR VARIOUS PHENOTHIAZINE DERIVATIVES

Compd.	Measured pKa	pKa Reported in Lit.[a]
Promazine hydrochloride	9.39	9.52
Mepazine hydrochloride	9.25	...
Chlorpromazine hydrochloride	9.21	9.30
Thioridazine hydrochloride	9.45	...
Pyrathiazine hydrochloride	9.36	8.96
Fluphenazine dihydrochloride[b]	8.05	...
Trifluoperazine dihydrochloride[b]	8.36	...
Promethazine hydrochloride	...	9.08
Ethopropazine hydrochloride	...	9.50

[a] Values reported by Marshall (8). [b] These values are for dissociation of the second basic group. For pK_1, values of 3.90 and 4.10 were obtained for fluphenazine and trifluoperazine, respectively.

of the compounds in hydroalcohol solutions containing as little as 10% v/v alcohol. Where calculations could be made from data collected both before and after the point of precipitation, good agreement was obtained between apparent pKa values.

Values of the apparent pKa for several compounds are reported in Table III.

Adsorption at Constant pH.—Adsorption of promazine hydrochloride and fluphenazine dihydrochloride was studied at pH 2.5 and 6.5. Phosphate buffers were prepared to contain 0.03 *M* total phosphate. For the pH 6.5 buffer, 4.23 Gm. of dibasic sodium phosphate and 2.0 ml. of hydrochloric acid were made to a volume of 1000 ml. with carbonate-free distilled water. For pH 2.5 buffer, 4.13 Gm. of monobasic sodium phosphate and 1.0 ml. of hydrochloric acid were made to 1000 ml. with carbonate-free distilled water. The pH of each buffer was brought exactly to the desired values, by addition of acid or base, before adjustment to volume. Stock solutions of each phenothiazine derivative in the appropriate buffer were prepared, and the isotherms were determined by the procedure described previously (1). The method employed here differed only with respect to the fact that buffer was used as the solvent for all systems. Limited adsorption experiments carried out in varying concentrations of phosphate buffer showed that the observed effects on adsorption were not due to competition between the buffer components and the phenothiazine derivative for sites on the adsorbent surface.

Effect of Electrolyte on Adsorption.—Adsorption of promazine by the various adsorbents was measured in the presence of 0.01 and 0.10 *N* sodium chloride. Stock solutions were prepared by dissolving promazine hydrochloride in the appropriate sodium chloride solution. Isotherms were determined in a manner similar to that described previously (1) except the appropriate sodium chloride solution was used as the solvent in place of distilled water. The pH of all samples was measured at the time of analysis with a Beckman model G pH meter equipped with glass indicator and calomel reference electrodes.

Effect of Temperature on Adsorption.—Adsorption was measured at 37.0° for chlorpromazine hydrochloride and thiopropazate dihydrochloride by the method described previously (1).

RESULTS AND DISCUSSION

All of the phenothiazine derivatives were adsorbed to a significant extent by the adsorbents tested. The experimental data were plotted according to the following form of the Langmuir equation:

$$\frac{C_{EQ}}{x/m} = \frac{1}{k_1 k_2} + \frac{C_{EQ}}{k_2}$$

where C_{EQ} is the concentration of the phenothiazine derivative remaining in solution at equilibrium, x/m is defined as the amount of compound adsorbed by the quantity of adsorbent used,[6] 1.00 Gm. for kaolin and talc, 0.100 Gm. for activated charcoal, and k_1 and k_2 are constants. The constant k_1 is sometimes called the adsorption coefficient and is related to the force of the interaction between the adsorbent and the bound molecules. The value of k_2 gives the maximum amount of compound which can be adsorbed by the weight of adsorbent used in the experiment. It must be assumed that only a monomolecular layer of solute molecules can be found in order to correctly apply the Langmuir equation.

The best straight line through the experimental data was calculated by the method of least squares. The values of the constants, k_1 and k_2, were obtained from the reciprocals of the slope and intercept values of the regression equation calculated from the experimental data. In each case, linear conformity of the data to the Langmuir equation was checked by graphic plots before calculating the regression equation. In most cases, the data could be expressed in linear form by the Langmuir equation. Values of the Langmuir constant, k_2, are summarized in Table IV. Figure 3 shows the Langmuir isotherms obtained for promazine hydrochloride and fluphenazine dihydrochloride which are typical of the over-all results.

Values of k_2 show that in unbuffered media, kaolin has the weakest adsorbent capacity. Talc possesses adsorbent capacity which is similar to kaolin, although approximately twice as great for any given compound. The similarity in adsorbent capacity between kaolin and talc is not especially surprising since both are basic aluminosilicates although they do differ with respect to physical structure and exchangable cations. Adsorption of all compounds by activated charcoal is much greater in magnitude. Compared on a weight basis, the adsorbent power of charcoal ranges from 20 to 80 times that of kaolin and talc.

Values for the adsorption coefficient, k_1, are not shown in Table IV. In most cases, adsorption approached surface saturation at very low concentration ranges. As a result, due to the lack of data at low degrees of surface saturation, calculation of k_1 values from intercept values of the Langmuir equation is potentially subject to some error. A procedure recommended by Finger *et al.* (9) is equally unsatisfactory for determining k_1 values from these data. The values of k_1 were found to increase from kaolin to talc to charcoal and were generally of a similar order of magnitude within a particular adsorbent series.

The intercept value of the Langmuir equation may also be used for comparing affinities between adsorbents and the phenothiazine derivatives. At the

[6] Since x/m is determined from concentration difference measurements, it is actually the apparent amount adsorbed. Adsorption of solvent by the adsorbent may also alter the solute concentration and hence produce some uncertainty in the value obtained for x/m. Due to the relatively large ratio of solvent to adsorbent in the test systems, the effect of solvent adsorption should be minimal and the values of x/m are considered to be subject to little error from this effect.

TABLE IV.—VALUES OF LANGMUIR CONSTANTS EXPRESSING ADSORPTION OF VARIOUS PHENOTHIAZINE DERIVATIVES BY KAOLIN, TALC, AND ACTIVATED CHARCOAL AT 20°

Compd.	Kaolin Adsorbent		Talc Adsorbent		Charcoal Adsorbent	
	$k_2 \times 10^3$	$k_1k_2 \times 10^{-3}$	$k_2 \times 10^3$	$k_1k_2 \times 10^{-3}$	$k_2 \times 10^3$	$k_1k_2 \times 10^{-3}$
Promazine	21.9	0.299	49.5	1.00	116.	4.08
Mepazine	26.2	0.119	52.9	10.3	91.7	4.76
Trimeprazine	23.0	0.175	47.8	1.15	91.7	2.53
Promethazine	22.4	0.227	44.8	1.11	103.	1.86
Ethopropazine	24.0	0.098	...[a]	...[a]	...[a]	...[a]
Pyrathiazine	18.9	0.267	39.7	1.09	101.	4.24
Chlorpromazine	24.0	0.194	51.8	0.744	102.	4.18
Methoxypromazine	12.0	−2.09	21.0	− .486	75.2	−3.65
Trifluprom azine	19.3	1.39	61.3	2.07	80.0	18.9
Acepromazine	21.8	0.476	35.2	0.825	90.9	1.93
Prochlorperazine	15.2	0.478	...[b]	...[b]	81.3	6.37
Thiopropazate	14.1	0.452	...[b]	...[b]	58.5	2.29
Trifluoperazine	14.5	1.07	...[b]	...[b]	65.8	1.58
Fluphenazine	15.1	0.239	...[b]	...[b]	64.9	3.32
Thioridazine	35.9	2.61	74.1	3.31	72.5	3.53

[a] Evidence indicated a decomposition of the sample with these adsorbents. [b] Data plotted according to the Langmuir equation produced a nonlinear curve, hence the value of the constants k_1 and k_2 could not be determined.

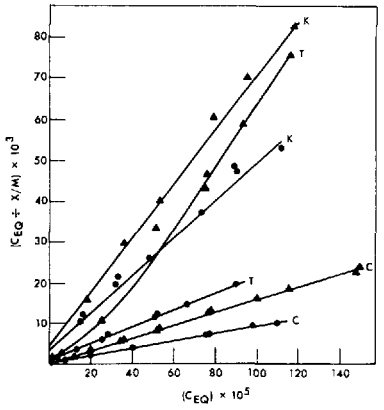


Fig. 3.—Langmuir plots for adsorption in simple aqueous media. Key: ●, promazine hydrochloride; ▲, fluphenazine dihydrochloride; K, kaolin; T, talc; C, activated charcoal. With exception of the curve expressing adsorption of fluphenazine dihydrochloride by talc, the various curves represent regression lines calculated by method of least squares.

lower equilibrium concentrations, *viz.*, at infinite dilution, the Langmuir equation reduces to

$$x/m = k_1k_2\, C_{EQ}$$

The product, k_1k_2, would be the initial slope of the adsorption isotherm and would, in a sense, be an equilibrium constant for adsorption of phenothiazine derivatives in systems well below saturation of the adsorbent surface. Thus, the k_1k_2 values should be useful in comparing the relative strength of interaction between the adsorbents and the various phenothiazine derivatives. Values of k_1k_2 taken from the reciprocals of intercept values of the Langmuir equations expressing experimental data are shown in Table IV. These values are subject to some of the uncertainty discussed for k_1 values, and it is seen that occasionally unexpectedly large variations within a particular adsorbent series are encountered. On the other hand, for a particular adsorbent, the k_1k_2 values are relatively similar and probably reflect essentially similar adsorption affinities. The values generally increase from kaolin to talc to charcoal. It must be remembered, however, that the k_1k_2 value only tells that the three adsorbents have different affinity for the compounds, and one cannot assume much about the mechanism of the interaction from these values.

Adsorption may occur as a result of several possible mechanisms (10–13). Certain mechanisms may involve a specific interaction between the surface of the solid and the adsorbed molecule. On the other hand, solutes which exhibit surface tension lowering properties are often strongly adsorbed by a wide variety of solids since these solutes often tend to accumulate at the solid–liquid interface as readily as at the solution–air interface (10, 12). Phenothiazine derivatives have been shown to exhibit surface tension lowering properties (14–16). Figure 4 presents plots of surface tension lowering *versus* extent of adsorption for the phenothiazine compounds shown in Fig. 1. The coefficient of correlation between extent of adsorption by activated charcoal and surface tension lowering is −0.843. This value is significant at the $P = 0.05$ level. Such correlation does not exist for kaolin and talc adsorbents. This suggests that the ability to accumulate at the solid–solution interface may be responsible for the charcoal–solute interaction. Such a condition is favored by the very large surface area of activated charcoal which offers an extensive interface to the solution phase.[7] The failure of talc and kaolin to exhibit adsorptive properties which correlate to surface tension lowering can be interpreted as indicating that the tendency of solute molecules to accumulate at an interface is not the sole reason why adsorption onto these substances takes place. Since surface areas of kaolin and talc are generally much smaller than for activated charcoal, a smaller interface is offered for accumulation of surface-active solute molecules. Adsorption by interfacial effects cannot be entirely ruled out for kaolin and talc, however, as it is possible that some adsorption occurs *via* this mechanism, but it is obscured by the effects of other factors also facilitating adsorption at the same time.

If adsorption of solute by charcoal is mediated primarily through ability of the solute to accumulate

[7] Cassidy reports (10) surface areas in the range of 15 M.²/Gm. for kaolin, and 500–1700 M.²/Gm. for activated charcoal. Talc could be expected to have a surface area somewere in the range below 50 M.²/Gm. While surface areas were not measured for the specific adsorbents used in this study, they would probably be of a similar order of magnitude.

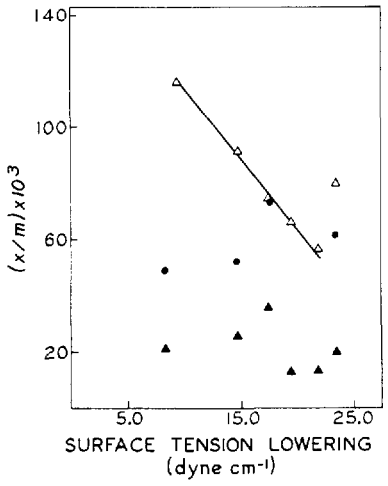


Fig. 4.—Limiting adsorptive capacity as a function of surface tension lowering produced by 1×10^{-2} M solutions of various phenothiazine derivatives. Key: △, charcoal adsorbent; ●, talc adsorbent; ▲, kaolin adsorbent.

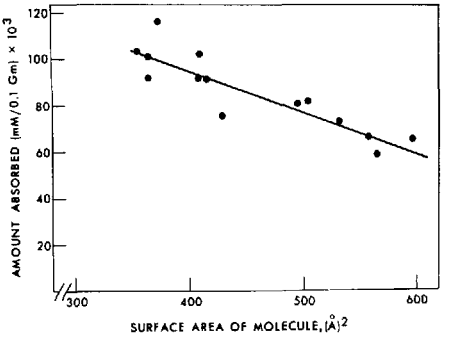


Fig. 5.—Limiting adsorptive capacity as a function of planar surface area of various phenothiazine derivatives adsorbed by activated charcoal.

at the solid–solution interface, a relationship might be expected to exist between the maximum extent of adsorption and the area occupied by the molecules in the interface. Figure 5 shows the apparent relationship between molecular surface area of the various phenothiazine derivatives and the Langmuir constant k_2.[8] The coefficient of correlation for the relationship in Fig. 5 is −0.861 and is significant at the $P = 0.001$ level. A coefficient of correlation equal to −0.886, significant at the $P = 0.01$ level, was obtained when k_2 values were compared to molecular volumes of the solutes. The total surface area of adsorbed molecules corresponds well with surface areas commonly encountered for activated charcoals. In the case of promazine, the total surface area of the molecules adsorbed at saturation of the adsorbent surface is 690 M.²/Gm. activated charcoal.

[8] Space-filling atomic models were used in calculating approximate surface areas of solute molecules. Areas were calculated on the basis of the smallest trapezoid which would contain the model. Molecular volumes were calculated as the smallest truncated pyramid which would contain the model.

Since the phenothiazine derivatives studied are capable of existence entirely or at least partially in a protonated form at the pH ranges of this experiment, it is possible that, in some cases, adsorption may be mediated by ion-exchange interactions. Both kaolin and talc particles would be expected to carry a negative charge (10) on their surface due to deficiencies in certain cations within the crystal structure of these substances. The negative charges are neutralized by exchangeable cations such as sodium and potassium. It is a well-established fact (10–13) that high molecular weight organic cations can displace these inorganic cations and preferentially adsorb to the particle surfaces. Such mechanisms would not account for the high adsorptive capacity of activated charcoal. Although certain charcoals can have some ion-exchange abilities (10), this cannot account for the high affinity of charcoal for solute molecules in the system under study.

The effect of electrolyte on promazine adsorption

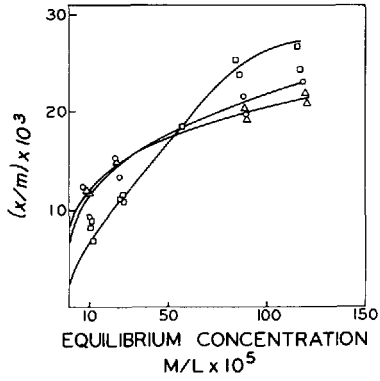


Fig. 6.—Isotherms for adsorption of promazine by kaolin. Key: ○, distilled water; △, 0.01 N sodium chloride; □, 0.10 N sodium chloride.

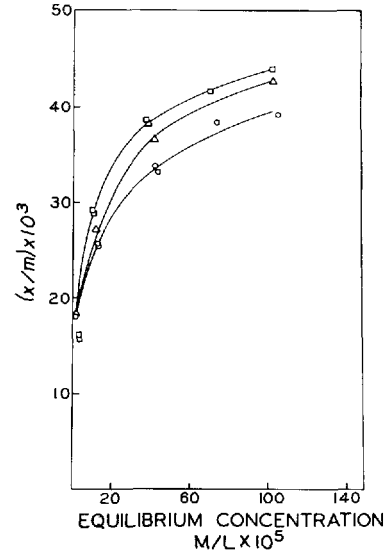


Fig. 7.—Isotherms for adsorption of promazine by talc. Key: ○, distilled water; △, 0.01 N sodium chloride; □, 0.10 N sodium chloride.

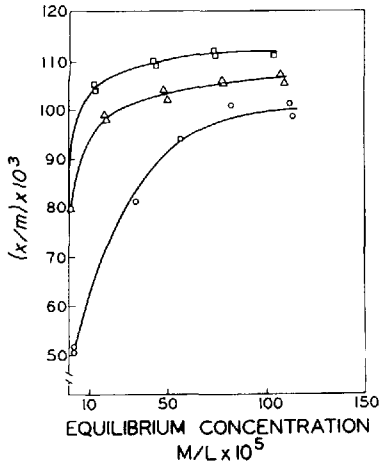


Fig. 8.—Isotherms for adsorption of promazine by activated charcoal. Key: O, distilled water; △, 0.01 *N* sodium chloride; □, 0.10 *N* sodium chloride.

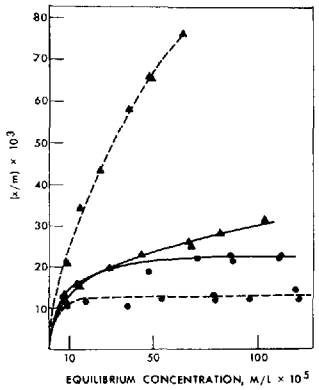


Fig. 9.—Adsorption of promazine hydrochloride (——) and fluphenazine dihydrochloride (- - - - -) by kaolin at pH 2.5 (●) and pH 6.5 (▲).

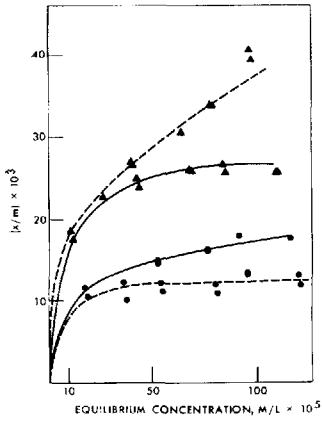


Fig. 10.—Adsorption of promazine hydrochloride (——) and fluphenazine dihydrochloride (- - - - -) by talc at pH 2.5 (●) and pH 6.5 (▲).

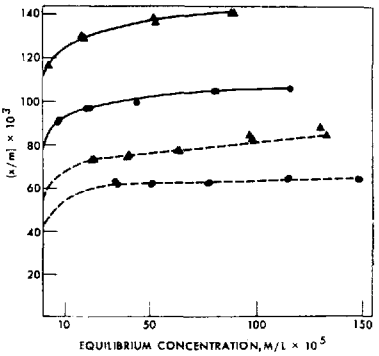


Fig. 11.—Adsorption of promazine hydrochloride (——) and fluphenazine dihydrochloride (- - - - -) by activated charcoal at pH 2.5 (●) and pH 6.5 (▲).

is shown in Figs. 6, 7, and 8. It is seen that increasing the electrolyte concentration increases the adsorptive capacity of the adsorbent.[9] In addition, at any given solute concentration below that necessary to achieve apparent saturation of the adsorbent surface, the extent of adsorption is increased. Measurements showed that pH remained unchanged in the presence of increasing amounts of sodium chloride. Sodium ion, as well as other electrolyte species, would be expected to compete with the protonated amine for anionic sites on the particle surface or for positions in the electrical double layer around the particles. Hence, addition of large amounts of sodium ion might be expected to decrease the extent of adsorption of the protonated species of the amine if ion-exchange mechanisms were responsible for adsorption (12, 13). Failure of sodium ion to displace the organic cation suggests that adsorption of the phenothiazine derivative is mediated through other than simple ion-exchange mechanisms alone or that even in great excess, sodium ion cannot displace the amine from the adsorption sites. The fact that adsorption of the phenothiazine derivative is actually increased by the sodium ion supports the idea that ion-exchange is not the sole mechanism for adsorption. Even if the sodium ion could not displace the solute, it should not increase adsorption if ion-exchange was solely responsible for adsorption of the phenothiazine derivative. Sodium chloride may be exerting its action through effects on solubility and other physical properties of the phenothiazine derivative. The apparent result is an increasing tendency of the phenothiazine derivative to accumulate at the solution–solid interface. Electrolyte may affect physical properties of the adsorbent as well.

The effect of 0.01 *N* sodium chloride on adsorption of promazine hydrochloride by kaolin is of interest. It may be possible to explain this effect on the basis that at low promazine hydrochloride concentrations, there is competition for exchange sites which leads to decreased adsorption. At higher promazine hydrochloride concentrations, the sodium ion is displaced. Increased adsorption at high concentrations of promazine hydrochloride may be due to effects of

[9] It may be noted that data presented in Figs. 6, 7, and 8 differ from Table IV. These experiments were carried out at a later date using different adsorbent to solvent ratios. It has been pointed out (10) that limiting adsorptive capacities may vary somewhat when measured at different adsorbent–solvent ratios. Data presented in Figs. 6, 7, and 8 should be considered separately from Table IV.

TABLE V.—pH OF VARIOUS UNBUFFERED ADSORBENT–SOLUTE MIXTURES[a]

Adsorbent–Solute System	pH of System at Max. Equilibrium Concn. of Solute	pH of System at Min. Equilibrium Concn. of Solute
Kaolin–promazine	4.6	5.0
Talc–promazine	7.6	8.6
Charcoal–promazine	4.0	4.2
Kaolin–fluphenazine	3.6	4.2
Talc–fluphenazine	4.1	7.8
Charcoal–fluphenazine	3.3	3.4

[a] The pH values for adsorbent suspensions containing no solute were kaolin, 4.3; talc, 8.4; and charcoal, 5.9.

sodium chloride on solubility, etc., as discussed. On the basis of present data, it does not appear possible to either prove or disprove ion-exchange as an important mechanism in adsorption of the phenothiazine derivative by kaolin and talc.

Results of adsorption studies at constant pH are presented in Figs. 9, 10, and 11. In all cases, adsorption is seen to be greater at pH 6.5 than at pH 2.5. A possible explanation for this pH effect could be involved with changes which occur in the relative amounts of protonated and nonprotonated amine present as pH increases. If the nonprotonated form of the phenothiazine derivative is better adsorbed as is often the case for systems of this type (10, 12), then adsorption would be expected to be greater at the higher pH. Inspection of pKa values in Table III shows that even at pH 6.5, only a relatively small portion of the total amine is in the nonprotonated form since this pH is several units below the apparent pKa. It is difficult to conceive that there would be enough free base present at pH 6.5 to exert a significantly greater driving force for adsorption as compared to lower pH levels. On the other hand, the equilibrium between protonated and nonprotonated forms is dynamic. As free base is removed by adsorption, it will be replaced from the large reservoir of protonated material. Adsorption could thus continue until equilibrium is established between the adsorbent surface and the free base in solution. The free base concentration is indeed many times greater at pH 6.5 than at the lower pH. It is not impossible that this difference may account for the greater adsorption at higher pH values.

A second possible explanation for the effect of pH might lie in the possibility that hydrogen ions and the protonated amine may compete for anionic sites on the adsorbent surface of, for positions in, the electrical double layer existing around the adsorbent particles.

As compared to kaolin and talc, adsorption of phenothiazine derivatives by charcoal is less strongly influenced by hydrogen-ion concentration, although some charcoals have a moderate electrostatic charge and some affinity for hydrogen ions (10). It is probable that the observed pH effects for charcoal result from increases in the amount of nonprotonated form of the phenothiazine derivative present at the higher pH levels. The nonprotonated forms of the phenothiazine derivatives have low water solubility and hence would have a greater tendency to accumulate at the solid–solution interface than would the protonated form.

Adsorption of solute by talc is further illustrative of pH effects on adsorption. Adsorption of promazine by talc is greatest from unbuffered solution. The reason for this becomes apparent if one considers the pH of the unbuffered systems containing talc (Table V). The pH of unbuffered suspensions containing talc is usually greater than pH 6.5, and, hence, it is not surprising that absorption from unbuffered media is even greater than from the pH 6.5 system. When Figs. 9 and 10 are compared, it is seen that under conditions of controlled pH, kaolin is actually a better adsorbent than is talc. Data in Table IV, which show talc to be a stronger adsorbent in unbuffered media, are primarily a result of the more alkaline pH of the talc systems. It appears that at a given fixed pH, there is actually not much difference between talc and kaolin with respect to adsorbent capacity.

The talc–fluphenazine dihydrochloride system shows interesting pH effects (Fig. 12). In unbuffered solutions, the more acidic proton on the fluphenazine, pKa 3.90, causes production of a relatively low pH at higher solute concentrations, and the fluphenazine is adsorbed less strongly as a result. In fact, in systems containing talc, adsorption of fluphenazine from unbuffered solutions at the higher concentration ranges is very nearly identical to adsorption from buffered solution at pH 2.5. Due to the pH effect, isotherms for fluphenazine in unbuffered talc systems, as well as for other dibasic phenothiazine derivatives, pass through a maximum with increasing solute concentration (Fig. 13). Monobasic phenothiazine derivatives such as promazine, pKa 9.38, are unable to lower the pH of talc systems to an extent that similar pH effects are observed in unbuffered media.

The fact that the ionic charge on the fluphenazine molecule is approximately doubled at pH 2.5, as compared to pH 6.5, must also be considered when explaining pH effects on adsorption of the dibasic compounds. If ionic interactions are responsible for

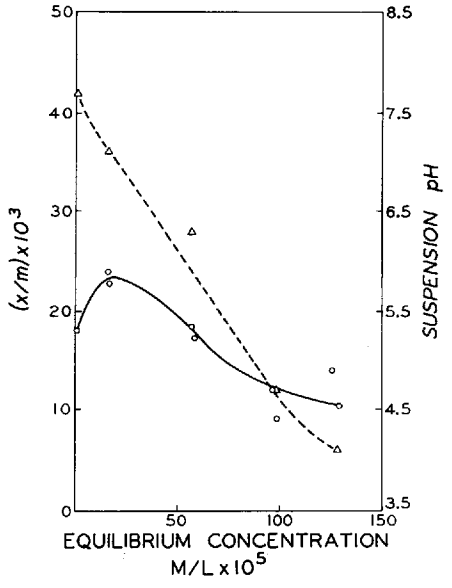


Fig. 12.—Relationship between the extent of adsorption of fluphenazine dihydrochloride by talc (O) and suspension pH (△).

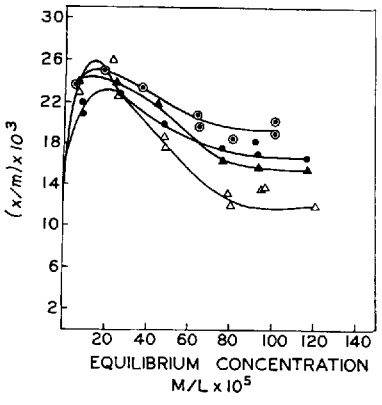


Fig. 13.—Isotherms for adsorption by talc in unbuffered media. Key: ⊙, prochlorperazine dihydrochloride; ●, trifluoperazine dihydrochloride; ▲, fluphenazine dihydrochloride; △, thiopropazate dihydrochloride.

adsorption, the compounds could potentially neutralize twice as many sites on the adsorbent surface at the lower pH and hence adsorption would be reduced to about 0.5 its value at the higher pH range. Inspection of the data shows that adsorption is decreased by a much greater factor at the lower pH. The adsorption of monobasic compounds is also decreased at low pH, even though there could be no change in the charge of the solute molecule. Thus, changes in total charge on the solute molecule do not appear to cause the observed pH effect on adsorption.

The relationship between pH and the extent of adsorption by talc lends support to the idea that the pH effect is due to changes in the amount of free base present. At pH 8.0, the midrange of the talc systems, 4% of the total amine content is in the base form. At pH 6.5, only about 0.13% exists in this form.

If adsorption is strongly influenced by the amount of free base present in the system, a correlation might be expected to exist betwen pKa and adsorption. Plots of apparent pKa *versus* the Langmuir constant, k_2, failed to show any correlation between these parameters. Likewise, apparent pKa and values for k_1k_2 could not be correlated. The lack of correlation is not surprising in view of the relatively slight differences between apparent pKa values for the compounds tested (Table III).

It is not possible to state on the basis of information obtained from these pH studies whether the observed effects are due to one specific certain mechanism. The fact that considerable adsorption occurs at pH 2.5 supports the hypothesis that adsorption of the protonated form also occurs to a considerable extent. Adsorption of solute by kaolin and talc is probably mediated through a combination of mechanisms.

Increasing temperature generally decreases the extent of adsorption. However, it is not uncommon to find that adsorption increases with temperature in certain systems (11, 12). Temperature can affect several properties of the solute molecules and the adsorbent (Table VI), as well as the adsorption interaction *per se*. The mechanism by which temperature increases adsorption in the systems under study is not known. Further investigations of the temperature effects are planned.

The effect of electrolyte and hydrogen-ion concentration is interesting with respect to potential implications regarding the effect of adsorption on the availability of certain drugs for absorption from the gastrointestinal tract. It has already been established (2) for a similar clay-type adsorbent and activated charcoal that adsorbents may alter the availability of promazine for absorption from the gastrointestinal tract. Within the stomach, the presence of hydrogen ion may cause desorption, thus favoring absorption of the drug. The electrolyte environment existing throughout the gastrointestinal tract would probably favor absorption and retard release of adsorbed drug to the fluids at the absorption site. It should be noted that the effect of electrolyte on promazine adsorption was predicted incorrectly (2). While this does not alter the validity of the research, statements made previously regarding the probable effect of electrolyte on release of promazine should be reconsidered.

It is also important that electrolyte not only changes the maximum amount of drug which can be adsorbed, but also increases the amount adsorbed at any given concentration below that which achieves surface saturation. Thus, for talc and activated charcoal, the plateau region corresponding to surface saturation is achieved at a much lower solute concentration. In terms of potential *in vivo* effects, this may be quite important. According to hypotheses presented previously (2), the amount of drug which can exist free in equilibrium with a given amount of adsorbate may be a determinant factor in modifying drug uptake from the gastrointestinal tract when adsorbents are present. These effects will bear further investigation. The reader should refer to *Reference 2* for additional discussions concerning the effects of adsorption on drug availability.

SUMMARY

It can be stated that the adsorption interaction between medicinally active phenothiazine derivatives and charcoal occurs to a significant extent in all cases studied. The mechanisms of the interaction cannot be completely defined from data obtained in experiments described in this paper. Adsorption

Table VI.—Effect of Temperature on Adsorption of Chlorpromazine Hydrochloride and Thiopropazate Dihydrochloride

	Limiting Adsorption Capacity of Adsorbent mM × 10³					
	Kaolin		Talc		Activated Charcoal	
Compd.	20°	37°	20°	37°	20°	37°
Chlorpromazine hydrochloride	24.0	27.1	51.8	70.4	102	110
Thiopropazate dihydrochloride	14.1	33.9	...[a]	...[a]	58.5	80.0

[a] The Langmuir plots for adsorption of thiopropazate dihydrochloride by talc were nonlinear and hence a limiting adsorptive capacity could not be calculated. The effect of temperature appeared to be negligible in this case, however, and the two isotherms were virtually identical.

by charcoal appears to be mediated primarily by physical forces and probably occurs as a result of the tendency of the solute to accumulate at solution–solid interfaces. This tendency to accumulate at the solution–solid interface is similar to the demonstrated surface tension lowering effects of the phenothiazine derivatives.

Adsorption of phenothiazine derivatives by clay-type materials such as kaolin and talc is more complex in nature and is probably mediated through several different mechanisms simultaneously. Mechanisms based on simple electrostatic charge interactions cannot alone explain the process. Molecular size and interfacial properties of the solute do not appear to correlate with adsorption in systems containing kaolin or talc in simple aqueous media.

The results of this experiment show that adsorption of phenothiazine derivatives is dependent upon both hydrogen-ion and electrolyte concentration. Hydrogen-ion concentration is inversely related to the extent of adsorption. The effect of hydrogen-ion may be either to determine the amount of nonprotonated form which is present or to compete with the protonated form for adsorption sites. It is probable that adsorption of phenothiazine derivatives by talc and kaolin is subject to both of these effects. The presence of sodium chloride generally increases adsorption. This is probably due to effects on various physical properties of the solute which increase its tendency to accumulate at the solution–solid interface. The failure of sodium chloride to depress adsorption tends to discount simple ion-exchange as a major mechanism of adsorption.

The effects of pH and electrolyte concentration have important implications with respect to the effect of adsorbents in altering availability of promazine for absorption from the gastrointestinal tract.

The results of these experiments will be useful in further studies of the effect of adsorbents on drug availability. They should also be of use in interpretation of drug–adsorbent interactions in general.

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Inositol N.F.

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An investigation of the suitability of inositol as a base for chewable tablets has been conducted by studying and comparing pertinent properties of inositol, mannitol, lactose, and a lactose–sucrose mixture (9:1). The amounts of moisture absorbed by the finely powdered materials, granulations made from these powders with the aid of selected binders, and tablets compressed from some of the granulations were determined (Karl Fischer method or difference in weight) before and after storage at selected relative humidities for specified periods of time. Tablets of similar weights and volumes were prepared from inositol as well as mannitol granulations and evaluated for hardness and dissolution times before and after aging and compared. Representative chewable tablets utilizing mannitol and inositol as bases were prepared for vitamins, antacids, and for acetylsalicylic acid and evaluated for compressability, hardness, dissolution, taste, and appearance. The experimental data indicate that inositol, due to its nonhygroscopic nature, chemical inertness, nontoxicity, physical stability, superior mouthfeel, and texture can be beneficially employed as the base for the formulation of chewable tablets.

ONE OF the recent modifications in tablet dosage forms has led to the development of the chewable tablet, a compressed dosage form, conveniently carried and self-administered, which can be chewed or sucked without the aid of external liquid (1, 2). Daoust and Lynch (1) state that the ideal chewable tablet must be nonhygroscopic and chemically stable, that it

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by charcoal appears to be mediated primarily by physical forces and probably occurs as a result of the tendency of the solute to accumulate at solution–solid interfaces. This tendency to accumulate at the solution–solid interface is similar to the demonstrated surface tension lowering effects of the phenothiazine derivatives.

Adsorption of phenothiazine derivatives by clay-type materials such as kaolin and talc is more complex in nature and is probably mediated through several different mechanisms simultaneously. Mechanisms based on simple electrostatic charge interactions cannot alone explain the process. Molecular size and interfacial properties of the solute do not appear to correlate with adsorption in systems containing kaolin or talc in simple aqueous media.

The results of this experiment show that adsorption of phenothiazine derivatives is dependent upon both hydrogen-ion and electrolyte concentration. Hydrogen-ion concentration is inversely related to the extent of adsorption. The effect of hydrogen-ion may be either to determine the amount of nonprotonated form which is present or to compete with the protonated form for adsorption sites. It is probable that adsorption of phenothiazine derivatives by talc and kaolin is subject to both of these effects. The presence of sodium chloride generally increases adsorption. This is probably due to effects on various physical properties of the solute which increase its tendency to accumulate at the solution–solid interface. The failure of sodium chloride to depress adsorption tends to discount simple ion-exchange as a major mechanism of adsorption.

The effects of pH and electrolyte concentration have important implications with respect to the effect of adsorbents in altering availability of promazine for absorption from the gastrointestinal tract.

The results of these experiments will be useful in further studies of the effect of adsorbents on drug availability. They should also be of use in interpretation of drug–adsorbent interactions in general.

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must disintegrate smoothly at a satisfactory rate, have a pleasant taste and mouthfeel, and leave no unpleasant aftertaste.

Since the excipient constitutes usually a large proportion of the substance of the tablet, the physical characteristics of the dosage form are necessarily influenced by those of the excipient, although not all to the same degree.

Extensive studies (1, 3) have been conducted on sugars and sugar alcohols in order to determine their suitability as bases for chewable tablets with the results indicating that mannitol possesses all the desirable characteristics of the "preferred excipient." No evaluation has been reported on the use of cyclic polyhydroxic alcohols for this purpose, although it was noted that inositol possesses many of the same desirable characteristics and properties (4–6). Inositol N.F. is an odorless, nonhygroscopic, chemically stable, sweet-tasting, white, crystalline material having a negative heat of solution (7) and is soluble to the extent of 1 Gm. in 5.7 ml. of water at 25°. This cyclic alcohol is widely distributed in nature being found in nearly all living cells investigated (8) and has demonstrated no toxicity.

With this information in mind and with the intent of possibly improving a well-accepted dosage form while, at the same time, extending the usefulness of an abundant natural product, a study was undertaken to determine the suitability of a cyclic polyhydroxic alcohol, inositol N.F., as a base for chewable tablets. The investigation included a comparison of some of the more pertinent properties of inositol, granulations of inositol, and tablets made from inositol with those corresponding properties of some accepted excipients.

EXPERIMENTAL

Materials.—The mannitol N.F. and inositol N.F. were obtained in powdered form from Mann Research Laboratories and the lactose U.S.P. was purchased from City Chemical Corp. Other materials used included sucrose U.S.P., gelatin U.S.P., potato starch,[1] acacia U.S.P., methylcellulose U.S.P. (1500 cps.), alcohol U.S.P., magnesium stearate U.S.P., magnesium trisilicate U.S.P., aluminum hydroxide,[1] aspirin U.S.P., liquid glucose U.S.P., and sodium saccharin N.F. The vitamins used in the preparation of the multivitamin tablets were U.S.P. grade as obtained from Mann Research Laboratories.

Preparation of Powders.—The finely powdered mannitol, inositol, and lactose were passed through a 60 mesh screen in preparation for the moisture absorption studies and prior to the preparation of the various granulations.

[1] Obtained from J. T. Baker Chemical Co., Phillipsburg, N. J.

Preparation of Granulations.—Four series of granulations were prepared using mannitol, inositol, lactose, and lactose–sucrose (9:1) as bases according to the following formula: base, 993 Gm.; sodium saccharin, 7 Gm.; binder solution, as required.

Five granulations were prepared from each of the four bases utilizing purified water, 10% acacia solution, 10% gelatin solution, 10% starch paste, and diluted alcohol U.S.P. as the binder solutions. The volumes of granulating solutions required for the granulation of 500 Gm. of each base are shown in Table I.

The dry and previously mixed materials were moistened with the binder solutions and kneaded by hand until masses were formed. The wetted masses were then passed through an Erweka F.G.S. granulator fitted with a screen having 1.5 mm. openings (approximately 14 mesh). The resulting wet granules were dried in a Glatt TR-2 fluidized bed dryer at 40° with a gate opening of 2.5 for a period of 30 min. for the mannitol and inositol granulations, and for 50 min. for those granulations containing lactose and the lactose–sucrose mixture. The increased drying times for the lactose and lactose–sucrose granulations were necessary in order to reduce the moisture content to an acceptable level as determined by the Karl Fischer method. The moisture content of each of the dried granulations expressed as averages of triplicate determinations is shown in Table I. After drying, the granules were passed through a No. 18 screen and stored in airtight containers for future use.

Additional series of granulations were prepared both from mannitol and from inositol, but using the No. 14 sieve as the final size in order to obtain larger granules. Similar granulations were prepared with 2% liquid glucose added as an additional binder solution. These granulations also were reserved for further study.

Compression of Granulations.—Portions (1000 Gm.) of each of the prepared granulations were mixed with portions (10 Gm.) of magnesium stearate as lubricant with the aid of an Erweka cube mixer. The granulations were then compressed into tablets on a Colton model 204 four-station rotary press equipped with a model 205 induced die feeder and 0.5 in. flat-faced, beveled-edged punches. The representative weight, hardness, thickness, and dissolution times of the resulting tablets are shown in Table II. The dissolution times were determined with the aid of a U.S.P. apparatus and taken at the time when all of the tablet passed through the screen of the apparatus.

Hygroscopicity Studies.—*Powdered Materials.*—Accurately weighed samples (approximately 100 mg. each) of the powdered inositol, the powdered mannitol, and the powdered lactose were placed in open wide-mouth glass vials and stored for 96 hr. in relative humidities of approximately 65 and 100%. Previous experiments indicated that similar samples of the inositol reached maximum moisture concentration in less than 72 hr. The controlled humidity chambers were prepared using desiccators containing saturated solutions of suitable salts (9) and maintaining the temperature at 24° ± 2° with relative humidities of approximately 10, 51, 65, 84, 95, and 100% being produced.

The moisture contents of the powdered materials

TABLE I.—PROPERTIES OF SOME GRANULATIONS PREPARED WITH DIFFERENT BINDERS AND BASES

Binder	Base	Vol. of Binder Soln. Used, ml./500 Gm.	% Moisture After Drying	Hardness of Granules	Percentage Fines[c]
Acacia, 10% soln.	Inositol	110	3.3[a]	Hard	20.0
	Mannitol	125	3.7[a]	Hard	20.0
	Lactose	107	7.4[b]	Hard	12.5
	Lactose + sucrose (9:1)	80	7.7[b]	Hard	13.0
Gelatin, 10% soln.	Inositol	105	3.6[a]	Hard	13.1
	Mannitol	112	3.5[a]	Hard	17.1
	Lactose	100	6.4[b]	Hard	15.1
	Lactose + sucrose (9:1)	74	6.6[b]	Hard	13.0
Starch, 10% soln.	Inositol	130	2.3[a]	Fairly hard	13.0
	Mannitol	140	3.1[a]	Fairly hard	20.0
	Lactose	100	6.2[b]	Hard	17.0
	Lactose + sucrose (9:1)	75	7.9[b]	Hard	15.5
Diluted alcohol U.S.P.	Inositol	155	3.0[a]	Soft	28.0
	Mannitol	170	2.1[a]	Very soft	38.0
	Lactose	125	6.4[b]	Soft	21.3
	Lactose + sucrose (9:1)	86	5.7[b]	Fairly hard	23.0
Water	Inositol	140	2.5[a]	Very soft	32.0
	Mannitol	155	3.0[a]	Very soft	41.0
	Lactose	112	8.2[b]	Soft	49.0
	Lactose + sucrose (9:1)	63	7.4[b]	Fairly hard	38.0

[a] Drying time, 30 min.; Glatt TR-2 dryer; temp., 40°; gate opening, 2.5. [b] Drying time, 50 min.; Glatt TR-2 dryer; temp., 40°; gate opening, 2.5. [c] Amount of material passing through a No. 40 sieve.

TABLE II.—SOME PHYSICAL PROPERTIES OF TABLETS[a] PREPARED FROM GRANULATIONS OF INOSITOL OR MANNITOL

Binder, 10% Soln.	Base	Tablet, Wt., Gm. ± S.D.	Thickness, mm. ± S.D.	Dissolution Time, min. ± S.D.	Hardness of Tablets (Kg. Stokes) Initial	Aged 2 Mo.	Aged 6 Mo.
Acacia	Inositol	0.620 ± 0.013	3.77 ± 0.02	26.6 ± 1.0	5.0	6.8	13.5
	Mannitol	0.623 ± 0.010	3.76 ± 0.01	30.0 ± 1.6	5.0	8.0	11.0
Gelatin	Inositol	0.555 ± 0.018	3.88 ± 0.07	36.6 ± 2.0	6.5	9.2	13.5
	Mannitol	0.557 ± 0.017	3.89 ± 0.01	40.6 ± 2.0	7.5	10.4	13.0
Starch	Inositol	0.590 ± 0.017	3.70 ± 0.07	41.6 ± 2.0	2.5	7.6	11.0
	Mannitol	0.583 ± 0.024	3.75 ± 0.06	46.1 ± 1.0	3.0	4.0	6.5

[a] Compressed using 0.5 in. beveled edge punches (flat-faced).

TABLE III.—MOISTURE CONTENT (%) IN POWDERS[a] AFTER STORAGE[b] AT SELECTED RELATIVE HUMIDITIES

Base	Relative Humidity 65%	100%
Inositol	1.00	2.93
Mannitol	0.99	2.66
Lactose	5.16	5.13

[a] Sample size approximately 100 mg. of finely divided material. [b] Stored for 96 hr. at 24° ± 2°.

so treated were determined by the Karl Fischer method (10) with the aid of a Lab Industries aquametry apparatus. The results, averages of triplicate determinations, are shown in Table III.

Granulations.—Each of the granulations was analyzed for moisture content when freshly prepared (Table I) and after storage for 96 hr. in the controlled humidity chambers (Table IV). The moisture contents of powders and granulations were determined by the Karl Fischer method with minor modifications. A measured portion of anhydrous methanol (60 ml.) was titrated to the Karl Fischer end point, after which 10 ml. of this solution was removed and retained in a pipet. The sample for analysis was then washed with this 10 ml. of solution through a funnel into the receiver of the aquametry apparatus. The mixture was stirred magnetically for 2 min., after which it was titrated to a Karl Fischer end point which remained stable for 30 sec.

Tablets.—The extent of moisture absorption or desorption by tablets was determined by the differences in weights observed before and after storage for 20 days in the controlled humidity chambers.

The tablets of mannitol and inositol prepared using selected binder solutions were accurately weighed, stored for 20 days in the previously described controlled humidity chambers, then reweighed. The percentage by weight of moisture absorbed or desorbed was calculated from the change in weight of the tablets. The results are shown in Table V.

Preparation of Representative Tablets.—Three different types of tablets, antacid, multivitamin, and aspirin tablets, were prepared using inositol and mannitol as bases. The formula (11) for the antacid tablets was modified by the use of gelatin solution as the binder and the omission of the disintegrator. In one batch of the antacid tablets inositol was used in place of mannitol; batches of both types of tablets were prepared, compared, and evaluated. The results are shown in Table VI.

Aspirin tablets were prepared according to a published formula (12) modified by the use of 10% gelatin solution as the binder and the omission of a

disintegrator. Batches of tablets were prepared with both inositol and mannitol as bases and were compared and evaluated. The results are shown in Table VI.

Two batches of multivitamin tablets were prepared with mannitol as the base in one and with inositol as the base in the other. The published formula (11) was modified through use of 10% gelatin solution as the binder and the omission of the disintegrator. The prepared tablets were compared and evaluated with the results being shown in Table VI.

DISCUSSION AND RESULTS

In order to limit the maximum particle size and minimum specific surfaces for the powders examined for their hygroscopic nature, the materials were passed through a 60-mesh sieve. This procedure was considered advisable to ensure more uniform and complete moisture absorption by the powdered materials. In order to reduce the time required for maximum absorption of moisture the weights of the samples exposed at the various relative humidities were limited to approximately 100 mg. each, which were spread in thin layers.

The results of the experiment, as shown in Table III, indicate that for the range of relative humidities studied, the hygroscopic nature of inositol is very similar to that of mannitol, the hygroscopicity of both being much less than that of lactose.

A comparison of the physical properties of the various granulations (Table I) shows that the inositol granulations required less binder solution than did the mannitol granulations and that both of these required significantly more binder solution than did the others tested. The hardness of the granules and the percentages of fines in the granulations indicated that a strong binding material must be used to make satisfactory granulations from

TABLE IV.—MOISTURE CONTENT OF GRANULATIONS AFTER STORAGE FOR 96 hr. AT SELECTED RELATIVE HUMIDITIES (% ± S.D.)

Binder, 10% Soln.	Base	Relative Humidity 10%	51%	65%	84%	95%	100%
Acacia	Inositol	1.51 ± 0.17	1.64 ± 0.17	1.70 ± 0.21	2.16 ± 0.25	3.83 ± 0.30	10.10 ± 0.29
	Mannitol	1.83 ± 0.19	2.00 ± 0.16	3.75 ± 0.20	3.85 ± 0.32	5.03 ± 0.16	11.40 ± 0.47
	Lactose	9.68 ± 0.37	9.96 ± 0.19	10.30 ± 0.10	11.30 ± 0.10	12.20 ± 0.36	13.20 ± 0.20
	Lactose + sucrose (9:1)	8.20 ± 0.40	9.20 ± 0.25	9.40 ± 0.30	9.61 ± 0.36	11.80 ± 0.23	X[a]
Gelatin	Inositol	2.61 ± 0.38	3.80 ± 0.26	4.02 ± 0.40	4.08 ± 0.19	4.09 ± 0.28	13.20 ± 0.42
	Mannitol	2.17 ± 0.10	3.73 ± 0.36	5.03 ± 0.28	5.20 ± 0.36	8.31 ± 0.22	16.51 ± 0.19
	Lactose	7.90 ± 0.31	8.20 ± 0.24	8.30 ± 0.15	9.20 ± 0.23	11.20 ± 0.17	13.00 ± 0.29
	Lactose + sucrose (9:1)	8.30 ± 0.22	8.98 ± 0.38	10.40 ± 0.32	11.20 ± 0.33	12.80 ± 0.32	X[a]
Starch	Inositol	2.75 ± 0.10	3.84 ± 0.30	5.80 ± 0.36	5.87 ± 0.36	5.89 ± 0.33	12.50 ± 0.10
	Mannitol	2.60 ± 0.10	5.91 ± 0.60	6.10 ± 0.44	7.22 ± 0.37	7.35 ± 0.33	16.50 ± 0.11
	Lactose	7.98 ± 0.28	8.14 ± 0.34	8.15 ± 0.35	8.64 ± 0.29	11.90 ± 0.44	17.18 ± 0.42
	Lactose + sucrose 9:1	8.00 ± 0.28	8.06 ± 0.23	8.47 ± 0.37	9.68 ± 0.22	13.62 ± 0.31	X[a]

[a] X, liquifaction.

TABLE V.—GAIN OR LOSS OF MOISTURE OF TABLETS AFTER STORAGE[a] AT SELECTED RELATIVE HUMIDITIES EXPRESSED AS PER CENT OF WEIGHT OF FRESHLY PREPARED TABLET

Binder, 10% Soln.	Base	Relative Humidity 10%	51%	65%	84%	95%
Acacia	Inositol	(0.18)[b]	0.30	0.95	1.56	2.90
	Mannitol	(0.23)	0.16	0.70	1.50	2.50
Gelatin	Inositol	(0.10)	0.25	1.05	2.00	3.27
	Mannitol	(0.40)	0.23	1.00	1.60	3.30
Starch	Inositol	(0.10)	0.13	0.60	1.19	1.60
	Mannitol	(0.13)	0.13	0.61	0.90	1.70

[a] Equals 20 days at 24° ± 2°. [b] = Values in parentheses signify loss in weight.

TABLE VI.—SOME PHYSICAL PROPERTIES OF REPRESENTATIVE TABLETS

Type	Base	Wt., Gm. ± S.D.	Thickness, mm. ± S.D.	Dissolution Time, min. ± S.D.	Hardness, Kg. Stokes Initial	Hardness, Kg. Stokes Aged 2 Mo.
Antacid	Inositol	0.680 ± 0.025	4.22 ± 0.02	45.0 ± 1.6	6.0	6.5
	Mannitol	0.687 ± 0.036	4.67 ± 0.01	52.0 ± 1.7	6.0	7.5
Aspirin	Inositol	0.791 ± 0.032	4.72 ± 0.02	61.0 ± 0.7	6.0	6.0
	Mannitol	0.788 ± 0.041	4.88 ± 0.02	65.0 ± 1.3	7.0	7.5
Multivitamin	Inositol	0.341 ± 0.011	4.03 ± 0.01	15.0 ± 0.2	1.5	1.0
	Mannitol	0.336 ± 0.017	4.20 ± 0.01	22.0 ± 0.3	2.0	1.5

inositol and mannitol. The data of Table I also reveal that the inositol and mannitol granulations are dried more easily and thoroughly than the others examined.

The studies of the absorption of moisture were conducted on those granulations prepared with stronger binding solutions with the results (Table IV) showing that the inositol granulations generally absorbed less moisture than did those others tested. The inositol and mannitol granulations both showed little tendency to absorb moisture even at 95% relative humidity, with the inositol granulations demonstrating significantly less absorption of moisture at all the higher relative humidities. It should be noted that both of these granulations showed a sharp increase in moisture content and that the lactose–sucrose (9:1) granulation liquified at 100% relative humidity. This demonstrated that low degree of hygroscopicity can be expected to enhance the stability of solid dosage forms prepared from the inositol granulations.

The compression of the inositol and mannitol granulations into tablets was accompanied by considerable capping. Attempts to modify the granulations by decreasing the percentage of fines and by increasing the granulation size to 14 mesh produced no significant improvement in compressibility. Although the compression characteristics of both granulations were greatly improved by the addition of 2% liquid glucose as an auxiliary binder, the tablets prepared by these granulations were not studied because of the known and undesirable hygroscopic nature of liquid glucose. Suitable tablets were prepared from the original granulation (18 mesh) but with reduced hardness as measured by a Stokes hardness tester.

The hardness of these tablets increased considerably on aging, as indicated in Table II. The dissolution times of the prepared tablets show that those prepared from the inositol granulations dissolved in a shorter period of time than did those prepared from the mannitol granulations. This quality, although not too important in "chewable tablets," does deserve mention.

The period of time that the tablets prepared with inositol and mannitol as excipients were stored at controlled relative humidities was limited by mold growth to 20 days. The behavior of the tablets in respect to moisture absorption compares very closely, with each series demonstrating almost identical absorption as shown in Table V.

The problem of capping did not occur during the compression of representative tablets in which the bases, inositol and mannitol, were diluted with the therapeutically active substances. Excellent antacid and aspirin tablets were easily prepared having smooth, glossy surfaces and having equally elegant appearances, but the multivitamin tablets could not be compressed to a hardness above 2.0 Kg. When special granulations using methylcellulose and alcohol as binders were prepared and compressed, the maximum hardness achieved was 2.5 Kg. as measured by the Stokes hardness tester. All of the vitamin tablets had sufficient surface hardness such that chipping, powdering, and breaking did not occur.

The dissolution times of these representative tablets prepared with inositol as the base were consistently less than the corresponding tablets prepared with the mannitol.

One of the important qualities of mannitol which contributes to its utility as a base for chewable tablets is the ability to mask the unpleasant tastes of some drugs. It was noted that inositol possesses this same characteristic as reported by randomly selected and untrained subjects. In addition, inositol has a mouthfeel as comparably pleasant and cool as that described for mannitol.

CONCLUSIONS

1. Inositol N.F. possesses those properties described as characteristic of the ideal excipient for chewable and other compressed tablets.
2. Granulations prepared from inositol N.F. generally absorbed less moisture from varied environmental conditions than did granulations prepared from mannitol N.F. and other excipients.
3. Tablets prepared from inositol granulations compare favorably with those prepared from mannitol granulations in respect to appearance, taste, dissolution times, low degree of moisture absorption, and hardness.
4. The results of the study indicate that further evaluation of tablets prepared from granulations of inositol N.F. in order to determine stability, drug release characteristics, and drug availability is warranted.
5. In view of the results of this study, inositol N.F. should be seriously considered as an excipient for chewable and other compressed tablets.

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Production of Pantothenic Acid and Inositol by *Chlorella vulgaris* and *C. pyrenoidosa*

By ROBERTSON PRATT and EVELYN JOHNSON

Two unicellular green algae, *Chlorella vulgaris* and *C. pyrenoidosa*, were compared with respect to pantothenic acid and inositol content at different times during a 3-week culture cycle. Pantothenic acid was found in the cells and in the external medium at all times: the concentration in the cells (mmcg./mg. dry weight) decreased sharply toward the end of the first week and then less abruptly through most of the remaining 2 weeks. However, due to increase in the cell mass, the absolute yield (mmcg./ml. harvested culture) increased. The concentration in the external medium, after a relatively small decline during the first week, rose substantially and at the end of the culture period far exceeded that in the cells. Inositol content of the cells increased slowly throughout the culture period. No extracellular inositol was detected at any time. *C. pyrenoidosa* excels *C. vulgaris* as a source of both compounds.

PREVIOUS investigations have revealed that the unicellular green algae, *Chlorella vulgaris* and *C. pyrenoidosa*, compare favorably with conventional vegetable dietary sources of several important vitamins, *e.g.*, biotin, folic acid, niacin, pyridoxine, riboflavin, and thiamine (1, 2). Morimura has reported consistent variations in vitamin content at different stages during the division and reproduction cycle of *C. ellipsoidea* grown in synchronous culture for short periods (3), and others have found that the vitamin content of *C. vulgaris* and of *C. pyrenoidosa* changes during 3 weeks of normal nonsynchronous growth (1, 2). Under the latter conditions, the content of biotin, folic acid, riboflavin, and thiamine (relative to dry weight of the cells) rises to a maximum and then declines during the 3-week culture period while the content of niacin and pyridoxine decreases continuously. However, because of the 16- to 17-fold gain in total cell mass, the absolute yield (mmcg./ml. of harvested culture) of all these vitamins, except folic acid, increases throughout the 3 weeks. The absolute yield of folic acid tends to decrease during the later portion of the culture period.

Of the six vitamins above, only riboflavin occurs in substantial quantities in the supernatant culture medium, the amount sometimes equaling or exceeding that in the cell mass after 3 weeks. The folic acid and the niacin content of the supernatant may be approximately 5% of that in the cells; the corresponding figure for pyridoxine may approximate 20%. Neither thiamine nor biotin appears in the supernatant in concentrations detectable by the microbiologic assay methods that were employed.

The present report deals with the pantothenic acid and inositol content of *Chlorella*. The rationale for pursuing this line of investigation has been reviewed by the authors (1, 2, 4) and others (5, 6). The view has been expressed that the vitamin content of *Chlorella* is sufficient to give the alga "premium value . . . as human or animal food" but is insufficient to render the plants suitable for recovery of vitamin concentrates (7). However, the latter point, being merely a matter of technology and of the economics of supply and demand, could well be subject to revision in the future.

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EXPERIMENTAL

Chlorella Cultures and Procedures

The pedigree of the strains of *C. vulgaris* and of *C. pyrenoidosa* employed and the procedures adopted as standard for working with them in this laboratory have been described in detail elsewhere (4). In brief, experimental cultures were inoculated with a sufficient number of organisms from a 4-day liquid culture (inoculated from the most recent of a series of at least six 4-day cultures) to give an initial population of 100 cells/mm.^{3} (100,000/ml.); light was supplied continuously from a Mazda source (intensity 600 f.c. at the position of the culture vessels); aeration was provided by a mixture consisting of 5% CO_2 and 95% air continuously passed through the cultures in finely dispersed bubbles; and the temperature was 20.5 ± 0.5°. The specific source of illumination and the geometry of the culture vessels have been described (4). The culture medium consisted of KNO_3, 0.025 *M*; $MgSO_4 \cdot 7H_2O$, 0.02 *M*; KH_2PO_4, 0.018 *M*; $FeSO_4 \cdot 7H_2O$, 5×10^{-5} *M*; potassium citrate, 5×10^{-5} *M*; Zn (as $ZnSO_4 \cdot 7H_2O$), 0.4 p.p.m.; Cu (as $CuSO_4 \cdot 5H_2O$), 0.004 p.p.m.; Mn (as $MnSO_4 \cdot 4H_2O$), 0.4 p.p.m.; and B (as H_3BO_3), 0.02 p.p.m.

Microbiologic Assays

Pantothenic Acid.—Harvested *Chlorella* cells were steamed for 30 min. in 0.1 *M* sodium acetate buffer at pH 6.8 and enzymatically digested by exposure for 4 hr. at 37° to a chicken liver extract[1]

[1] Prepared as described by Nielands and Strong (8).

TABLE I.—PANTOTHENIC ACID AND INOSITOL CONTENT IN *C. vulgaris* AND *C. pyrenoidosa*

Days	*C. vulgaris* 5	7	11	14	18	21
Vitamin	A. Content Expressed as mmcg./mg. Dry Wt.					
Panto. ac., cells	27.79	13.18	3.76	2.71	1.51	3.09
Panto. ac., supnt.	12.64	8.89	10.06	12.05	12.35	14.72
Panto. ac., total	40.43	22.07	13.82	14.76	13.86	17.81
Inositol	1,550	1,860	1,640	1,680	2,090	2,290
Days	*C. pyrenoidosa* 5	7	11	14	18	21
Vitamin	A. Content Expressed as mmcg./mg. Dry Wt.					
Panto. ac., cells	16.09	9.20	2.85	2.35	3.13	3.16
Panto. ac., supnt.	15.21	10.03	14.19	15.62	22.21	26.32
Panto. ac., total	31.30	19.23	17.04	17.97	25.34	29.48
Inositol	2,030	1,950	1,880	2,300	2,140	2,370
Days	*C. vulgaris* 5	7	11	14	18	21
Vitamin	B. Content Expressed as mmcg./ml. of Culture					
Panto. ac., cells	9.89	10.47	8.11	7.46	6.82	13.30
Panto. ac., supnt.	4.66	7.40	21.80	33.04	44.58	73.22
Panto. ac., total	14.55	17.87	29.91	40.50	51.40	86.52
Inositol	650	1,510	3,620	5,170	7,610	9,510
Days	*C. pyrenoidosa* 5	7	11	14	18	21
Vitamin	B. Content Expressed as mmcg./ml. of Culture					
Panto ac., cells	6.89	8.30	7.12	7.65	14.90	16.88
Panto. ac., supnt.	6.49	10.55	33.15	49.75	101.40	135.50
Panto. ac., total	13.38	18.85	40.27	57.40	116.30	152.38
Inositol	890	1,870	4,810	7,550	9,680	12,250

TABLE II.—PANTOTHENIC ACID AND INOSITOL CONTENT OF *C. vulgaris* RELATIVE TO THAT OF *C. pyrenoidosa* AT DIFFERENT HARVEST TIMES

	Days											
	A. mmcg./mg. Dry Wt.						B. mmcg./ml. Culture					
Vitamin	5	7	11	14	18	21	5	7	11	14	18	21
Pantothenic ac., cells	1.73	1.43	1.32	1.15	0.48	0.98	1.44	1.26	1.14	0.98	0.46	0.79
Pantothenic ac., supnt.	0.83	0.89	0.71	0.77	0.56	0.55	0.72	0.70	0.66	0.66	0.44	0.54
Pantothenic ac., total	1.29	1.15	0.81	0.82	0.55	0.60	1.09	0.96	0.74	0.71	0.44	0.57
Inositol	0.76	0.95	0.87	0.73	0.98	0.97	0.73	0.81	0.75	0.68	0.79	0.78

and alkaline phosphatase.[2] Cellular debris was removed by filtration and the residual extract was assayed for pantothenic acid, using *Lactobacillus plantarum* ATCC 8014 as the test organism in bacto-pantothenate medium U.S.P. (9).

Inositol.—Inositol was extracted according to the method of Wooley as modified by Snell (10). This involved refluxing the cells in 18% HCl for 6 hr., evaporating *in vacuo* to near dryness, and neutralizing. After filtration, the extract was assayed for inositol, using *Saccharomyces carlsbergensis* ATCC 9080 as the test organism in bacto-inositol assay medium (11).

Reliability of the Assays.—Two extracts were prepared from each sample at each harvest time, and each extract was assayed (in duplicate) at two levels. The S.D. for each such set of eight values was determined. All values for pantothenic acid were within the range ±10% of the mean. All inositol assay values for any given single extract were within the same range but to include all S.D. values from multiple extracts the range was ±15%.

RESULTS AND DISCUSSION

The over-all data, averaged from results of three successive experiments, are presented in Table I. Inositol was found only in the cell mass. At none of the six harvest times spaced over the 3-week culture period could it be detected, by the microbiologic assay technique employed, in the culture fluid. In this respect, its pattern of distribution is similar to that found previously for biotin and thiamine (2). In contrast substantial amounts of pantothenic acid occurred in the culture milieu, and during the latter half of the growth period the concentration in the medium was several times that in the cells, whether results were expressed relative to the dry weight of the cell mass (Table I, A) or in terms of absolute yield (Table I, B).

Examination of Table I, A, reveals a distinct difference in the patterns of pantothenic acid and of inositol content of the cells, relative to dry weight, in both species of *Chlorella*. Pantothenic acid is present in relatively high concentrations during the early phases of the culture period, and then the concentration decreases sharply between the fifth and the seventh days after which it continues to decrease, although less abruptly, for about another week to week and a half when it tends to reach a minimum and then to rise slightly during the remaining few days.

Inositol displays much less variation in level, increasing less than 50% between days 5 and 21 in *C. vulgaris* and approximately 15% in *C. pyrenoidosa*. These figures are in sharp contrast to the decrease of about 95% in pantothenic acid concentration between days 5 and 18 in *C. vulgaris* and of about 85% between days 5 and 14 in *C. pyrenoidosa*.

[2] Obtained from the Mann Research Laboratories, New York, N. Y.

The concentration of pantothenic acid in the culture milieu, expressed in relation to dry weight of the cells, swings through a smaller range but it too decreases (about 30%) between days 5 and 7, after which it rises slowly during the following 2 weeks. At the end of the culture period, it is substantially higher than at day 5 and is from 5 to 8 times higher than in the cells of *C. vulgaris* and of *C. pyrenoidosa*, respectively, at that time.

Despite the decrease in pantothenic acid content and the relatively stable content of inositol in the cells, relative to dry weight of the cells, the absolute yield of both compounds (Table 1, B) increases during the 3-week growth period because of the large (16- to 17-fold) increase in total cell mass. This is true also for the total yield of pantothenic acid, *i.e.*, content in cells plus that in supernatant (Table I, B).

The data summarized above differ in two significant respects from those found earlier for other vitamins. First, the abrupt decrease in concentration of pantothenic acid in the cells (Table I, A) and in total production of pantothenic acid (cells + supernatant), expressed relative to dry weight of cells, has not been observed in analyses for other vitamins. Changes in the concentration of other vitamins, from high to low (or vice versa), during the culture period occur gradually and more or less uniformly over a period of several days (1, 2).

Second, the high levels of pantothenic acid in *C. vulgaris* relative to those in *C. pyrenoidosa* during the early days of growth seem unique. In the authors' studies of eight vitamins, only one other (niacin) was found which occurs in higher concentration in *C. vulgaris* than in *C. pyrenoidosa*. In that case the difference in favor of the former species ranged from about 8% at 1 week to about 47% after 3 weeks, when results were calculated in terms of dry weight, *i.e.*, mmcg. of vitamin/mg. dry weight of cells, although when results were expressed in absolute terms, *i.e.*, mmcg./ml. harvested culture, the yield from *C. vulgaris* exceeded that from *C. pyrenoidosa* by 14 to 18%, irrespective of the time of harvest. In contrast, at day 5 the concentration of pantothenic acid in cells of *C. vulgaris* exceeds that in cells of *C. pyrenoidosa* by as much as 73% when expressed in terms of dry weight (Table II, A) and by 44% when calculated as absolute yield from the cells (Table II, B). This advantage of *C. vulgaris* decreases during the next several days but the species still holds a 15% lead at the end of the second week (Table II, A). However, by the end of the third week the two species are essentially equal in pantothenic acid content of the cells. Examination of Tables I and II reveals that the advantage *C. vulgaris* seems to hold over *C. pyrenoidosa* in pantothenic acid content of the cells stems in part from the fact that in cultures of the latter species relatively more of the total pantothenic acid content is found in the external medium. This is shown more specifically by Table III. Thus, except for the first week of the growth period the total pantothenic acid (cells + supernatant) is substantially higher in cultures of *C. pyrenoidosa* than in those of *C. vulgaris*, and the difference in favor of the former increases with time (Tables I and II). At each harvest time (except one) the percentage of the total pantothenic acid found in the supernatant was greater in cultures of *C. pyrenoidosa* than in those of *C. vulgaris* (Table III).

TABLE III.—PANTOTHENIC ACID IN RESIDUAL CULTURE SOLUTION EXPRESSED AS PER CENT OF THE TOTAL (CELLS + SUPERNATANT) CONTENT

Harvest Day	*C. vulgaris*	*C. pyrenoidosa*
5	31.3	48.6
7	40.1	52.2
11	72.8	83.3
14	81.6	86.9
18	89.1	87.6
21	82.6	89.3

It is suggested that the high concentration of cellular pantothenic acid in cultures of *C. vulgaris* relative to *C. pyrenoidosa* at day 5 followed by continuous diminution of the apparent advantage of the former thereafter is essentially a syndrome that stems coincidentally from the culture conditions and the harvest times chosen. Others have shown that in cultures of *C. ellipsoidea* undergoing synchronous growth the cells are largest during the "ripening and maturing" stage which precedes release of daughter cells and that this is a stage of development when pantothenic acid content of cells is greatest (3, 12). At that stage the cells have high respiratory activity, suggesting enhanced formative metabolism and an important role for pantothenic acid as a component of coenzyme A (12). Concomitant with the increase in cell size and in pantothenic acid content, there is a striking decrease in the ratio of dry weight/volume during the early ripening phase, suggesting more active absorption of water by the algal cells during that process (13). The accompanying decrease in glucosamine content of the cell wall (14) may indicate significant alteration in the permeability and other characteristics of the cell.

In the present work it was observed that early harvests, in contrast to later ones, appeared to contain a greater percentage of larger cells, perhaps comparable to the "ripening and maturing" stage of development reported by others to precede release of daughter cells. Accordingly, a study was made of the size distribution and settling rates of cells harvested at different times. The results will be the subject of a later communication, and it will suffice for the present to mention that the percentage of "large" cells, *i.e.*, 6 μ or more in diameter does indeed vary during the growth period, increasing approximately threefold between the time of inoculation and the third day and nearly fivefold in the first 5 days. By the seventh day postinoculation, the percentage (but not the absolute number) of "large" cells has decreased to a value below that found in the inoculum.

To determine rate of settling, cells were spun down, washed with 0.002 *M* K_2SO_4, spun down again, and resuspended in 2% NaCl. Transmission was determined with a Klett-Summerson colorimeter. (Initial Klett reading of suspensions = 200.) The decrease in Klett values after 1 hr. for suspensions prepared from 3-, 4-, 7-, and 11-day harvests was 140, 70, 41, and 20, respectively, and suggests that cells from the younger cultures were less dense, and thus remained in suspension longer, than those from older cultures. This is consistent with the observation of a decrease in the ratio dry weight/volume during the "ripening" stage (13).

In view of the foregoing observations of size distribution and settling rates in our cultures con-

TABLE IV.—PANTOTHENIC ACID AND INOSITOL CONTENT OF *Chlorella* AND OF SOME CONVENTIONAL FOODS (mcg./100 Gm. OF EDIBLE PORTION)[a]

Item	Pantothenic Acid	Inositol
C. vulgaris, dried 5-day harvest	2,779	155,000
C. vulgaris, dried 7-day harvest	1,318	186,000
C. vulgaris, dried 21-day harvest	309	229,000
C. pyrenoidosa, dried 5-day harvest	1,609	203,000
C. pyrenoidosa, dried 7-day harvest	920	195,000
C. pyrenoidosa, dried 21-day harvest	316	237,000
Beans, dried lima	830	?
Beef, liver	5660–8,180	340,000
Beef, muscle	1,100	11,500
Bread, whole wheat	570	64,400–103,000
Broccoli	1,400	?
Cabbage	?	95,000
Cauliflower	920	?
Cheese	350–960	25,000
Eggs	2,700	22,000
Milk, whole	290	50,000
Oats	1,300	100,000
Oranges	340	210,000
Peas, fresh	600–1,040	162,000
Peas, dried	2,800	?
Peanuts, roasted	2,500	180,000
Potatoes, white	400–650	29,000
Potatoes, sweet	940	66,000
Salmon	660–1,100	17,000
Soy beans	1,800	?

[a] Values for food from Sebrell, W. H., and Harris, R. S., "The Vitamins," vol. III, Academic Press Inc., New York, N. Y., 1954; and "Nutritional Data," 5th ed., H. J. Heinz, Pittsburgh, Pa., 1962.

sidered in conjunction with the very detailed observations on physical characteristics of cells at different stages of development cited in the literature (3, 12), it seems reasonable to suggest that, under the conditions of the experiments in this laboratory (relatively small inoculum, adaptation to new medium, different light intensity, etc.), there may be a tendency toward partial or simulated synchronized growth at the time of the first harvest—day 5. Expressed another way, it may be plausible to suggest that a larger percentage of cells of the total population divides about the third to fifth day postinoculation than at earlier or later times. Since *C. pyrenoidosa* develops more rapidly than *C. vulgaris* during the early phases of culture and enters the log phase and reaches the stationary phase sooner (1, 4), it is not unreasonable to assume that at the time of first harvest (day 5) cultures of *C. vulgaris* were just approaching or entering the critical time of the major burst of division (percentagewise, although not in terms of total number of cells dividing or produced) and that cultures of *C. pyrenoidosa* had already passed that peak.

Bearing in mind that pantothenic acid level is greatest in cells in the "ripening and maturing" stage of development (3, 12), the above hypothesis is consistent with the higher cell content of that constituent found in *C. vulgaris* in the first half of the culture period. It is interesting to note also that Morimura (3) reported formation of pantothenic acid to be related primarily to the process of "ripening" rather than directly to the process of photosynthesis or "growing." If greatest production occurs during the phase preceding release of daughter cells, it may not be unreasonable to assume that upon release of the daughter cells some of the vitamin would also be released to the external medium. If such does in fact occur, since *C. pyrenoidosa* cultures develop more rapidly than those of *C. vulgaris*, it could account for the higher percentage of the total pantothenic acid found in the external medium of the *C. pyrenoidosa* cultures (Table III).

The pantothenic acid and inositol content of the two *Chlorella* species and of several conventional dietary items is shown in Table IV. In comparing *Chlorella* with the other items listed, it should be remembered that data for *Chlorella* are for dried cells but that values of the other items (except lima beans, oats, dried peas, peanuts, and soy beans) are for fresh material. Therefore, since the water content of *Chlorella* is about 90%, values expressed in terms of fresh weight would be about 10% of those recorded.

SUMMARY

In cultures of *C. vulgaris* and of *C. pyrenoidosa* the cellular content of inositol, expressed relative to dry weight of the cells, increased throughout the 21-day culture period—the increase being less than 50% between days 5 (first harvest) and 21 in *C. vulgaris* and about 15% in *C. pyrenoidosa*. The increase in total yield (mmcg./ml. of harvested culture) was approximately 14-fold during the same period in both species. At all harvest times, *C. pyrenoidosa* exceeded *C. vulgaris* in inositol content. At no harvest time was inositol detected in the external culture medium.

In contrast, pantothenic acid was recovered from the cells and from the external culture solution at all harvest times. The concentration in the cells, relative to dry weight decreased sharply during the first week, then less abruptly for another week or week and a half. Except during the first week, when there was a decline, the concentration in the external solution increased throughout the culture period, and at terminal harvest the concentration in the external medium was severalfold that in the cells.

During the first week postinoculation *C. vulgaris* excelled *C. pyrenoidosa* in yield of pantothenic acid, but after that initial period the relative positions of the two species as sources of the vitamin were reversed.

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Mechanism of Action of Phenolic Disinfectants VII

Factors Affecting Binding of Phenol Derivatives to *Micrococcus lysodeikticus* Cells

By JOSEPH JUDIS

Whole cells of *Micrococcus lysodeikticus* ATCC 4698 bound approximately 2–4 per cent of phenol-^{14}C (P-^{14}C), 20 per cent of 2,4-dichlorophenol-^{14}C (DCP-^{14}C), and 30 per cent of *p-tert*-amylphenol-^{14}C (PTAP-^{14}C) when exposed to the latter in trace amounts. Protoplasts bound approximately 20 per cent less, respectively, of each of the phenol derivatives. With increasing pH (from 4.9 to 9.6), whole cells bound decreasing amounts of DCP-^{14}C, but binding of P-^{14}C and PTAP-^{14}C was relatively constant over the same pH range. Human serum decreased binding of DCP-^{14}C and PTAP-^{14}C by *M. lysodeikticus* cells and a similar effect, although less marked, was found with crystalline human serum albumin. The decrease was proportional to protein concentration. Human serum γ globulin in the same concentration as serum albumin did not reduce binding of the labeled phenols to the bacterial cells while a sonic disintegrate of *M. lysodeikticus* cells caused some reduction in binding.

A BASIC ASSUMPTION in previous work (1–6) on the mechanism of action of phenol disinfectants has been that the lipophilic properties of the latter lead to association of these disinfectants with microbial lipids and exertion of biological activity at such sites in which lipid is contained, *e.g.*, the cell membrane. In support of this assumption has been the work on uncoupling halophenols, the biological action of which has been correlated with lipid solubility (7, 8). However, in the light of the recent work by Weinbach and Garbus (9–11) on the restoration of halophenol-uncoupled oxidative phosphorylation by serum albumin and binding of halophenols by serum albumin and mitochondrial protein, it seemed imperative to re-examine the role of proteins in the bactericidal effects of phenolic disinfectants. Organic matter, including serum (12, 13), has long been known to interfere with the activity of germicides. One of the features generally listed for the ideal disinfectant is activity in the presence of organic matter and in a number of the commonly used testing procedures, activity is determined in the presence of serum. Yet, very few studies have been made on possible associations between serum proteins and disinfectants, especially phenolic disinfectants.

The ability of proteins to bind chemical substances was observed a number of years ago, and much of the work has centered on binding of small ions, dyes, fatty acids, and surface-active ions (14–18) by plasma albumin and studies have been carried out on the binding of thyroid hormones, especially of thyroxine by serum proteins (19). In previous studies (3), the binding of certain phenolic disinfectants by bacterial cells was demonstrated and the affinity of the latter for these disinfectants was related to antibacterial potency. This laboratory felt it would be useful to extend the studies to determine the extent to which several entities affect this binding process—namely, bacterial cell walls, human serum, human serum albumin, and pH. *Micrococcus lysodeikticus* was chosen as the test organism because of its well-characterized cell wall (20, 21) and the occurrence of lipid primarily in its cell membrane (22).

MATERIALS AND METHODS

Bacterial Culture.—*M. lysodeikticus* ATCC 4698 was used throughout and was maintained on nutrient agar. Cells for experimental use were grown on a medium with the following composition: yeast extract, 10 Gm.; bacto-tryptone, 10 Gm.; dibasic potassium phosphate, 2 Gm.; distilled water, to make 1 L. Glucose was sterilized separately as a 50% w/v solution and added aseptically at the time of inoculation. Cultures were started by inoculation of 7 ml. of the culture medium, supplemented with 0.1 ml. of 50% glucose solution, and grown on a rotary shaker for 24 hr. All incubations were at 37°. The same medium in 200-ml. quantities contained in 500-ml. conical flasks was inoculated with 0.5 ml. of the test tube culture and supplemented with 0.5 ml. of 50% glucose solution. These cultures were shaken for 24 hr., the cells recovered by centrifugation, washed twice with distilled water, and resuspended in distilled water to a concentration equal to approximately 15 mg. dry weight per ml. Each reaction mixture contained 0.6 ml. of this cell suspension.

The radioactive derivatives used in this study were synthesized by New England Nuclear Corp., Boston, Mass., and had the following specific activities: phenol-^{14}C, 50 μc./3.04 mg.; 2,4-dichlorophenol-^{14}C, 50 μc./12 mg.; and *p-tert*-amylphenol-^{14}C, 50 μc./30.4 mg. The label in all three

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Previous paper: Commager, H., and Judis, J., *J. Pharm. Sci.*, 54, 1436(1965).

compounds was uniformly distributed in the rings. Phenol was used in aqueous solution while the other derivatives were dissolved in 0.1% sodium hydroxide and all stock solutions contained 5 μc./ml. Radioactivity was assayed in a Nuclear-Chicago liquid scintillation system. All radioactive samples were diluted to a total volume of 2 ml. with water and added to 13 ml. of scintillation solution of the following composition: naphthalene, 120 Gm.; PPO (2,5-diphenyloxazole), 7 Gm.; dimethyl POPOP (1,4-bis-2-4 methyl-5 phenyloxazolylbenzene), 50 mg.; and *p*-dioxane to make 1 L. Enough counts were accumulated to give a nine-tenth error of less than 5%.

Preparation of Protoplasts.—Six-tenths of a milliliter of cell suspension was placed in a centrifuge tube, the cells were sedimented by centrifugation, and suspended in 0.9 ml. of 1.2 *M* sucrose in 0.01 *M* phosphate buffer (pH 6.8). Then 0.1 ml. of lysozyme solution, 1 mg./ml., was added and the mixture incubated for 30 min. at room temperature (24°). After centrifugation the protoplasts were suspended in the reaction mixtures described below. Whole cell suspensions were made up in the same reaction mixtures in the controls comparing binding of phenol derivatives. A control to determine extent of protoplast formation consisted of cells treated with lysozyme in the absence of sucrose. Complete clearing of the cell suspension resulted.

Experimental Procedure.—In the experiments involving comparison of binding of the phenol derivatives by whole cells and protoplasts, the reaction mixtures were prepared as follows: protoplasts were suspended in a mixture of 1.5 ml. of 82% w/v sucrose, 0.3 ml. of 0.1% sodium hydroxide, 0.9 ml. of 0.067 *M* phosphate buffer, pH 6.7, and 0.2 ml. of radioactive phenol or phenol derivative. Stock solutions of the latter (5 μc./ml.) were diluted 1:20 with distilled water before use. The reaction mixtures were incubated for 20 min. at room temperature, centrifuged for 5 min. at 15,000 r.p.m., and aliquots of the pellet and supernatant assayed for radioactivity. Whole cells were treated with the same reaction mixtures. The final pH value of the reaction mixtures was 7.2.

In the studies on the effect of pH on binding of phenol derivatives to whole cells, the reaction mixtures consisted of 0.9 ml. of 0.067 *M* phosphate buffer, 0.6 ml. of cell suspension, 0.2 ml. of radioactive phenol derivative solution (stock solutions diluted 1:10 with distilled water), and 1.3 ml. of water. The pH values of the phosphate buffers were chosen to give the desired final pH of the reaction mixtures. In those experiments in which human serum, human serum albumin, or other proteins were used, the solution of the latter replaced a corresponding volume of distilled water. Reaction mixtures contained either 0.3 ml. of human serum, 0.3 ml. of crystalline human serum albumin (42 mg./ml.), or 0.3 ml. of γ globulin (42 mg./ml.). The general experimental procedure was the same. Human serum was obtained from Difco Laboratories or Grand Island Biological Co. (tissue culture grade), and human serum albumin (crystallized 4X), γ globulin, and lysozyme were products of Nutritional Biochemicals Corp. The sonic disintegrate was prepared by treating a suspension of bacterial cells with a Branson sonic disintegrator, model 125, for 15 min. at maximum energy output, followed by centrifugation at 15,000 r.p.m. The supernatant contained 8 mg. of protein per ml. using crystalline human serum albumin as a standard and the Folin-Ciocalteau reagent for protein assay. Reaction mixtures contained 0.3 ml. of the sonic disintegrate in 3 ml. of total volume.

Table I.—Binding of Phenol Derivatives to Cells and Protoplasts of *M. lysodeikticus*

Phenol Derivative	Total c.p.m. Added	Radioactivity, % Associated with: Cells	Protoplasts
Phenol-^{14}C	12,300	2.33	1.78
2,4-Dichlorophenol-^{14}C	80,563	17.9	13.7
p-tert-Amylphenol-^{14}C	14,860	30.5	23.6

RESULTS AND DISCUSSION

In general, the same patterns of binding of phenol and the derivatives used were obtained as with *Escherichia coli* (3) in that the more potent the phenol derivative as a germicide, the higher the proportion of the latter was found to be associated with the cells (Table I). Protoplasts bound somewhat less of each of the phenol derivatives than whole cells, in all cases amounting to a decrease of approximately 20%. This observation would suggest that cell walls do bind a portion of the phenol derivatives and that the mucopeptide complex is capable of associating with these compounds. In Gram-positive bacteria, the cell wall constitutes approximately 20% of dry weight (22), corresponding to the proportion of radioactivity with which they apparently associated.

Table II.—Effect of Human Serum on Binding of 2,4-Dichlorophenol-^{14}C and *p-tert*-Amylphenol-^{14}C by *M. lysodeikticus* Cells

Phenol Derivative	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells: Human Serum Control	Added	% of Control
2,4-Dichlorophenol-^{14}C	158,266	4.9	34,776	14,504	41.8
	158,266	6.1	34,090	14,358	42.1
	158,266	7.3	30,152	11,852	39.3
	158,266	9.3	16,914	6,524	38.6
	158,266	9.6	11,814	5,806	49.1
p-tert-Amylphenol-^{14}C	142,093	4.9	49,766	26,346	52.9
	142,093	6.1	50,928	24,780	48.7
	142,093	7.3	50,692	24,924	49.2
	142,093	9.3	48,088	23,486	48.8
	142,093	9.6	50,428	29,320	58.1

TABLE III.—EFFECT OF CRYSTALLINE HUMAN SERUM ALBUMIN ON BINDING OF 2,4-DICHLOROPHENOL-^{14}C AND *p-tert*-AMYLPHENOL-^{14}C BY *M. lysodeikticus* CELLS

Phenol Derivative	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells—Serum Albumin		
			Control	Added	% of Control
2,4-Dichlorophenol-^{14}C	153,748	4.9	33,912	20,974	61.8
	153,748	6.1	32,180	14,382	44.7
	153,748	7.3	29,136	9,314	32.0
	153,748	9.6	13,046	6,984	53.5
p-tert-Amylphenol-^{14}C	133,866	4.9	44,478	36,486	82.0
	133,866	6.1	43,080	27,520	63.9
	133,866	7.3	44,352	25,120	56.6
	133,866	9.6	43,378	22,642	52.2

Binding of 2,4-dichlorophenol-^{14}C was affected by changes in pH of the reaction mixture (Fig. 1), while the other compounds were bound to the same extent between pH's of 4.9 to 9.6. These observations suggest that the ionized form of a phenolic compound is bound considerably less than the unionized form. 2,4-Dichlorophenol has a Ka of 7.08×10^{-9} (23) while the other compounds have Ka values of about 10^{-10} (24–26). Thus, the latter compounds would be unionized over the range of pH values used, while 2,4-dichlorophenol would exist in the ionized form to a considerable extent above neutrality. Many organic compounds, especially weak acids, have been shown to possess greater biological activity in the unionized form (13). In contrast, however, Weinbach and Garbus claim that the ionized form of uncoupling halophenols possesses the greatest biological activity (10).

From Table II, it can be seen that human serum decreased the binding of 2,4-dichlorophenol-^{14}C and *p-tert*-amylphenol-^{14}C to *M. lysodeikticus* cells. The effects of pH variation were similar in the presence and absence of serum in the case of 2,4-dichlorophenol-^{14}C, while pH had little effect on binding of *p-tert*-amylphenol-^{14}C in the absence or presence of human serum. It would thus appear that pH changes affect the phenol derivative but not the binding potency of human serum proteins. At the highest pH value used, a slight, but consistent decrease was observed in the interference by human serum with binding of the two phenol derivatives. This may be due to the change in serum albumin structure found at extreme pH's (14), and in this altered form, perhaps serum albumin is unable to associate with the phenol derivatives. Treatment of mitochondria with 8 *M* urea or guanidine, which caused protein denaturation, was found to cause release of bound pentachlorophenol, providing an example in which change in protein structure changed the latter's ability to associate with a phenol derivative (9).

The major protein in human serum is albumin, and thus it seemed appropriate to determine whether albumin in the same concentration in which it is normally found in human serum could duplicate the effects observed with the latter. From the data in Table III, it is clear that human serum albumin does interfere with the association of the two phenol derivatives tested and the bacterial cells, but the extent of interference is less than that found with whole serum. It is conceivable that other components of serum, such as lipids and lipoproteins with which phenolic compounds would tend to associate, might account for the effects of whole serum. The effects of pH variation were also different with human serum albumin as compared to whole serum in that increasing the pH caused a decrease in the amount of *p-tert*-amylphenol-^{14}C associated with the cells in the presence of albumin beyond the effect of the latter. This pH effect was not found with whole serum. Since the phenol derivative would be unlikely to ionize at any of the pH's used, the effect thus must be on the albumin. Variation in pH was shown to have no effect on binding of *p-tert*-amylphenol-^{14}C to *M. lysodeikticus* cells (Fig. 1).

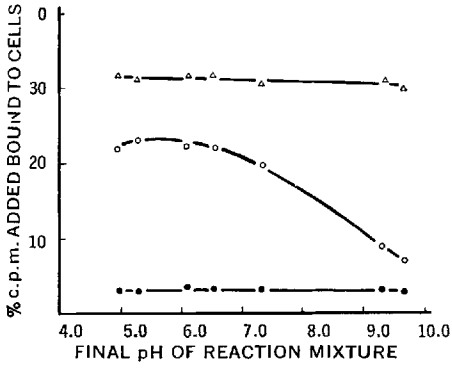

Fig. 1.—The effect of pH on binding to *M. lysodeikticus* cells. Key: ●, phenol-^{14}C; ○, 2,4-dichlorophenol-^{14}C; △, *p-tert*-amylphenol-^{14}C.

In a preliminary fashion, the ability of proteins in general to interfere with the association of phenol derivatives and cells was surveyed. In Tables IV and V, human serum γ globulin and a sonic disintegrate of *M. lysodeikticus* cells were examined for their ability to duplicate the effects observed with human serum and human serum albumin. γ Globulin, in a concentration equal to that of human serum albumin used in previous experiments, had no effect on the amounts of 2,4-dichlorophenol-^{14}C or *p-tert*-amylphenol-^{14}C associated with the cells. The sonic disintegrate, however, did cause some decrease in association of radioactivity with the cells. This suggests that certain bacterial proteins have an affinity for these phenol derivatives and in further work, specific bacterial proteins, as isolated by electrophoresis, are being examined for their phenol derivative-binding properties. Weinbach and Garbus (11) surveyed a large number of proteins for ability to associate with uncoupling phenols and found this effect limited to serum albumin and mitochondrial protein. They found, further, that there was a definite relationship between the amounts of human serum albumin and that of

phenol derivative bound (11). In Table VI, it can be seen that while not stoichiometric, there is a definite relationship between concentration of human serum or human serum albumin and decrease in radioactivity associated with the bacterial cells.

At least two hypotheses suggest themselves as explanations for the ability of serum or serum albumin to decrease association of phenol derivatives with bacterial cells. It is possible that the proteins compete with the bacterial cells for the phenol derivatives and the relative proportions of each determine how much of the phenol derivatives will be found in the cells. An alternative hypothesis would hold that the proteins coat the bacterial cells, making a portion of the latter's surface unavailable for penetration by the phenol derivatives. The latter hypothesis was tested experimentally in a preliminary fashion by preparing the same reaction mixtures containing albumin at various pH's and adding radioiodinated (^{131}I) serum albumin. These reaction mixtures contained 2,4-dichlorophenol-^{14}C only. With an increase in pH, as expected, there was a decrease in the amount of ^{14}C associated with the cells in the presence or absence of serum or serum albumin. One would also expect, if the second hypothesis is valid, that the amount of ^{131}I associated with the cells due to binding of albumin would be higher with higher pH values if it were competing with the phenol for the cell surface. The reverse, however, was found in that at the lowest pH tested (4.9), the cells contained the most ^{131}I. The amount of ^{131}I associated with the cells in all cases, was very low, equal approximately to the same proportion that the pellet of cells constituted of the reaction mixtures, except at pH 4.9, where it was about 3 times as high. It would thus appear, that the most attractive hypothesis at this time is that serum albumin is able to bind phenol derivatives.

Any general hypothesis for the mechanism of action of phenolic disinfectants would have to explain the observations of the effect of pH on binding of the former. Weinbach and Garbus (10) concluded that the ionic form of the halophenol uncoupling agents they studied was the active form and the interaction between the halophenol and the binding sites on the albumin molecule, amino groups, was an anion–anion interaction. Yet, phenol and *p-tert*-amylphenol, especially the latter, are bound to whole cells and protoplasts at high pH's at which alkylated phenols would not ionize to any considerable degree.

Perhaps the suggestion by Davis and Dubos (27) that serum albumins associated with negatively

TABLE IV.—EFFECT OF HUMAN SERUM γ GLOBULIN AND *M. lysodeikticus* SONIC DISINTEGRATE ON BINDING OF 2,4-DICHLOROPHENOL-^{14}C BY *M. lysodeikticus* CELLS

Addition	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells: Control	Protein Added	% of Control
Human serum γ globulin	152,562	4.9	33,420	34,874	101.6
		6.1	32,484	33,664	103.6
		7.3	28,556	29,118	102
		9.6	17,384	18,724	107.7
Sonic disintegrate of *M. lysodeikticus* cells	157,734	4.9	31,236	27,858	89.2
		6.1	30,750	28,328	92.1
		7.3	27,352	24,662	90.2
		9.6	17,094	13,836	80.9

TABLE V.—EFFECT OF HUMAN SERUM γ GLOBULIN AND *M. lysodeikticus* SONIC DISINTEGRATE ON BINDING OF *p-tert*-AMYLPHENOL-^{14}C BY *M. lysodeikticus* CELLS

Addition	Total c.p.m. Added	pH of Reaction Mixture	Total c.p.m. Bound to Cells: Control	Protein Added	% of Control
Human serum γ globulin	108,422	4.9	37,122	38,004	102.3
		6.1	35,750	38,224	106.9
		7.3	38,035	38,446	101.0
		9.6	35,974	36,510	101.5
Sonic disintegrate of *M. lysodeikticus* cells	140,395	4.9	39,084	31,546	80.7
		6.1	38,740	31,352	80.4
		7.3	36,188	32,798	90.6
		9.6	39,732	32,240	81.1

TABLE VI.—EFFECT OF DIFFERENT CONCENTRATIONS OF HUMAN SERUM AND CRYSTALLINE HUMAN SERUM ALBUMIN ON BINDING OF 2,4-DICHLOROPHENOL-^{14}C AND *p-tert*-AMYLPHENOL-^{14}C TO *M. lysodeikticus* CELLS

Addition[a]	2,4-Dichlorophenol-^{14}C[b] Total c.p.m. Bound to Cells	% of Control	*p-tert*-Amylphenol-^{14}C[c] Total c.p.m. Bound to Cells	% of Control
None	28,954	...	40,740	...
Human serum				
0.1 ml.	12,130	41.9	26,508	65.1
0.3 ml.	6,040	20.9	15,460	37.9
Crystalline human serum albumin				
4.2 mg.	15,380	53.1	33,266	81.7
8.4 mg.	10,944	37.8	27,534	67.6
12.6 mg.	8,830	30.5	23,966	58.9

[a] Per 3 ml. reaction mixture. Final pH was 7.3. [b] A total of 162,163 c.p.m. was added to the reaction mixtures. [c] A total of 153,846 c.p.m. was added to the reaction mixtures.

charged molecules due to the ω amino groups in lysine residues and with lipophilic chains through the side chains of leucine residues should lead to the conclusion that this protein has not only different binding capacities for different compounds, but also different mechanisms by which it binds. If bacterial proteins rather than lipids are the compounds with which phenolic disinfectants associate, it should be possible to demonstrate as much binding of the latter to bacterial proteins as to whole cells as was shown by Weinbach and Garbus (10, 11) with mitochondria and mitochondrial protein in their studies with uncoupling halophenols. These possibilities are currently being tested by examining reaction mixtures containing serum proteins or bacterial proteins and phenol derivatives for complexes using Sephadex chromatography, electrophoresis, density gradient ultracentrifugation, difference spectrophotometry, and reduction in bactericidal and bacteriostatic effects. It is felt that an intimate knowledge of the reaction between phenol derivatives and organic matter such as serum proteins would be useful in the design of disinfectants, the activity of which would not be affected by organic matter. An ultimate goal in these studies is a complete knowledge of the site(s) of action of phenolic germicides in bacterial cells.

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Molecular Orbital Calculations of the Electronic Structure of the Sydnones

By LEMONT B. KIER and EDWARD B. ROCHE*

The electronic structure of the sydnone ring has been calculated from an ω-HMO technique. Several new parameters for the calculation have been derived, in conjunction with previously used parameters. Charge densities derived have yielded accurate calculations of dipole moments. Energy levels have yielded values which correlate well with observed U.V. maxima. A general discussion of the calculated structures is presented.

OUR INTEREST in the medicinal chemical aspects of the mesoionic compounds known as the sydnones has required a further understanding of the chemistry of these aromatic heterocycles (1–6). Recent reviews of these compounds have summarized their chemical and physical properties (7–10) but have not added much basic information concerning their electronic structure and the electronic changes involved in their reactions. This information is essential if the relationships between their structure and pharmacological activity is to be evaluated.

A classical resonance theory or valence bond approach to the electronic picture of the sydnones is not illuminating since, embodied within the definition of a mesoionic compound is the requirement that no covalent structure can be drawn for the system (11). Furthermore, it has been shown that at least 20 polar structures can be drawn for phenylsydnone (12). The total number of such

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TABLE I.—PARAMETERS

Atom	h	Bond	k
N (pyrrole type)	1.5	C—N (pyrrole type)	0.8
H_3 (conjugated methyl)	−0.5	N—O	0.5
≡C— (conjugated methyl)	−0.1	N—N	0.9
		H_3≡C— (conjugated methyl)	2.8

TABLE II.—DIPOLE MOMENTS

Compd.	Calcd. μ (D)	Exptl. μ (D)	Ref.
Pyrrole	1.85	1.80	(25, 26)
N-Methylpyrrole	2.16	1.92	(44)
Toluene	0.38	0.39	(45)
Furazan	3.10	3.38	(46)
Isoxazole	2.79	2.76	(47, 48)
N-Methylpyrazole	2.34	2.28	(49)
N-Phenylpyrazole	2.30	2.0	(49)
3-Phenylsydnone	6.47	6.48	(50)
3-Methylsydnone	7.06	7.31[a]	...

[a] This value was determined by the authors employing the method described by Smyth (51). Dielectric constants were determined on a General Radio Co-type No. 1610-A capacitance bridge.

structures which must be considered in a valence bond approach is many more, and the real problem arises in weighting each structure in the overall equation for the wave function of the molecule. Thus, the valence bond or classical resonance theory approach to the electronic structure of sydnones presents immense difficulties.

A more reasonable approach to the solution of the wave equation of the system is the LCAO–MO (linear combination of atomic orbitals–molecular orbital) method.

The approximations involved and the various techniques employed for solution of electronic structures have been adequately described (13–15). A number of studies of this type have been done on the sydnones, using simple Hückel procedures (16–18). The results of these calculations failed to reproduce physical experience, such as dipole moment. This failure undoubtedly was partly a result of a poor selection of heteroatom parameters, certainly unrefined at the time these calculations were made, and the failure of the simple Hückel method to consider electron correlation.

In previous calculations performed in this laboratory, the authors have empirically treated the the effect of electron correlation within the framework of the Hückel method (19, 20). This has been done by employing a disposable parameter which serves to alter the Coulomb integrals of the atoms as a function of the calculated charge densities, q, of the respective atoms (21). The procedure, known as the "ω-technique," produces new values of the Coulomb integral, in terms of β_0, the standard resonance integral for the carbon-carbon bond in benzene (22).

$$\alpha_i = \alpha_0 + \omega\, q_i\, \beta_0$$

This technique involves iterating the calculation until self-consistency is achieved in the charge densities at each position in the molecule. The value of ω used in this study was 1.4, which corresponds to the value used by several other authors (23, 24) and appears to give results that correlate well with experimental data.

PARAMETERS

An essential feature for the successful use of this method of calculation is the choice of parameters for heteroatoms. The parameter used to modify the Coulomb integral of a heteroatom, h_x, must reflect its modified electronegativity and electronic configuration with respect to an aromatic carbon atom. This modification is again made in terms of the standard resonance integral, β_0

$$\alpha_x = \alpha_0 + h_x\, \beta_0$$

A second parameter, k_{xy}, is employed to reflect the alteration in electron exchange between the heteroatom in question and its neighbors with respect to an aromatic carbon-carbon bond:

$$\beta_{xy} = k_{xy}\, \beta_0$$

In previous work (19, 20) the authors have approached this problem with the desire to obtain calculated values of eigenfunctions and eigenvalues of heteroatom-containing molecules which would adequately approximate chemical and physical experience. These calculations have resulted in the close approximation of dipole moments from eigenfunctions (19) and relative chemical reactivities from eigenvalues (20). The ability to produce these results from the same set of heteroatom parameters lends a measure of confidence in their values.

In the present work, these same values have been used with only one modification. In the previous study on dipole moments (19) an experimental value of 1.54 *D* for pyrrole and a model for the nitrogen atom identical to that for the nitrogen of aniline were used. It is now felt that the value of 1.80 *D* for pyrrole is a better experimental value (25, 26). Also, the model for the nitrogen atom must be reevaluated. The unshared electron pair on nitrogen of aniline is not in a pure *p* orbital; hence the Coulomb integral should reflect a greater electronegativity than that of the nitrogen in pyrrole (27). Furthermore, since the pure *p* orbital of nitrogen in pyrrole is more available for delocalization, a higher resonance integral should be used for the C-N bond. This improved model with the accompanying parameters has permitted us to calculate suc-

cessfully the dipole moments of pyrrole and *N*-methylpyrrole. (See Table I for parameters and Table II for dipole moments.)

Although the heteroatom model has previously been used for the treatment of a methyl group on an aromatic ring (28), the authors felt that a more realistic model of this group is provided by the conjugative model (29) in which the electron distribution of both the hydrogens and the methyl carbon atom is specifically designated and the methyl hydrogens are collected into a pseudo π orbital. Using the parameter values proposed by Coulson and Crawford (30) for this model, the authors have again successfully calculated the dipole moment of toluene.

For the calculation of the electronic structure of the sydnone molecule, two new parameters had to be introduced, and their values had to be consistent with other parameters employed. These parameters were the resonance integral for the Ṅ-Ö bond ($k_{\dot{N}\ddot{O}}$) and the resonance integral for the Ṅ-N̈ bond ($k_{\dot{N}\text{-}\ddot{N}}$). The value of 0.6 has been derived for $k_{\dot{N}\ddot{O}}$ for use in simple Hückel calculations (18). The value of 0.5 was found to reproduce successfully the value of the dipole moments of furazan and isoxazole. For the parameter $k_{\dot{N}\ddot{N}}$, the value of 0.87 has been derived from a proportionality to overlap integrals (31). The value of 0.9 has been employed successfully to calculate the dipole moments of *N*-methylpyrazole and *N*-phenylpyrazole.

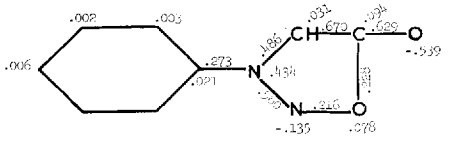


Fig. 1.—3-Phenylsydnone. Numbers shown are calculated π-bond orders and charge densities.

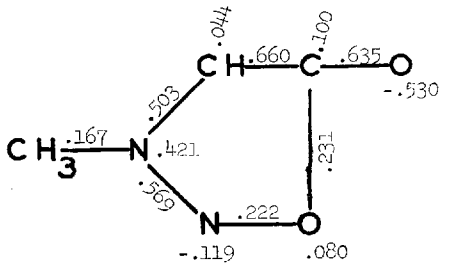


Fig. 2.—3-Methylsydnone. Numbers shown are calculated π-bond orders and charge densities.

CALCULATIONS

The computations were made using the computer program previously described (20). Due to the greater complexity of some of the sydnones, convergence of charge densities to self-consistency required a substantially larger number of iterations. The versatility of this program permitted this with no difficulty. The values from consecutive calculations of the charge densities were tested until an increment no larger than 0.001 electron existed between the final iteration and the average of all preceding calculations. The test was simultaneously applied to all atoms by the program. The calculated charge densities for the ring atoms are shown for 3-phenylsydnone in Fig. 1 and for 3-methylsydnone in Fig. 2. The π-bond order for each of these compounds was calculated from the eigenfunctions and is shown for the appropriate bonds in Figs. 1 and 2.

The dipole moments were calculated from charge densities as previously described (19) and are listed in Table II along with experimental values.

From the eigenvalues of the matrices the $M_m \rightarrow M_{m+1}$ transition energies were calculated. These are recorded in Table III along with the corresponding wavelengths for a series of variously substituted sydnones. The energy levels in units of β for 3-phenylsydnone and 3-methylsydnone are listed in Table IV. The values of ΔM, in units of β, are plotted against the corresponding frequencies of maximum absorption in Fig. 3.

DISCUSSION

As can be seen from Table II, the agreement between the calculated and experimental dipole moments for the sydnones is excellent. This, in the authors' opinion, attests to the reasonableness of the values for the charge densities calculated (32).

Several observations can be made from an inspection of the charge densities and bond orders of 3-phenyl and 3-methylsydnone (Figs. 1 and 2). The exocyclic oxygen atom in both compounds bears a high charge density, being over 0.15 electron higher than what was calculated for butyrolactone (33). This clearly implies significant participation from all ring atoms, in the total delocalization of the π-electrons. The number 3 nitrogen atom bears a high positive charge, particularly in the case of the 3-phenylsydnone. This observation, combined with the highest bond order calculated, between the carbon atoms, indicates the substantial participation of

TABLE III.—MAXIMUM U.V. ADSORPTION OF THE SYDNONE RING AND CALCULATED VALUES OF ΔM

Compd.	$\lambda_{max.}$, mμ	ν, cm.$^{-1}$	$\Delta M(\beta)$	Ref.
1. 3-Methylsydnone	290	34483	0.481	(41)
2. 3-Phenyl-4-ethylsydnone	307	32573	0.451	(41)
3. 3-(*p*-Tolyl)-4-methylsydnone	307	32573	0.450	(41)
4. 3-Phenylsydnone	310	32258	0.465	(41)
5. 3-(*m*-Tolyl)sydnone	310	32258	0.465	(41)
6. 3-(*β*-Pyridyl)sydnone	312	32051	0.461	(41)
7. 3-(*β*-Naphthyl)sydnone	315	31746	0.457	(52)
8. 3-Phenyl-4-acetylsydnone	326[a]	30675	0.417	...
9. 3,4-Di-(*p*-tolyl)sydnone	338	29586	0.405	(41)
10. 3,4-Di-phenylsydnone	340	29412	0.405	(52)

[a] The $\lambda_{max.}$ of this compound was determined by the authors on a Cary model 15 U.V. spectrophotometer in ethanol.

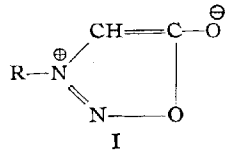
TABLE IV.—ENERGY LEVELS FOR 3-PHENYL AND 3-METHYLSYDNONE IN UNITS OF β

3-Phenylsydnone	3-Methylsydnone
−2.025	
−1.268	
−1.040	−3.164
−0.996	−1.228
−0.060	−0.058
0.405	0.423
0.951	1.381
1.004	2.101
1.380	2.951
1.931	3.294
2.802	
3.218	

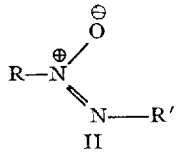
valence bond representation, I, in the total wave equation of the molecule, a conclusion also reached by dipole moment studies of Hill and Sutton (34).

A comparison of the two calculated structures indicates that the phenyl group in the 3 position makes a greater mesomeric contribution of electrons to the sydnone ring than the 3-methyl group. The phenyl ring is deactivated at all positions by the sydnone ring; hence electrophilic attack on the benzene ring would be expected to be retarded. This is borne out by experiment (12, 35). This fact may in reality be the result of electron withdrawal by the sydnone ring rather than donation by the phenyl ring. The phenyl ring is known to be an electron withdrawing group in most systems (36).

In the work cited previously (35), the sydnone ring was found to be comparable to the β-azoxy group (II), with respect to its activating effect on nucleophilic displacement reactions on p-chlorophenyl derivatives. The proposed transition state in reactions of this type is stabilized by electron-withdrawing substituents (37). The comparison between these two substituents is also observed in electrophilic substitutions on benzene in which both groups are deactivating. This is not true for α-azoxy substituents (35).



I



II

This relationship between the sydnone ring and β-azoxy groups lends further support for the charge distributions shown in Fig. 1. A larger bond order between the two nitrogens would also be required for conjugation with the phenyl ring leaving the bond order between nitrogen and oxygen somewhat deficient. This situation might also be expected from electronegativity considerations (35).

Further support for the bond order calculated between nitrogen and oxygen (Fig. 1) is found in a comparison of bond lengths. An X-ray study of p-bromophenyl sydnone has shown (38, 39) the length of the N—O bond to be 1.34 Å. The bond length of the N—O single bond has been reported to be 1.46 Å. while that of an N=O double bond is 1.14 Å. (40). Thus, in general terms, the N—O bond in the sydnone ring is largely of a single bond nature which corresponds favorably with the relatively low π-bond order calculated for these compounds.

To determine the reliability of the eigenvalues obtained from solution of the secular equations, an examination of the ultraviolet spectra of a number of substituted sydnones seemed appropriate. A single peak in the 290 to 350 mμ region has been attributed to the sydnone ring (41). This maximum is quite intense ($\epsilon > 4700$) and experiences a bathochromic shift from 3-alkyl to 3-aryl substitution indicating an extended interannular conjugation (Table III) (42). Increase in delocalization also appears to be more effective with the aryl substituent in the 4-position of the sydnone ring than in the 3-position. This is expected from the increased ability of carbon π-orbitals to participate in electron delocalization compared to that of the nitrogen.

Other bands in the spectra of aryl-substituted sydnones appear at lower wavelength and have been attributed to benzenoid K absorption (41).

General considerations of ultraviolet spectra of aromatic compounds within the framework of molecular orbital calculations have led to the general conclusion that the more intense peaks at longer wavelengths are due to transitions occurring between the highest filled energy level (m) and the lowest unoccupied level ($m+1$) (43). In empirical LCAO–MO calculations, such as are presented here, the over-all electronic transition energy is considered to be larger than any energy differences due to configuration interaction or singlet-triplet splitting (43). The transition is then taken to be the perpendicular Franck-Condon transition. The frequency of such a transition is given by

$$h\nu = E^* - E$$

where E^* is the energy of the excited state, and E is the energy of the ground state.

In the total π-energy expressions from LCAO–MO calculations, the energy of the two states are given by

$$E^*_\pi = n\alpha + M^*\beta$$

$$E_\pi = n\alpha + M\beta$$

Thus, the frequency should be proportional to the difference in the coefficients of β:

$$h\nu = (M^* - M)\beta = \Delta M\beta$$

This operation is equivalent to taking the difference between M_m and M_{m+1}.

Table III shows the $\lambda_{max.}$ and ΔM for a number of substituted sydnones, and Fig. 3 shows the relationship between the frequency in cm.$^{-1}$ and ΔM.

The correlation of these data is good. The correlation coefficient of 0.931 indicates that about 86.6% of the variance is accounted for. The maximum deviation of the experimental $\lambda_{max.}$ from that calculated by the correlation equation from Fig. 3

$$\nu(\text{cm.}^{-1}) = 60533\,(\Delta M) + 4794$$

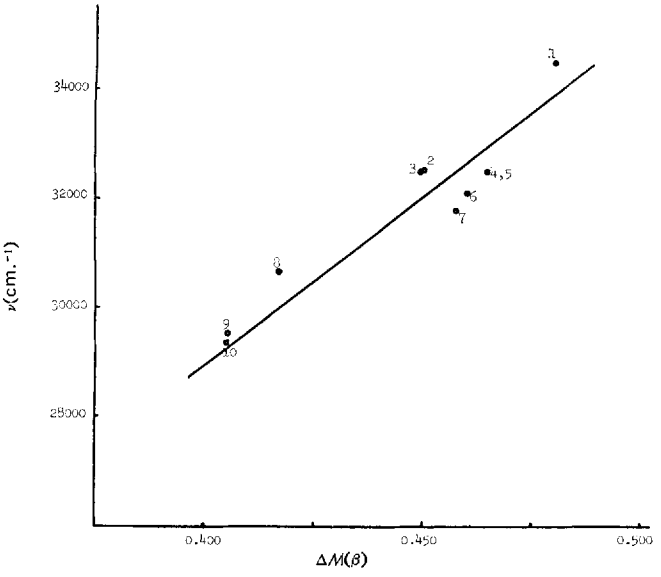


Fig. 3.—Correlation plot of the frequency of maximum absorption of the sydnone ring *vs.* the calculated ΔM in units of β.

is ± 7 mμ, and the average deviation of points from the line is ± 0.010 ΔM units. Thus, it appears that the relative energy levels calculated by the present method are in good agreement with the major electronic transition energy of the substituted sydnone rings.

The deviations from calculated values are not unexpected considering the empirical nature of the calculations. In the ω-HMO technique, no distinction is made between the geometry of the ground and excited state or between singlet and triplet state. A perpendicular Franck-Condon transition is assumed which may involve a promotion to a higher level than the lowest available level in the excited state.

Further error is involved with the nonexplicit consideration of electron repulsion. When the energy difference between the ground and excited state is small, the energy levels will interact. This repulsion will result in a larger separation between the levels. More sophisticated methods employing configuration interaction are required to take this phenomenon into account.

Through an application of the ω-HMO technique in conjunction with parameters previously derived (19) and parameters derived in this present work, the dipole moments of some representative heterocyclic systems including 3-methyl and 3-phenylsydnone have been successfully calculated (Table II). These same parameters have also given relative energy levels that correspond well with the maximum ultraviolet absorptions of the variously substituted sydnone rings (Table III and Fig. 3). Thus, the authors feel that the values of these parameters when employed in this type of an LCAO–MO calculation produce a reasonable electronic structure of the sydnones.

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Gas Chromatographic Analysis of Trichloroethanol, Chloral Hydrate, Trichloroacetic Acid, and Trichloroethanol Glucuronide

By EDWARD R. GARRETT and HOWARD J. LAMBERT*

A sensitive and specific assay has been developed for trichloroethanol, chloral hydrate, trichloroacetic acid, and trichloroethanol glucuronide utilizing gas chromatography and electron capture detection. Trichloroethanol and chloral hydrate are extracted from alkalinized water or urine with ether, and a portion of the ether extract is assayed with chlorobutanol and chloroform as internal standards. Trichloroacetic acid and trichloroethanol glucuronide are not extracted under these conditions. The trichloroacetic acid is decarboxylated as the potassium salt and the resultant chloroform extracted into ether and assayed. The trichloroethanol glucuronide is enzymatically hydrolyzed and the hydrolysate assayed for the trichloroethanol formed. Sensitivities obtained were 0.5 mcg. of trichloroethanol and chloral hydrate (3.3 and 3.02 mμmoles, 10^{-9} moles, respectively), 1.0 mcg. of trichloroacetic acid (6.1 mμmoles) in 2.0 ml. of sample and 0.5 mcg. of trichloroethanol glucuronide (1.54 mμmoles) in 3.0 ml. of sample. Statistical analysis of assays on urinary mixtures of the four compounds demonstrates the reliability of the method.

THERE ARE no sensitive specific assays for the hypnotic drug trichloroethanol (TCE), its precursor chloral hydrate (CH), or its detoxification products trichloroacetic acid (TCA) and trichloroethanol glucuronide (TCE-G), in the available literature.

Extant methods (1–3) are mostly modifications of the colorimetric method of Fujiwara (4) and were not sufficiently sensitive or specific for a planned pharmacokinetic study of TCE in this laboratory. Friedman and Cooper (2) obtained a sensitivity of 20 mμmoles of TCA and CH and 100 mμmoles of TCE in mixtures but found that some tissue preparations interfered with chromophore production as much as 25% and lessened the reliability of the Fujiwara procedure. Leibman and Hindman (3) improved the assay sensitivity down to 5 mμmoles of TCA and CH and 15 mμmoles of TCE, but separation of the components prior to reaction would be required as the procedure was nonspecific. A gas chromatographic separation of CH, TCA, and chlorobutanol, but not TCE, with a sensitivity of 306, 302, and 281 mμmoles, respectively, has also been reported (5). This sensitivity of detection is not as good as the modified Fujiwara (2, 3).

The purpose of this investigation was to develop a specific procedure for the quantitative determination of TCE and its metabolites separately and in mixtures with gas-liquid chromatography and the electron capture detector. If possible, this method would have greater sensitivity than those previously reported.

EXPERIMENTAL

Materials.—The TCE (Calbiochem, Inc.) used in the preparation of calibration curves and synthetic mixtures in water and urine was redistilled at 152° before use.

Chloral hydrate U.S.P., chlorobutanol U.S.P., and TCA (reagent grade and 5% solution) were obtained from the Fisher Scientific Co. Ether (Baker) was redistilled over sodium before use. The purity of these components was verified chromatographically, and only one peak was observed.

The sodium salt of trichloroethanol glucuronide

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A sensitive and specific assay has been developed for trichloroethanol, chloral hydrate, trichloroacetic acid, and trichloroethanol glucuronide utilizing gas chromatography and electron capture detection. Trichloroethanol and chloral hydrate are extracted from alkalinized water or urine with ether, and a portion of the ether extract is assayed with chlorobutanol and chloroform as internal standards. Trichloroacetic acid and trichloroethanol glucuronide are not extracted under these conditions. The trichloroacetic acid is decarboxylated as the potassium salt and the resultant chloroform extracted into ether and assayed. The trichloroethanol glucuronide is enzymatically hydrolyzed and the hydrolysate assayed for the trichloroethanol formed. Sensitivities obtained were 0.5 mcg. of trichloroethanol and chloral hydrate (3.3 and 3.02 mμmoles, 10^{-9} moles, respectively), 1.0 mcg. of trichloroacetic acid (6.1 mμmoles) in 2.0 ml. of sample and 0.5 mcg. of trichloroethanol glucuronide (1.54 mμmoles) in 3.0 ml. of sample. Statistical analysis of assays on urinary mixtures of the four compounds demonstrates the reliability of the method.

THERE ARE no sensitive specific assays for the hypnotic drug trichloroethanol (TCE), its precursor chloral hydrate (CH), or its detoxification products trichloroacetic acid (TCA) and trichloroethanol glucuronide (TCE-G), in the available literature.

Extant methods (1–3) are mostly modifications of the colorimetric method of Fujiwara (4) and were not sufficiently sensitive or specific for a planned pharmacokinetic study of TCE in this laboratory. Friedman and Cooper (2) obtained a sensitivity of 20 mμmoles of TCA and CH and 100 mμmoles of TCE in mixtures but found that some tissue preparations interfered with chromophore production as much as 25% and lessened the reliability of the Fujiwara procedure. Leibman and Hindman (3) improved the assay sensitivity down to 5 mμmoles of TCA and CH and 15 mμmoles of TCE, but separation of the components prior to reaction would be required as the procedure was nonspecific. A gas chromatographic separation of CH, TCA, and chlorobutanol, but not TCE, with a sensitivity of 306, 302, and 281 mμmoles, respectively, has also been reported (5). This sensitivity of detection is not as good as the modified Fujiwara (2, 3).

The purpose of this investigation was to develop a specific procedure for the quantitative determination of TCE and its metabolites separately and in mixtures with gas-liquid chromatography and the electron capture detector. If possible, this method would have greater sensitivity than those previously reported.

EXPERIMENTAL

Materials.—The TCE (Calbiochem, Inc.) used in the preparation of calibration curves and synthetic mixtures in water and urine was redistilled at 152° before use.

Chloral hydrate U.S.P., chlorobutanol U.S.P., and TCA (reagent grade and 5% solution) were obtained from the Fisher Scientific Co. Ether (Baker) was redistilled over sodium before use. The purity of these components was verified chromatographically, and only one peak was observed.

The sodium salt of trichloroethanol glucuronide

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(hereafter referred to as TCE-G) was isolated from rabbit urine by the method of Seto and Schultze (6).

Assay solutions of TCA were prepared by diluting the 5% stock solution and the final concentration verified by titration. TCE, CH, and TCE-G were weighed to within 0.1 mg., transferred to a clean 100-ml. volumetric flask, and brought to volume with water or urine.

Apparatus.—An F&M model 700 gas chromatograph with either a flame ionization or electron capture detector and equipped with a Minneapolis Honeywell recorder and disc integrator was used.

Column.—The column for calibration curve determination and studies with mixtures was an 8 ft. × 1/4 in. stainless steel tube containing 20% Carbowax 20 M on 60–80 mesh Chromosorb W. Other columns investigated but not chosen due to incomplete peak resolution or tailing were a 4 ft. × 1/4 in. stainless steel, 10% diethylene glycol succinate; a 4 ft. × 1/4 in. glass, 5% Carbowax 20 M and a 4 ft. × 1/4 in. glass, 5% Carbowax 20 M plus 2% H_3PO_4, all on 80–100 Diatoport S.

Flame Ionization Detector.—The temperatures used were: injection port, 120°; column, 100°; and detector, 290°. The flow rate of the helium carrier gas was 60 ml./min. Hydrogen and air flow rates 60 and 500 ml./min. (20 p.s.i.), respectively.

Electron Capture Detector.—The optimum temperatures were: injection port, 160°; column, 125°; and detector, 190°. Carrier gas (helium) flow rate was 60 ml./min. while the purge gas (90% argon–10% methane) was fixed at 140 ml./min.

Sample Assay

Trichloroethanol and Chloral Hydrate.—Five-microliter aliquots of aqueous solutions of TCE and CH were injected directly on-column when the flame ionization detector was used. For electron capture detection, 2 ml. of the solution was shaken with 2 ml. of diethyl ether in a 10-ml. rubber-sealed injectable vial and 5 μl. of the ether extract was assayed. The ether contained 1.6 mg./100 ml. of chlorobutanol and 0.004 mg./100 ml. of chloroform as internal standards.

Trichloroethanol Glucuronide.—One milliliter of water or urine containing TCE-G was added to 1.0 ml. of pH 4.5 acetate buffer (ionic strength = 0.1 *M*) containing 5 mg. of β-glucuronidase (beef liver, 360 Fishman units/mg., Calbiochem). The mixture was sealed and allowed to react at 37.5° overnight. Two milliliters of diethyl ether (plus chlorobutanol) was then added, the mixture was shaken, and a 5-μl. aliquot of the ether extract assayed for TCE using the electron capture detector. No analysis was run on TCE-G using flame ionization.

To determine the purity of the TCE-G isolated, 1 ml. of a 5.14 mg./100 ml. solution was hydrolyzed overnight at 80° with 1 ml. of concentrated hydrochloric acid. The TCE formed was extracted into 2 ml. of ether, a 5-μl. aliquot of the ether extract assayed, and the assay value compared with the theoretical yield. The stated enzyme concentration was optimum in that it gave results equivalent to acid hydrolysis after overnight reaction at pH 4.5, 37°.

The identity of the glycone obtained by enzymatic hydrolysis was verified by thin-layer chromatography. Twenty microliters of the hydrolysate were spotted on a Silica Gel GF_{254} (Brinkmann Instruments) plate of 0.4-mm. thickness along with reference spots of D-glucuronic acid, D-glucuronolactone, TCE, and TCE-G. The plates were then developed in 1-butanol–acetic acid–water (4:5:1) up to approximately 10 cm. The plates were then dried, sprayed with concentrated sulfuric acid, and heated until carbonization was complete. The hydrolysate was resolved into two spots with R_f values corresponding to D-glucuronic acid (0.50) and a small amount of unhydrolyzed TCE-G (0.81). No D-glucuronolactone (R_f = 0.71) was found. The volatility of TCE prohibited its visualization by this method.

Trichloroacetic Acid.—A chromatographic peak for TCA could not be resolved with the authors' columns. Alkaline decarboxylation produced chloroform which was extracted with ether and assayed with the electron capture detector. One milliliter of 0.1 *M* KOH was added to 1 ml. of water or urine containing TCA in a 10-ml. injectable vial. Two milliliters of diethyl ether (no internal standards present) was then added, the vial was sealed and heated at 100° for exactly 30 min. The vial was then cooled in an ice bath, allowed to reach room

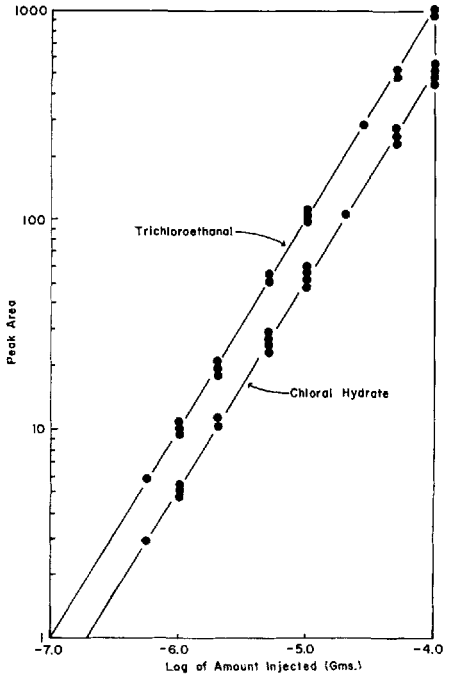

Fig. 1.—Calibration curves for 4-μl. aliquots of aqueous trichloroethanol and chloral hydrate solutions assayed with the flame ionization detector.

TABLE I.—DISTRIBUTION COEFFICIENTS FOR THREE NONPOLAR SOLVENTS AND AQUEOUS SOLUTIONS OF CHLORAL HYDRATE AND TRICHLOROETHANOL

	Pentane/H_2O	Benzene/H_2O	Diethyl Ether/H_2O
CH	0.003	0.03	0.191
TCE	0.34	3.5	22

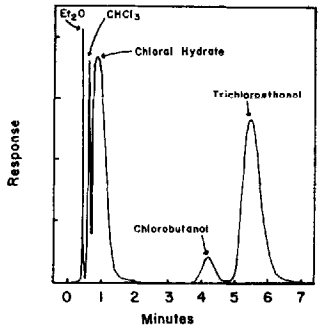

Fig. 2.—A typical chromatogram for a 5-μl. aliquot of a 1:1 ether extract of a solution of chloral hydrate and trichloroethanol (1.40 and 1.36 mg./100 ml., respectively). Pulse = 15. Sensitivity: 0.001 of maximum.

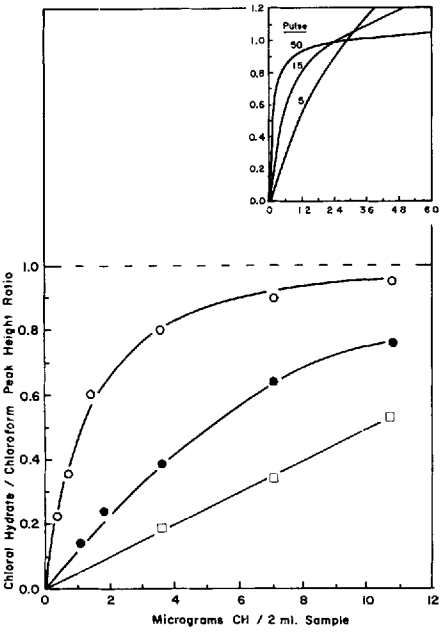

Fig. 3.—Calibration curves for chloral hydrate at three pulse intervals. Sensitivity: 0.001 of maximum. Insert shows the calibration curves for amounts of chloral hydrate up to 60 mcg./2 ml. sample. One microgram = 6.04 mμmoles. Key: ○, pulse = 50; ●, pulse = 15; □, pulse = 5.

temperature, and 5 μl. of the ether extract assayed, using the electron capture detector for the chloroform formed. No analysis was run for TCA using flame ionization.

Mixtures.—A 10-ml. sample of urine containing TCE, CH, TCA, and TCE-G was brought to pH 7.0 by dropwise addition of concentrated KOH. The volume change was negligible. Two milliliters of this urine was removed and assayed for TCE and CH. A 2.0-ml. sample was then extracted with six 10-ml. portions of ether to remove the TCE and CH, divided in half, and the 1-ml. portions assayed for TCA and TCE-G.

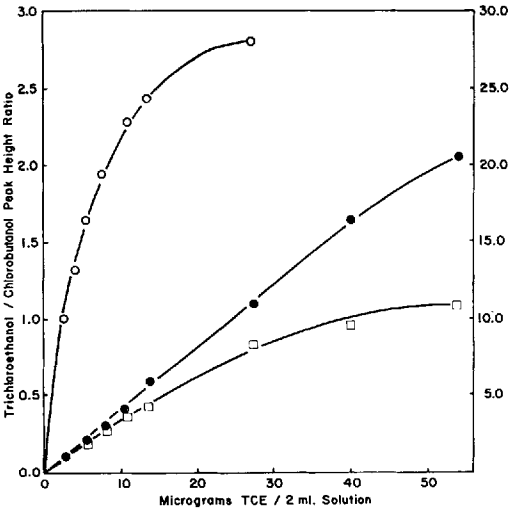

Fig. 4.—Calibration curves for trichloroethanol at three pulse intervals. Sensitivity: 0.001 of maximum. Pulse = 50, ordinate on left; pulse = 5 and 15, ordinate on right. One microgram = 6.6 mμmoles. Key: ○, pulse = 50; ●, pulse = 5; □, pulse = 15.

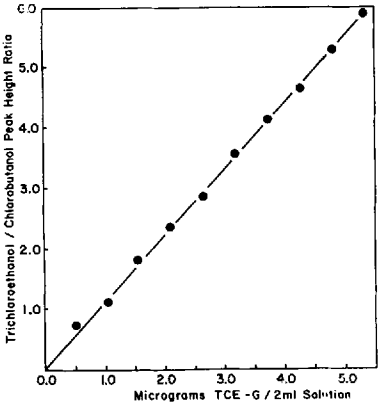

Fig. 5.—Calibration curve for trichloroethanol glucuronide based on the assay of trichloroethanol obtained by enzymatic hydrolysis. Pulse = 15, sensitivity: 0.001 of maximum. One microgram = 3.08 mμmoles.

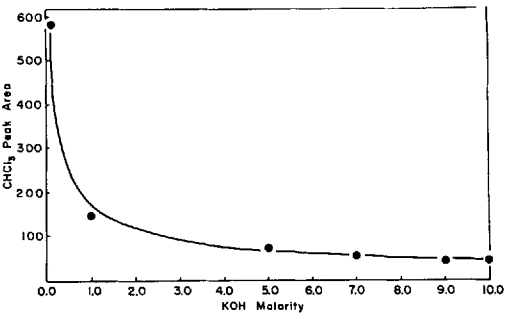

Fig. 6.—Effect of potassium hydroxide concentration on the amount of ether-extracted chloroform after trichloroacetic acid decarboxylation.

RESULTS AND DISCUSSION

Flame Ionization Detector.—The calibration curves for TCE and CH obtained by injecting 4-μl. aqueous aliquots are seen as Fig. 1. The sensitivity of flame ionization detection was only slightly greater than that obtained with the Friedman-Cooper assay. Therefore, this was not the method of choice.

Electron Capture Detector—Chloral Hydrate and Trichloroethanol.—Since direct injection of aqueous solutions gave poor peak reproducibility with electron capture detection, extraction with a nonpolar solvent was used. The distribution coefficients for several solvents are located in Table I. Figure 2 is a typical chromatogram of ether-extracted CH and TCE, with chloroform and chlorobutanol as internal standards. Better resolution of the CH and chloroform peaks could be obtained by decreasing either the carrier gas flow rate or the column temperature, but only at the expense of prolonging the retention time and increasing the width of the TCE and chlorobutanol peaks.

The potential across the detector cell was applied by the square wave pulse technique (7). The intervals available between $^3/_4$ μsec. voltage pulses were 5, 15, 50, and 150 μsec. An increased linear relationship between response and concentration was obtained by using shorter pulse intervals when high concentrations of CH or TCE were used. This is demonstrated in Figs. 3 and 4, the calibration curves for CH and TCE, respectively. However, greater sensitivity was available when the longer pulse intervals were used. These pulse intervals were more advantageous at lower drug concentrations. Thus, the pulse interval used for assay would depend on the concentration of drug which is physiologically encountered.

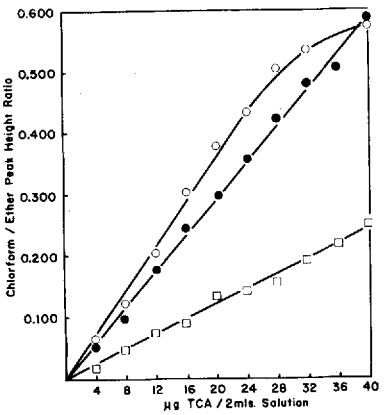

Fig. 7.—Calibration curve for trichloroacetic acid based on the assay of ether-extracted chloroform after alkaline (0.1 *M* KOH) decarboxylation. Sensitivity: 0.001 of maximum. One microgram = 6.1 mμmoles. Key: ○, pulse = 50; ●, pulse = 15; □, pulse = 5.

The assay parameter used for TCE and CH is peak height ratio (compound/internal standard). Although peak area ratios are conventionally used for compounds exhibiting broad peaks, peak height ratios gave less error when calibration curves using the two parameters were compared.

Trichloroethanol Glucuronide.—The acid hydrolysis yielded a TCE assay with peak height ratio of 6.0 at pulse = 15. Since this region of the pulse = 15 calibration curve (Fig. 4) showed a slight negative deviation from linearity, a second assay was run at pulse = 5, and a peak height ratio of 9.0 was obtained. These values corresponded to concentrations of 19.2 and 22.4 mcg. of TCE in 2 ml. of solution (87.2 and 100.3% of calculated theoretical yield) when compared with the calibration curves for TCE. Enzymatic hydrolysis at 37°, pH 4.5 with varying amounts of enzyme gave the same yield with 5 mg. of β-glucuronidase/2 ml.; 1.0 mg. of enzyme gave only 65.8% of theoretical yield.

The calibration curve for TCE-G is in Fig. 5. A pulse interval of 15 μsec. was used because it gave a linear response for physiological concentrations of TCE-G.

Trichloroacetic Acid.—The effect of KOH concentration on the assay was checked by decarboxylating a 1-ml. sample of 5 mg./100 ml. of TCA with 1.0 ml. of base ranging in concentration from 0.1 to 10 *M* and assaying after 30 min. for chloroform. The results of this experiment are depicted in Fig. 6.

Coulometric titration with a Cotlove chloridometer (Laboratory Glass and Instrument Corp.) demonstrated an increase in ionic chloride concomitant with the decrease of assayable chloroform from decarboxylation as the concentration of alkali was increased. For KOH concentrations of 1.0, 3.0, 5.0, and 10.0 *M*, the ionic chloride concentrations (and $CHCl_3$ peak heights) were 0.0296 (3.0), 0.0866 (1.20), 0.1104 (0.65), and 0.1626 meq./L.

TABLE II.—ASSAY DATA FOR LEVELS OF CHLORAL HYDRATE IN URINARY MIXTURES

TCE Level, mg./100 ml.	TCA Level, mg./100 ml.	CH/$CHCl_3$ Peak Ht. Ratio: CH, 0.14 mg./100 ml. — Day 1	CH, 0.14 mg./100 ml. — Day 2	CH, 1.40 mg./100 ml. — Day 1	CH, 1.40 mg./100 ml. — Day 2
0	0	.270, .270	.270, .283	1.025, 1.025	1.010, 1.025
0	5.00	.259, .270	.289, .260	1.025, 1.020	1.010, 1.010
0	3.75	.250, .270	.270, .289	1.010, 1.020	1.025, 1.020
0	1.25	.293, .257	.274, .260	1.010, 1.025	1.030, 1.020
1.36	0	.261, .276	.279, .282	1.020, 1.010	1.020, 1.030
1.36	5.00	.256, .243	.240, .259	1.010, 1.010	1.010, 1.020
1.36	3.75	.262, .268	.282, .260	1.010, 1.020	1.020, 1.020
1.36	1.25	.270, .261	.260, .278	1.020, 1.010	1.010, 1.010
0.136	0	.226, .236	.261, .253	1.010, 1.005	1.010, 1.005
0.136	5.00	.240, .240	.258, .253	1.003, 1.016	1.008, 1.004
0.136	3.75	.240, .247	.261, .265	1.020, 1.005	1.005, 1.002
0.136	1.25	.245, .251	.269, .269	1.020, 1.004	1.005, 1.004

(0.36), respectively. These data can explain the decrease in assayable chloroform with increased concentrations of alkali.

The first-order rate constants at the lowest KOH concentration (0.1 M) were checked at three different initial TCA concentrations. These rate constants were reasonably independent of the TCA concentrations, given in the parentheses, and were 15.0 (0.75×10^{-3} M), 13.8 (1.5×10^{-3} M), and 14.3 $\times 10^{-4}$ sec.$^{-1}$ (3.0×10^{-4} M). These values were in good agreement with the 13.2 $\times 10^{-4}$ sec.$^{-1}$ reported by Fairclough (8).

The 0.1 M KOH was used in the decarboxylation procedure since the greatest yield of chloroform was obtained in contrast to higher concentrations of alkali (Fig. 6).

The calibration curve for TCA (chloroform/ether peak height ratio *versus* concentration of TCA) is in Fig. 7. The use of the extraction solvent as an internal standard is deemed justifiable since the

TABLE III.—ANALYSIS OF VARIANCE FOR THE ASSAY[a] OF LOW LEVEL OF CHLORAL HYDRATE IN URINARY MIXTURES

Sources of Variation	d.f.	S.S.	M.S.	Components of Variance
Days (D)	1	0.0014	0.0014	$\sigma_S^2 + 2\sigma_E^2 + 24\sigma_D^2$
TCE (A)	2	0.0033	0.00165	$\sigma_S^2 + 2\sigma_E^2 + 4\sigma_{AB}^2 + 16\sigma_A^2$
TCA (B)	3	0.007	0.00023	$\sigma_S^2 + 2\sigma_E^2 + 4\sigma_{AB}^2 + 12\sigma_B^2$
TCE × TCA (AB)	6	0.0013	0.00021	$\sigma_S^2 + 2\sigma_E^2 + 4\sigma_{AB}^2$
Expt. error (E)	11	0.0011	0.0001	$\sigma_S^2 + 2\sigma_E^2$
Sampling error (S)	24	0.0026	0.0001	σ_S^2

[a] Assay data for chloral hydrate presented as CH/$CHCl_3$ peak height ratios (Table II).

TABLE IV.—SUMMARY OF SIGNIFICANT AND NONSIGNIFICANT EFFECTS (F TEST, 5% LEVEL) FOR ASSAYS OF CHLORAL HYDRATE, TRICHLOROETHANOL, AND TRICHLOROACETIC ACID IN MIXTURES

Drug	Level Tested	Significant Effects and F Values	Nonsignificant Effects
CH	0.14 mg./100 ml.	Days, 14.0[a] TCE levels, 8.25[b]	TCA, levels, TCE × TCA, exptl. error
CH	1.40 mg./100 ml.	TCE levels, 22.1[b]	TCA levels, TCE levels, TCE × TCA, exptl. error, days
TCE	0.136 mg./100 ml.	None	CH levels, TCA levels, CH × TCA, exptl. error, days
TCE	1.36 mg./100 ml.	None	CH levels, TCA levels, CH × TCA, exptl. error, days
TCA	1.25 mg./100 ml.	None	Treatments,[c] days, exptl. error
TCA	3.75 mg./100 ml.	Treatments,[c] 4.0[d]	Days, exptl. error
TCA	5.00 mg./100 ml.	Treatments,[c] 3.33[d]	Days, exptl. error

[a] Tabulated F (.05) = 4.84 for 1 and 11 d.f. [b] Tabulated F (.05) = 5.14 for 2 and 6 d.f. [c] A missing piece of data for all levels of TCA tested eliminated the isolation of the interactions for TCE levels, CH levels, and TCE × CH from the treatment sum of squares. The effect noted, therefore, is a pooled effect of all three of the above. [d] Tabulated F (.05) = 3.29 for 7 and 9 d.f.

TABLE V.—ISOLATED COMPONENTS OF VARIANCE AND PER CENT STANDARD DEVIATION FOR ASSAYS OF CHLORAL HYDRATE, TRICHLOROETHANOL, AND TRICHLOROACETIC ACID

Drug	Level Tested	Components of Variance × 10^6	$\sigma_{x_{ij}} \times 10^3$[a]	$\bar{\bar{X}}$[b]	S.D., %[c]
CH	0.14 mg./100 ml.	σ^2 days = 54.2 σ^2 TCE = 90.6 σ^2 TCE × TCA = 25.0 σ^2 sampling = 100	16.4	0.262	6.25
CH	1.4 mg./100 ml.	σ^2 TCE = 28.5 σ^2 error = 3.90 σ^2 sampling = 45.8	8.84	1.014	0.87
TCE	0.136 mg./100 ml.	σ^2 days = 5.4 σ^2 CH = 46 σ^2 CH × TCA = 84 σ^2 sampling = 210.0	18.6	0.885	2.1
TCE	1.36 mg./100 ml.	σ^2 days = 5340 σ^2 TCA = 650 σ^2 error = 5760 σ^2 sampling = 25890	194.0	6.326	3.1
TCA	1.25 mg./100 ml.	σ^2 error = 1.5 σ^2 sampling = 42.0	8.75	0.337	2.6
TCA	3.75 mg./100 ml.	σ^2 treatments = 18.8 σ^2 treatments = 79.5 σ^2 sampling = 105	13.6	0.574	2.36
TCA	5.0 mg./100 ml.	σ^2 error = 5.8 σ^2 treatments = 7.0 σ^2 sampling = 12.5	5.04	0.762	0.66

[a] $\sigma_{x_{ij}}$, the estimated standard deviation of a single assay. The square root of the sum of the isolated components of variance (σx^2_{ij}). [b] $\bar{\bar{X}}$, the mean assay value, over all treatments, for a given level of a drug. Presented for TCE as the TCE/chlorobutanol peak height ratio, for CH as the CH/$CHCl_3$ peak height ratio, and for TCA as the $CHCl_3$/ether peak height ratio. [c] Per cent standard deviation = $\sigma_{x_{ij}} \times 100/\bar{\bar{X}}$.

TABLE VI.—STATISTICAL ANALYSIS OF ASSAY DATA FOR MIXTURES OF TRICHLOROACETIC ACID AND TRICHLOROETHANOL GLUCURONIDE IN DOG URINE

Drug Assayed	Components of Variance × 10^6	$\sigma_{x_{ij}} \times 10^3$[c]	$\bar{\bar{X}}$[d]	S.D., %[e]
TCA[a]	σ^2 TCE-G = 8.0			
	σ^2 error = 50	7.6	0.99	0.76
TCE-G[b]	σ^2 TCA = 1870			
	σ^2 error = 10430	111.0	4.11	2.70

[a] TCA concentration = 7.0 mg./100 ml.; TCE-G concentrations = 5.14, 2.57, and 0.514 mg./100 ml. [b] TCE-G concentration = 5.14 mg./100 ml.; TCA concentrations = 7.0, 3.5, and 0.7 mg./100 ml. [c] $\sigma_{x_{ij}}$, the estimated standard deviation of a single assay. The square root of the sum of the isolated components of variance ($\sigma^2_{x_{ij}}$). [d] $\bar{\bar{X}}$, the mean assay value over all treatments. Presented for TCA as $CHCl_3$/ether peak height ratio and for TCE-G as the TCE/chlorobutanol peak height ratio. [e] Per cent standard deviation = $\sigma_{x_{ij}} \times 100/\bar{\bar{X}}$.

assay conditions (sealed vials) prohibit volatilization and the ether peak height is proportional to the volume injected.

Statistical Evaluation.—Three levels of TCE (0, 0.136, and 1.36 mg./100 ml.), three levels of CH (0, 0.140, and 1.40, mg./100 ml.), and four levels of TCA (0, 1.25, 3.75, and 5.0 mg./100 ml.) were combined in all possible mixtures (a total of 36) in dog urine and assayed randomly with two assays of each component per sample on each of two days. A typical series of results, those for 0.140 and 1.40 mg./100 ml. CH, are listed in Table II. Analyses of variance (9) of the assay data were obtained for each concentration of drug in the presence of the various concentrations of the other drugs. Such analyses were run on the raw assay data; for TCE, as TCE/chlorobutanol peak height ratios and for TCA, as chloroform/ether peak height ratios. A typical analysis of variance, for 0.140 mg./100 ml. CH in mixtures of various concentrations of TCE and TCA, is given in Table III. This analysis is based on data for this CH level given in Table II.

On each of the analyses of variance performed the presence of significant factors was determined by the F test at the 5% level (Table IV). The isolated components of variance are listed in Table V along with $\sigma_{x_{ij}}$, the standard deviation of a single assay (which is the square root of the sum of the isolated components of variance, $\sigma^2_{x_{ij}}$), the mean assay value over-all treatments $\bar{\bar{X}}$, and the per cent standard deviation of the assay ($\sigma_{x_{ij}} \times 100/\bar{\bar{X}}$). From this table it was observed that the major source of variation for all components assayed was due to sampling error. Experimental error was either absent or much smaller than that for sampling.

The variance due to the presence of TCE in assays for CH was postulated to be a result of formation of a soluble chloral alcoholate (10) which was then irregularly distributed between ether and urine. This could be corrected by running CH calibration curves in the presence of varying concentrations of TCE.

If one excludes the variation due to days noted in the assay of 0.140 mg./100 ml. CH by assuming a weighing error during sample preparation, the per cent standard deviation for the remaining drug concentrations is never greater than 3.1%. Hence, the assay is both reliable and reproducible.

Two similar experiments, the first with 7.0 mg / 100 ml. TCA solution in the presence of 5.14, 2.57, and 0.514 mg./100 ml. TCE-G and the second with 5.14 mg./100 ml. TCE-G in the presence of 7.0, 3.5, and 0.70 mg./100 ml. TCA, were also run. Analyses of variance were carried out on the assay data and the results are found in Table VI. No significant effect was found for either the presence of TCA in TCE-G assays or the presence of TCE-G in TCA analyses.

Finally, to determine possible assay variation when different experimental animals were used, analyses of variance were run on assay data for TCE, CH, and TCA in aqueous solution and in the urine of three different dogs (four urine samples per dog per day on two different days). The design had unequal numbers of replicates which prohibited isolation of components of variance. No significant difference was noted among different urines using the F test (5% level).

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Application of Membrane Filtration to Antibiotic Quality Control Sterility Testing

By FRANCES W. BOWMAN

Membrane filtration procedures for antibiotic sterility testing as described in the recently amended Antibiotic Regulations of the Food and Drug Administration (FDA) offer an improved approach for determining the sterility of antibiotics.

FOR ALMOST 20 years the Antibiotic Regulations[1] of the Food and Drug Administration for the sterility testing of antibiotics required 10 containers from each batch to be tested, six in thioglycollate broth for bacteria and four in Sabouraud fluid medium for molds and yeasts (1). In testing penicillin preparations, the antibiotic was inactivated by the addition of penicillinase to the thioglycollate medium. Since there were no suitable inactivators for the nonpenicillin antibiotics, no inactivator was added to the medium used for testing them, and for the most part any organisms detected were those highly resistant to the inhibitory action of the particular antibiotic.

DISCUSSION

To improve the sterility test, several possibilities were investigated. Serial dilution techniques to reduce the antibiotic to a subminimal inhibitory concentration were explored. This procedure is unsatisfactory since it also dilutes the contaminating organisms. Dilution of the antibiotic sample by large initial volumes of culture media was found to be both costly and impractical. Finally, a filtration procedure was developed employing a membrane filter composed of cellulose esters (2). The antibiotic preparations were first solubilized and the solution was filtered through a circular membrane filter approximately 47 mm. in diameter, with a porosity of 0.43–0.47 μ. Any organisms contaminating the preparation were trapped on the membrane, which was then washed with sterile water to remove any residual antibiotic without affecting the organism. Portions of the washed filter disk were placed in sterile fluid thioglycollate for 5 days at 32°.

Research on the application of the membrane filtration technique to the sterility testing of the antibiotic drugs produced practical methods for solubilizing and filtering each antibiotic preparation. Modifications were made to accommodate antibiotic powders and various formulations in which they are incorporated.

Use of the filtration technique for testing the tetracycline antibiotics eliminates two major problems. The antibiotic which would inhibit the growth of bacterial contamination, if present, is removed and degradation is avoided. When tetracyclines are added to thioglycollate medium, the breakdown products produce acidity, turbidity, and discoloration of the medium. The acidity prevents growth of most contaminating microorganisms, and the turbidity and dark color prevent visual observation of contamination.

Sodium novobiocin and amphotericin B also presented problems in that the slight acidity of Sabouraud broth rendered these antibiotics insoluble, again interfering with visual detection of growth. Filtration eliminates this difficulty. The antibiotics are dissolved in 0.1% peptone solution, filtered, and washed three times. For practical purposes no antibiotic is transferred to the Sabouraud broth because none remains on the membrane.

The procedures for the sterility testing for antibiotics published March 28, 1964 (3), gave membrane filtration procedures official status and culminated years of evaluating their merit. In addition to increasing the sensitivity of the test by the use of filtration procedures, the amended regulations increased the sample size to 20 representative units from each "filling operation" to be tested in both of the media previously mentioned. The term "filling operation" is defined as that period of time not longer than 24 consecutive hr. during which a homogeneous quantity of a drug is being filled continuously into market-size containers and during which no changes are made in the equipment used for filling. Both the direct method and the membrane filtration procedure are described in detail in the regulations. The direct method is used for insoluble preparations and the membrane filtration for soluble preparations. The membrane filtration procedure specifies that 300 mg. of a solid drug or 1 ml. by volume of a liquid drug from each of 20 immediate containers be aseptically transferred to 200 ml. of 0.1% (w/v) peptone solution. Since bacterial contamination is not related to the dosage of the antibiotic tested, a constant amount (weight or volume) of the antibiotic was selected for each sterility test. After complete solubilization, the antibiotic solution is filtered through a bacteriological membrane filter. All air entering the system is passed through air filters capable of removing microorganisms. Three 100-ml. portions of 0.1% peptone water are then filtered through the membrane to remove as much as possible of the residual antibiotic. A 17.5-mm. diameter disk is aseptically cut from the center of the filtering area and transferred to a sterile 38 × 200-mm. (outside dimensions) test tube containing approximately 90 ml. of sterile thioglycollate medium. The remaining portion of the membrane is transferred to a second similar tube containing approximately 90

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[1] The Antibiotic Regulations are issued under Section 507 of the Federal Food, Drug, and Cosmetic Act.

ml. of fluid Sabouraud medium. The tubes are incubated for 7 days at 32° and 25°, respectively, and then examined for visible growth. The batch meets the requirements of the test if no tube shows growth. If growth is observed in any tube, the test is repeated with twice the number of containers. The batch meets the requirements if no tube on the repeat test shows growth.

The regulations require that specific environmental tests to assess the suitability of the testing conditions be made frequently enough to assure the validity of the test results. The responsible head of each sterility testing laboratory must determine which methods are to be used for air analysis and what levels of microbial contamination of the air are significant and tolerable. Special devices for sampling air to determine its microbial content are helpful in evaluating the conditions of the areas in which the tests are performed. Some of the devices and techniques commonly used for the microbiological analysis of air are the settling plate technique, the sieve and slit-type samplers, and liquid impingement devices. Although settling plates are easy to use, they yield a limited amount of information, since only particles of certain dimensions will settle onto the plates. Another disadvantage is that the volume of air sampled cannot be measured. The liquid impingement samplers, which force a definite quantity of air through an impingement fluid, recover all of the airborne contamination in the volume of air sampled, thus giving more quantitative information. In addition to requiring tests to assess the area in which the sterility tests are performed, the regulations specify that tubes of thioglycollate to which penicillinase has been added must be tested for sterility, either prior to use or at the time of the test. If the environmental or other tests show sufficient evidence that the results obtained in the first or second official sterility tests are not valid, additional tests may be performed.

As previously noted, 0.1% peptone solution was chosen as the diluting fluid for the filtration procedure. Sterile distilled water or physiological saline had been used for many years to dissolve antibiotics prior to transferring them to the media in the sterility tests (4). The deleterious effect of distilled water on some microorganisms has been well documented in the literature (5). (The "British Pharmacopoeia" specifies sterile physiological saline for dissolving antibiotic powders, and U.S.P. XVII requires that a sterile diluent be used to dissolve solids for the general sterility test.) A diluting fluid which would minimize the destruction of small populations of vegetative cells during the pooling, solubilizing, and filtering of antibiotics was sought. A diluting fluid affording protection to vegetative cells is a necessity when a large number of filtration sterility tests are performed on the same day because each test cannot be carried to completion before another test is started. Therefore, the diluent must protect the vulnerable vegetative cells in an antibiotic milieu until they are safely separated from the antibiotic and transferred to the growth medium.

Straka and Stokes (6) reported that destruction of vegetative bacteria can be minimized by the use of 0.1% peptone solution. Jane-Williams (7) studied the survival of bacteria in six different diluents and found that 0.1% peptone solution was the only one that did not kill one or more of the test organisms.

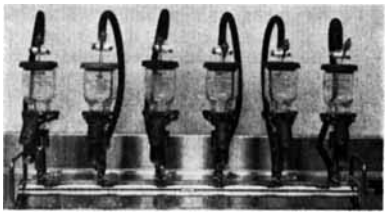

Fig. 1.—Sterility test unit.

EXPERIMENTAL

Equipment.—To accommodate the large number of samples tested daily for quality control by filtration techniques, new equipment had to be designed. The filtering apparatus used in developing the technique has been assembled by a manufacturing firm into a compact "sterility test unit" (as shown in Fig. 1) consisting of six separate filtering devices on a manifold. This unit was designed expressly for sterility testing of antibiotics and was produced to meet the specific requirements described in the Antibiotic Regulations.

The completely assembled unit is steam sterilized at 121° for 20 min., with stopcocks open and membranes in position in the funnels. It is brought into the filtering area where, after all stopcocks have been closed, the metal intake tube of the manifold is aseptically attached to an overhead bottle of sterile 0.1% peptone solution. The metal exhaust tube of the manifold is attached by means of a connecting hose to an empty bottle. The latter, in turn, is connected to a vacuum outlet. All exhaust valves are turned to an "off" position. The vacuum is then turned on, and the unit is ready for use.

Tests on Diluting Fluids.—The survival of organisms in antibiotics dissolved in 0.1% peptone solution, distilled water, and 0.85% sodium chloride solution was compared. No attempt was made to adjust the pH of the distilled water or saline. However, the pH ranged between 6.5 and 6.9 before the addition of the antibiotic, and the pH of the 0.1% peptone solution was 7.0 ± 1. Bacteriological peptone (Difco B 118) was used. The seed organisms were added to two flasks each containing 200 ml. of the given diluent in which the antibiotic powder was dissolved. One flask of each diluent was filtered immediately and the second flask was filtered after 1 hr. After the solutions containing the organisms were filtered through a 47-mm. diameter membrane having a porosity of 0.45 μ, the membranes were washed three times with 100 ml. of the individual diluent. Each membrane was then aseptically moved to the surface of sterile nutrient agar in sterile Petri dishes (20 × 100 mm.). The plates were covered with sterile porcelain tops, glazed on the outside, and incubated at 32° for 5 days. The colonies were counted and the survival was noted for each contact period, initially and after 1 hr. The amount of each antibiotic used was the amount required for the sterility test of that particular antibiotic in the Antibiotic Regulations. For example, 300 mg. of sodium penicillin G from 20 immediate containers is the amount pooled for a

TABLE I.—VIABILITY OF ORGANISMS RECOVERED FROM ANTIBIOTIC SOLUTIONS FILTERED IMMEDIATELY[a] AND AFTER 1 hr. AT 25°C.

Organism	Antibiotic	Amt., Gm.	Estimated No. Cells	Recovery After 5 Days at 32°C. Peptone, 0.1% Ini.	Peptone, 0.1% 1 hr.	Saline, 0.85% Ini.	Saline, 0.85% 1 hr.	Distilled Water Ini.	Distilled Water 1 hr.	Medium
S. aureus ATCC 6538P	Tetracycline hydrochloride	1.0	40	36	28	28	2	34	0	Peptone–casein agar
Serratia marcescens ATCC 14756	Tetracycline hydrochloride	1.0	20	18	16	16	10	18	14	Peptone–casein agar
Streptococcus pyogenes ATCC 8668	Tetracycline hydrochloride	1.0	50	34	22	2	0	6	0	Peptone–casein agar
Bacillus subtilis (spores)[b]	Dihydrostreptomycin sulfate	6.0	55	50	48	42	50	52	48	Peptone–casein agar
Aspergillus sp. (spores)	Polymyxin B′ sulfate	6.0	42	38	37	40	38	35	42	Sabouraud dextrose agar
S. aureus ATCC 6538P	Sodium penicillin G	6.0	30	20	28	18	0	12	4	Peptone–casein agar
Escherichia coli ATCC 11699	Sodium penicillin G	6.0	64	50	44	44	0	52	0	Bacto E.M.B. agar

[a] As soon as the organisms were added. [b] ATCC 6633 strain made resistant to dihydrostreptomycin.

test. Therefore, 6-Gm. portions were dissolved in each of two flasks containing 200 ml. of each of the three diluents. As shown in Table I, the survival of spores of either bacteria or fungi was not affected either by the nature of the diluent or by the time of exposure to the antibiotic solution. However, the vegetative cells added to the antibiotic solutions and immediately filtered survived to approximately the same extent in each diluent. When the filtration was delayed for 60 min., the survival rate was higher in 0.1% peptone solution.

Sodium Thioglycollate.—The fluids for solubilizing insoluble antibiotics should be nontoxic to microorganisms. Although no sterile zinc bacitracin products are in use, an aerosol spray can containing zinc bacitracin for topical use is tested. The Antibiotic Regulations require this product to contain no more than 10 microorganisms per container. To prepare it for the test it must be filtered through a membrane filter in the same way as samples tested for absolute sterility. Early attempts to solubilize zinc bacitracin (for sterility testing) were unsuccessful. For potency assay zinc bacitracin is dissolved in 0.1 *N* hydrochloric acid, but for sterility testing the acidic solution cannot be used. It was noted that zinc bacitracin is soluble in thioglycollate medium but not in Sabouraud medium. Investigation disclosed that the chelating properties of sodium thioglycollate were responsible for the solubilization. Consequently, 0.05% sodium thioglycollate is added to the 0.1% peptone solution to solubilize zinc bacitracin prior to filtration. Since the required concentration of sodium thioglycollate is the same as that in thioglycollate medium, there is no problem of toxicity to microorganisms when it is used as a component of the diluting fluid.

Polysorbate.—To overcome filtration difficulties with a tetracycline aerosol containing isopropyl myristate, 0.5% polysorbate 80 is added to the 0.1% peptone water. (Aerosols to be tested for sterility by using filtration procedures are sprayed into an empty sterile flask, and after the propellant evaporates, the residue is dissolved.)

Penicillinase.—To solubilize procaine penicillin G alone or in combination with streptomycin, the enzyme penicillinase is aseptically added to the peptone diluting fluid. Bowman (8) reported that procaine penicillin G could be solubilized rapidly by using a high-titer penicillinase. It has been found that approximately 337,000 Levy units of penicillinase will solubilize 6 Gm. of procaine penicillin G for the membrane filtration sterility test.

Isopropyl Myristate.—The use of isopropyl myristate as a diluent for petrolatum-based ointments was first suggested by Sokolski (9), who improved the recovery of viable cells from ointments by a filtration technique. Isopropyl myristate dissolves certain sterile petrolatum-based antibiotic ointments so that these preparations can also be tested by membrane filtration. The Antibiotic Regulations have been amended (10) to include a sterility test for sterile ophthalmic ointments containing bacitracin, neomycin, and polymyxin. Portions of 0.1 Gm. from each of 10 immediate ointment containers are aseptically transferred to 100 ml. of isopropyl myristate at 47°. The process is then repeated with another 10 ointment containers. After the ointment has dissolved, the solutions are filtered as described for the membrane filtration sterility test. Since it is difficult to wash residual antibiotics, especially neomycin, from membranes through which oleaginous preparations have been filtered, a rinse medium has been devised to help overcome this problem. The rinse medium contains bacteriological peptone and beef extract to protect the vegetative cells during the rinsing period. (Each filter is rinsed five times with 100 ml. of the rinse medium.) Polysorbate 80 is included in the rinse medium to decrease the filtration time, and sodium chloride 3% (w/v) is added to reverse the action of the neomycin which adheres to the membrane.

RESULTS AND CONCLUSIONS

As previously mentioned, the Antibiotic Regulations contain instructions for performing sterility tests by both membrane filtration and the direct method. Some antibiotics, sodium penicillin G for example, may be tested by either method. However, if the direct method is used, the ability of the penicillinase to inactivate all the penicillin in the

sample under test is checked by adding the proper amount of penicillin to another tube of thioglycollate medium containing the penicillinase and inoculating it with 1 ml. of a 1:1000 dilution of an 18–24 hr. culture of *Staphylococcus aureus* ATCC 6538P. Typical microbial growth must be observable after 24 hr.

The regulations also include any exceptions required for testing specific antibiotics. When 200 ml. of a 3% solution of an antibiotic cannot be filtered, as in the case of tetracyclines or chloramphenicol, 50-mg. portions are used from each of 20 containers instead of the 300 mg. required by the general method.

Investigational studies and experience have shown that the filtration procedure offers better assurance of the sterility of antibiotics. Bowman and Holdowsky (11), in a study of the survival of bacteria in dihydrostreptomycin sulfate solutions, showed the superiority of the membrane filter method over the direct method in recovering organisms sensitive to dihydrostreptomycin. Dihydrostreptomycin solutions were artificially contaminated with 50,000 spores/ml. of six different bacteria whose vegetative cells were sensitive to dihydrostreptomycin. When membrane filtration was used, organisms were consistently recovered at intervals up to 3 months (the duration of the testing period). The same solutions gave negative results when tested by the direct method.

Lightbown (12) reported on dihydrostreptomycin solution contaminated with up to 4000 viable particles per vial of a relatively resistant strain of *Klebsiella pneumoniae* or a sensitive strain of *S. aureus* and examined by the "British Pharmacopoeia" dilution method and by membrane filtration. With the resistant strain of *K. pneumoniae*, 34 samples out of 87 were found to be contaminated by the filtration test, while the dilution test detected no contamination; with *S. aureus* 54 samples out of 93 were found contaminated when tested by filtration, while the dilution test detected no contamination. He also reported that 7-year-old batches of dihydrostreptomycin sulfate tested by filtration procedures revealed the presence of contaminating organisms which had survived in the dry powder. The contamination had not been revealed by earlier dilution tests.

Twenty vials, each containing 10 ml. of a suspension of procaine penicillin G in dihydrostreptomycin sulfate solution, were tested in this laboratory and found contaminated by the filtration procedure. An additional 40 vials were retested by both the filtration method and the direct method. The membrane filtration method recovered the same contaminant, a Gram-positive bacillus. The direct method showed no growth in 40 tubes of thioglycollate or 40 tubes of Sabouraud medium after incubation for 7 days at 32° and 25°, respectively.

In the first year after the new sterility regulations became effective, a total of 8,190 sterility tests were performed. The membrane method was used for 4,743 and the direct method for 3,447. The filtration procedures were found to reduce the cost of sterility testing, the reduction being due primarily to significant savings of media and of manhours involved in the preparation of media. When a preparation was submitted for certification with 25 "filling operations" from one batch, the better coverage and the economy of the filtration method were apparent. Under the former regulations, 10 of these single-dose containers would have been tested, whereas under the new regulations, 1,000 were tested. Had this been a multiple dose container, only 500 would have been required, since each container could be used to furnish a test dose for both a thioglycollate and a Sabouraud tube. In any event, the direct method would have required 1,000 tubes of media, while the filtration method required only 50.

The membrane filtration sterility test has been officially accepted by the United Kingdom and the United States for antibiotic sterility testing. The "British Pharmacopoeia," 1963, requires filtration tests for testing the sterility of parenteral preparations of bacitracin, oxytetracycline, tetracycline, polymyxin, streptomycin, vancomycin, viomycin, and methicillin. For sterility tests of all antibiotics, U.S.P. XVII and N. F. XII refer to the Antibiotic Regulations of the Food and Drug Administration.

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Mechanisms of Antithyroidal Activity of Methimazole

By ROBERT E. BROCK and W. F. HEAD, JR.

Methimazole produces a significant inhibition of iodide ion absorption by the intact thyroid which may account for the major portion of its antithyroidal action. The principal intrathyroidal effect of methimazole is shown to be due to its reactivity with elemental iodine. It neither inhibits nor serves as a competitive substrate for the hydrogen peroxide–peroxidase enzyme system. Thyroxine is displaced from its serum binding sites by methimazole; however, this mechanism is not considered to be clinically important.

THIOUREA compounds, such as methimazole, are thought to exert their antithyroidal effect by competing with tyrosine for elemental iodine or by inhibiting the oxidative enzyme system which converts iodide ion to iodine. In either case, the formation of diiodotyrosine is prevented and the biosynthetic pathway to the formation of thyroxine is blocked.

In competing with tyrosine for iodine, the thiourea derivative is thought to enolize to form a sulfhydryl group which is easily oxidized by iodine to form a disulfide. Iodine is reduced to iodide ion which is incapable of iodinating tyrosine. Since this reaction proceeds at a rate several hundred times faster than the iodination of tyrosyl groups, competition should be quite favorable for diversion of iodine from organic binding (1, 2).

It has been concluded by Astwood (3) that inhibition of a peroxide–peroxidase enzyme system in the thyroid is the most likely mechanism of antithyroidal activity of the thiourea compounds. Alexander (4, 5) recently demonstrated the existence, in thryoid homogenates, of such an enzyme system which can perform the iodide oxidation. Randall (6) has shown that sulfhydryl compounds are capable of reacting with hydrogen peroxide and that the rates of reaction are accelerated in the presence of peroxidase. It was concluded that sulfhydryl compounds do not inhibit peroxidase, but rather serve as substrates, competing successfully with other substrates. It was not demonstrated that sulfhydryl groups could successfully compete with iodide ion for the peroxide–peroxidase system.

Others have studied the inhibition of secretion or activity of hormonal TSH (7, 8) and the inhibition of the utilization of thyroid hormones at the tissue level (9, 10). The controversial studies of the mechanisms of action of thiourea antithyroid compounds have been reviewed by Pitt-Rivers (11), Astwood (1), and Trotter (12).

The purpose of this study was to determine the extent of contribution to antithyroidal activity by various possible mechanisms of action of methimazole, a typical thiourea derivative. The modes of action investigated included both extrathyroidal and intrathyroidal mechanisms.

EXPERIMENTAL

Reaction of Iodine with Methimazole and Tyrosine.—The course of reaction between iodine and a mixture of methimazole and tyrosine was determined spectrophotometrically. Iodine was allowed to react with methimazole, tyrosine, and a mixture of the two. All reactions were performed at room temperature and completion noted by the discharge of the yellow iodine color. Each of the reactants was prepared in 0.001 *M* concentration in phosphate buffer, pH 7.4.

As shown in Table I, the molar ratio of methimazole–tyrosine–iodine used was 4:1:2 which provides a stoichiometric quantity of iodine to the reactions. In the presence of a mixture of methimazole and tyrosine, iodine was added last to provide a competitive effect and was present in sufficient quantity to react completely with only one component.

After reaction, all samples were diluted to 500 ml. with phosphate buffer and their ultraviolet absorption spectra determined from 275 to 215 mμ using a Beckman model DK-2A recording spectrophotometer. The results are shown in Fig. 1.

The spectrum of the reaction product of iodine with a mixture of methimazole and tyrosine (spectrum *3*) compares very closely with the spectrum of the reaction product of iodine with methimazole to which tyrosine was added after reaction (spectrum *5*). If iodine had reacted with tyrosine to the ex-

TABLE I.—REACTION COMPONENTS

Sample	0.001 *M* Methimazole, ml.	0.001 *M* Tyrosine, ml.	0.001 *M* Iodine, ml.	Buffer, ml.
1	0	2.5	5	10
2	10	0	5	2.5
3	10	2.5	5	0
4	10[a]	2.5	5	0
5	10	2.5[b]	5	0

[a] Added after reaction of tyrosine with iodine was complete. [b] Added after reaction of methimazole with iodine was complete.

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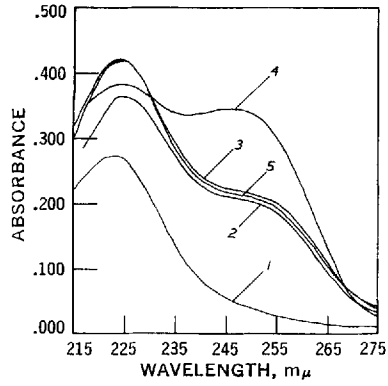

Fig. 1.—Ultraviolet spectra of methimazole and tyrosine reaction products with iodine. Key: *1*, tyrosine–iodine; *2*, methimazole–iodine; *3*, tyrosine and methimazole–iodine; *4*, tyrosine–iodine with added methimazole; *5*, methimazole–iodine with added tyrosine.

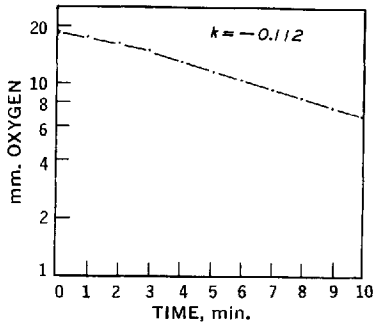

Fig. 2.—Catalytic decomposition of hydrogen peroxide by iodide ion.

clusion of methimazole, an absorption spectrum similar to spectrum *4* would have resulted. A mixture of reaction products would have resulted in an absorption spectrum of intermediate character. It is apparent that iodine in the presence of a mixture of methimazole and tyrosine reacts almost exclusively with methimazole.

To further verify that tyrosine failed to react with iodine in the presence of methimazole, the competitive reaction, containing twice the quantity of tyrosine, was repeated. Ultraviolet spectra indicated that methimazole had completely utilized the available iodine. Chromatography of this sample on Whatman No. 1 paper in a descending *n*-butanol–acetic acid (9:1)–water system failed to separate mono- or diiodotyrosine using 0.2% ninhydrin as detector. It was established that this system was capable of separating and detecting as little as 0.5% of the theoretical quantity of diiodotyrosine which could have been formed.

Interaction of Iodide Ion and Methimazole with Hydrogen Peroxide–Peroxidase Enzyme System.—The oxidation of iodide ion, methimazole, and a mixture of the two with hydrogen peroxide in the presence of peroxidase was studied using the manometric technique of Randall (6). All reactions were performed in the Warburg apparatus at 37.5°. The main chamber of the flask contained 1 ml. of either 0.06 *M* potassium iodide, 0.06 *M* methimazole, or both potassium iodide and methimazole in 0.06 *M* concentrations. To the flask were added 0.6 ml. of peroxidase (1 unit/ml.) and 0.4 ml. phosphate buffer, pH 7.4. After temperature equilibration for 10 min., 0.5 ml. of 0.06 *M* hydrogen peroxide was added from one sidearm and the reaction allowed to continue for the desired period of time. Catalase, 0.075 mg. contained in 0.5 ml., was added from the second sidearm to stop the reaction by decomposing the remaining hydrogen peroxide. Oxygen evolution, directly proportional to the hydrogen peroxide remaining in the flask, was read directly from the manometer after 2 min. All solutions were prepared using phosphate buffer, pH 7.4. Pressures (mm. oxygen) reported are averages of a minimum of four determinations corrected to a single manometer tube and corrected for barometric pressure changes. Reaction rates reported were all calculated using a first-order expression since the data obtained corresponded closely to this order.

In the reaction of iodide ion with hydrogen peroxide–peroxidase, a steady evolution of oxygen was noted before the addition of catalase. This was due to the catalytic decomposition of hydrogen peroxide by iodide ion as described by several investigators (13–15). This effect began after a brief induction period and continued until the hydrogen peroxide was decomposed by the addition of catalase. During the induction period, iodide ion is oxidized to a steady-state concentration of iodine. When this concentration is reached, hydrogen peroxide decomposition begins with no further iodine generation. In measuring oxygen pressures at given time intervals, it was first necessary to determine the catalytic oxygen pressure. The catalase solution was then added and the total oxygen pressure recorded after 2 min.

The difference between the catalytic oxygen pressure at various time intervals and the original peroxide oxygen pressure which was shown to be constant over the time involved is a measure of the total theoretical oxygen remaining if no other reactions are occurring. From the resulting data, as shown in Fig. 2, the rate of the catalytic decomposition of hydrogen peroxide can be calculated. This rate, $k = -0.112$, is in good agreement with the literature value (13).

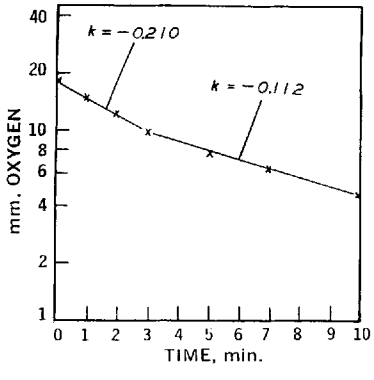

Fig. 3.—Oxidation of iodide ion and catalytic decomposition of hydrogen peroxide.

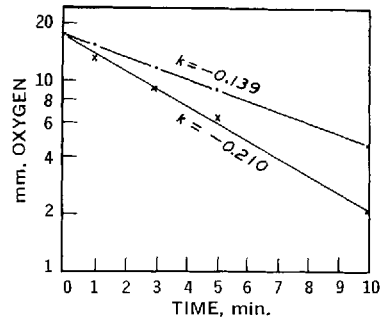

Fig. 4.—Reaction of hydrogen peroxide–peroxidase with methimazole and methimazole–iodide ion. Key: —·—·, methimazole and hydrogen peroxide–peroxidase; —×—×, methimazole, iodide ion, and hydrogen peroxide–peroxidase.

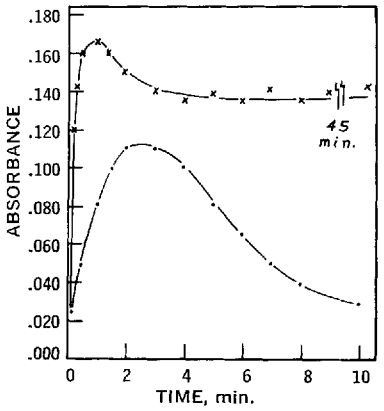

Fig. 5.—Iodine generation by hydrogen peroxide–peroxidase. Key: —×—×, iodide ion and hydrogen peroxide–peroxidase; —·—·, iodide ion, methimazole, and hydrogen peroxide–peroxidase.

The difference between the catalytic oxygen pressure and the total measured oxygen pressure after addition of catalase is equivalent to the actual peroxide oxygen remaining. These data (Fig. 3) show the initial oxidation of iodide ion to iodine during the induction period ($k = -0.210$) and the catalytic decomposition effect ($k = -0.112$). Beyond the induction period, only catalytic decomposition is occurring as evidenced from the rate constant.

Methimazole and iodide–methimazole substrates did not produce a catalytic peroxide decomposition. The rates of these reactions could be followed by direct measurement of residual oxygen pressure at a given time as shown in Fig. 4. Methimazole reacts with the hydrogen peroxide–peroxidase system at a slower rate ($k = -0.139$) than a mixed iodide–methimazole substrate. The rate constant of the latter reaction is the same as that calculated for the oxidation of iodide ion to a steady-state concentration of iodine. Since no catalytic decomposition was observed and the reaction rate was uniform over the time period measured, it would seem likely that the primary reaction is the oxidation of iodide ion in an attempt to gain a steady-state concentration of iodine which, in the presence of methimazole, cannot be attained.

The same ratio of reactants (methimazole–iodide ion–peroxide = 2:2:1) at 6 times the previous concentration also failed to display the catalytic peroxide decomposition effect. The same result was obtained at ratios of 2:2:2 and 2:2:3. At ratios of 2:2:4, 2:2:5, and 2:2:6, catalytic oxygen evolution began after a 6 to 8-min. induction period.

Further verification of iodine generation in a mixed iodide–methimazole system was obtained spectrophotometrically. During the manometric studies, a transient pale yellow color was noted in the first few minutes of reaction. The iodide ion reaction and the iodide–methimazole reaction with hydrogen peroxide–peroxidase were repeated as described and the absorbance at 400 mμ measured using a Bausch & Lomb Spectronic 20 spectrophotometer. As shown in Fig. 5, in the absence of methimazole, a steady-state concentration of iodine is reached within 2 to 3 min. and remains throughout the period of observation. In the presence of methimazole, iodine is produced; however, it never reaches a steady-state concentration. It gradually decreases in concentration probably by reaction with methimazole.

Inhibition of Thyroidal Absorption of Iodide Ion.—Methimazole has been observed to inhibit iodide ion accumulation in euthyroidal humans in doses of 0.5 mg. (16). Williams and Coker (17) have demonstrated that iodide ion absorption by surviving thyroid slices is completely inhibited upon incubation for 2 hr. in a solution containing 5 mcg. of methimazole. The following study was performed to quantitate this effect in the intact thyroid.

Sixteen male, white, Sprague-Dawley rats of the same ages, weighing from 220 to 337 Gm. (average 292 Gm.) were used for tests and controls. Eleven received 0.4 mg./Kg. of methimazole intraperitoneally 12 hr. and again 3 hr. prior to the administration of approximately 7.4 μc. of sodium radioiodide.[1] Five rats used as controls received only sodium radioiodide. Exactly 2 hr. after the administration of sodium radioiodide, the animals were exsanguinated under ether anesthesia. The thyroid glands were immediately removed, transferred to planchets, and counted for 5 min. using a Tracerlab Versamatic II scaler with a Tracerlab scintillation detector, P-20-D, and a 1 × 1.5 in. NaI (Tl) crystal. All counts were corrected for background. The results, shown in Table II, indicate an inhibition of thyroidal iodide ion absorption by methimazole of 80.9%.

Displacement of Thyroxine from Serum Binding Sites.—Diphenylhydantoin has been shown to lower the serum-bound iodine in both epileptic and normal subjects (18). To determine if methimazole exhibits the same property, a dialysis procedure was employed. Test materials were sodium diphenylhydantoin, methimazole, and histamine phosphate. Histamine was used because of its similarity in size and ring structure to methimazole. All were used in equimolar concentrations.

Four 5-ml. portions of fresh dog serum were placed in each of four preboiled 6-in. lengths of cellophane dialysis tubing which were tied at one end. To each was added 1.25 μc. of thyroxine-^{131}I contained in 0.2 ml. The serum control tube was diluted with 5 ml. of phosphate buffer, pH 7.4. To

[1] Oriodide. Abbott Laboratories, Radiopharmaceuticals Division, Oak Ridge, Tenn.

TABLE II.—THYROIDAL IODIDE ION ABSORPTION BY CONTROL AND METHIMAZOLE-TREATED RATS

Rats	^{131}I, c.p.m. Administered	Mean ± σ ^{131}I, c.p.m. Absorption	% ± σ ^{131}I Absorption
Controls	1.68×10^6	57,104 ± 12,941	3.4 ± 0.77
Methimazole-treated	1.68×10^6	10,941 ± 3,270	0.65 ± 0.02

TABLE III.—DISPLACEMENT OF THYROXINE FROM DOG SERUM BINDING SITES

Dialysis Time, hr.	Serum Sample			
	Control	Methimazole	Diphenylhydantoin	Histamine
1	2.0%	2.2%	2.6%	2.0%
4	2.8	3.7	4.0	2.1
8	5.1	29.0	33.7	4.0
12	10.2	75.0	83.2	5.1

the remaining three tubes were added 25 mcg. of methimazole, 60 mcg. of sodium diphenylhydantoin, and the equivalent of 24.3 mcg. of histamine, respectively, each in 5-ml. volume. This quantity of methimazole approximates physiological concentrations. The tubes were tied and each placed in separate capped jars containing 20 ml. of phosphate buffer, pH 7.4. The jars were agitated in a Dubnoff incubator at 37.5°. One hundred-microliter samples of the buffer were removed at 1, 4, 8, and 12-hr. intervals, placed in planchets, and counted using a scintillation detector.

Only if thyroxine is displaced from its protein binding sites will it dialyze into the phosphate buffer medium. The per cent displacement can be calculated from the count rate in the external buffer at a given interval and the known activity added to the serum. The results in Table III indicate that thyroxine is displaced by methimazole and sodium diphenylhydantoin. Histamine did not show this effect, perhaps indicating that a urea or thiourea moiety is required.

DISCUSSION

Methimazole has been shown to compete with tyrosine for elemental iodine so effectively that detectable levels of iodinated tyrosine derivatives are not formed. It neither inhibits nor serves as a preferential substrate for the hydrogen peroxide–peroxidase enzyme system. Iodide ion in the presence of methimazole and the enzyme system is converted to elemental iodine which apparently reacts with methimazole as it is formed. These data indicate that the avidity of methimazole for elemental iodine is the prime basis for its intrathyroidal activity.

It was found that a reduction in the uptake of iodide ion on the order of 80% by the intact thyroid followed the administration of methimazole. Since iodide ion absorption is the first step leading to the biosynthesis of thyroxine, this mechanism of action must be considered very significant and probably more important than the intrathyroidal effects.

In physiological concentrations, methimazole and sodium diphenylhydantoin were shown to displace thyroxine from its serum binding sites. Although sodium diphenylhydantoin causes a reduction in serum-protein bound iodine, it does not produce a clinical hypothyroidism. Therefore, it would appear unlikely that methimazole exerts a significant portion of its antithyroidal effect at this level of thyroid function.

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Kinetics of Nitrous Acid Induced Reserpine Fluorescence

By R. P. HAYCOCK, P. B. SHETH*, T. HIGUCHI†, W. J. MADER, and G. J. PAPARIELLO

The kinetics of the reaction of reserpine with nitrous acid has been studied. The results show a two-step process with nitrous acid reacting with protonated reserpine to form an intermediate complex which under the influence of hydrogen ions forms a colored fluorescent product, identified as 3-dehydroreserpine. A rate expression was derived and reaction rates computed.

MANY CHEMICAL reactions used for the production of chromogenic or fluorogenic products in organic analysis are never studied beyond the point of practical utilization. Yet, further knowledge of their chemistry is desirable to extend their use.

The reaction of reserpine with nitrous acid to produce a fluorogenic-colored product has a long history of effective analytical service. It was first reported on by Szalkowski and Mader (1), who utilized its chromogenic characteristics for the determination of reserpine in pharmaceutical preparations. The procedure was later modified by Banes *et al.* (2–4) and subsequently approved as the official method (5) for pharmaceutical preparations. Since fluorometric techniques are extremely sensitive, a procedure which considered this property of the reaction was adopted by the Association of Official Agricultural Chemists for the microdetermination of reserpine in feeds (6–8). Although there is a wealth of published data on its analytical applications, there seem to have been no earlier attempts to ascertain the mechanism and kinetics of the reaction. In the present investigation, the fluorescent method has been used to study the kinetics involved in treating reserpine with nitrous acid.

EXPERIMENTAL

All fluorescent measurements were made on an Aminco-Bowman spectrophotofluorometer. As previously reported (6), the excitation maximum for the nitrous acid induced reserpine fluorescence is 390 mμ and the fluorescent maximum is 510 mμ.

In studying the effect of nitrous acid on the reaction rate, the initial concentrations of reserpine and of hydrochloric acid in the reaction flask were kept constant at 3.16×10^{-7} moles $L.^{-1}$ and 0.24 mole $L.^{-1}$, respectively. However, the concentration of the nitrous acid was varied from 1.74×10^{-3} to 5.80×10^{-5} moles $L.^{-1}$. All reactions were run and all measurements were taken at room temperature. The procedure was as follows.

Pipet 15 ml. of a 60% chloroform–40% methanol solution containing 4.8 mcg. of reserpine into a 25-ml. volumetric flask. Add several milliliters of methanol. Mix well. Add 0.5 ml. of hydrochloric acid and the desired number of milliliters of sodium nitrite solution (0.1% in 50% methanol–50% water). Dilute to mark with methanol and shake well. Measure the fluorescent development as a function of time.

In studying the effect of hydrochloric acid on the reaction rate the initial concentration of reserpine and nitrous acid in the reaction flask were kept constant at 3.16×10^{-7} moles $L.^{-1}$ and 2.9×10^{-4} moles $L.^{-1}$, respectively. However, the concentration of the hydrochloric acid was varied from 4.8×10^{-2} to 4.8×10^{-1} moles $L.^{-1}$. The procedure as described above was followed with the exception that the milliliters of hydrochloric acid added were varied while the milliliters of sodium nitrite solution added were kept constant.

RESULTS AND DISCUSSION

Postulated Reaction.—It is suggested that the reaction which takes place when one induces fluorescence in reserpine by use of nitrous acid is a two-step reaction which can be expressed as follows:

$$\text{protonated reserpine} + \text{HONO} \overset{K_1}{\rightleftharpoons} [\text{protonated reserpine} : \text{HONO}] \xrightarrow[k_2]{H^+} \text{3-dehydroreserpine (fluorescent product)}$$

That only the protonated reserpine species is involved in the reaction is quite obvious when one considers that the pKa of reserpine is 6.6 (9) and the reaction flask pH never exceeds 1.5.

3-Dehydroreserpine is considered to be the fluorescent product and the following evidence has been gathered to support this contention. (A) The wavelengths for excitation and fluorescence, *viz.*, 390 mμ and 510 mμ, are common to the nitrous acid induced reserpine fluorogen and the natural fluorogen of 3-dehydroreserpine. (B) The fluorescent yields at the above conditions are almost exactly the same for 3-dehydroreserpine and the reaction fluorophor. (C) On different paper chromatographic systems, *viz.*, (*a*) mobile phase, 10% pyridine–90% chloroform, immobile phase, 1%

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benzoic acid in formamide; (*b*) mobile phase, chloroform saturated with formamide, immobile phase, 1% benzoic acid in formamide, the R_f value for the product of the reaction mixture was the same as that for 3-dehydroreserpine.

Nitrous Acid Effects.—Figure 1 is a logarithmic plot of the concentration of unreacted reserpine, expressed as the final fluorescence (F_∞) minus the fluorescence at any time, t, (F_t) *versus* time. This plot demonstrates that at all nitrous acid concentrations a linear relationship is obtained. Thus, this reaction is an apparent first-order reaction with respect to reserpine concentration, *i.e.*,

$$-\frac{d(\mathrm{R})}{dt} = k_{\mathrm{obs.}}[\mathrm{R}_t] \qquad \text{(Eq. 1)}$$

It should be noted that one can use an expression such as $F_\infty - F_t$, because the final fluorescent intensity has been determined to be a certain value which is independent of nitrous acid concentration over a wide concentration range. Figure 2 demonstrates the relationship between the rate of fluorescent formation and nitrous acid concentration. It is seen that with increasing amounts of nitrous acid there is a corresponding increase in the rate of conversion of reserpine to the fluorophor. It is also seen that all the lines asymptotically approach the same final value.

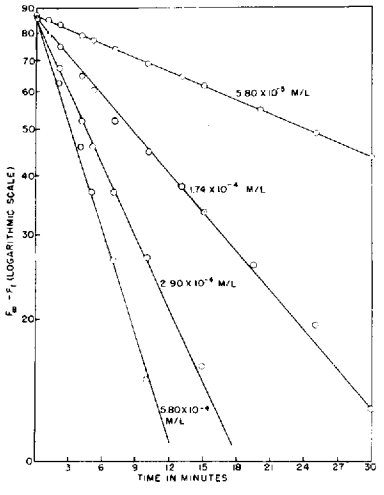

Fig. 1.—First-order plot for loss of reserpine in induced fluorescence reaction at various nitrous acid concentrations.

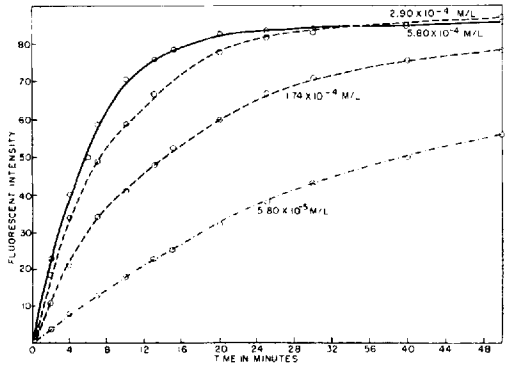

Fig. 2.—Relationship between rate of fluorophor formation and nitrous acid concentration.

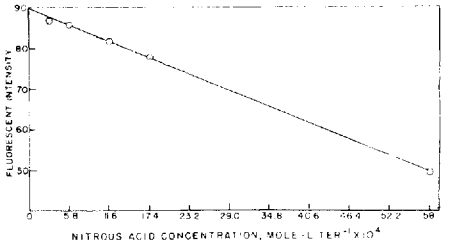

Fig. 3.—Suppression of fluorescent yield as a function of nitrous acid concentration.

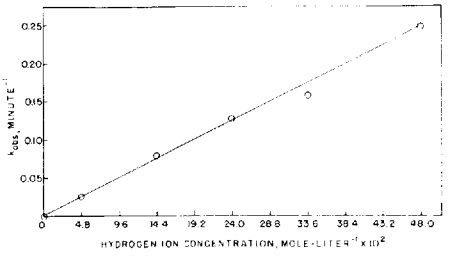

Fig. 4.—Relationship between observed rate constant and hydrogen ion concentration.

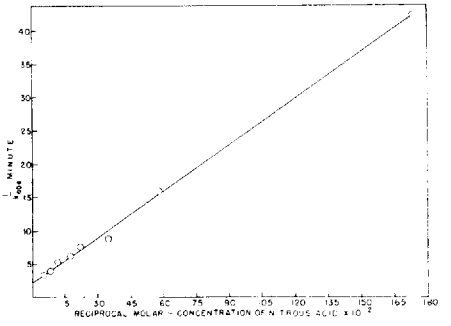

Fig. 5.—Relationship between the reciprocal observed rate constant and the reciprocal nitrous acid concentration.

However, if one extends this study to nitrous acid concentrations greater than 2.90×10^{-4} moles $L.^{-1}$ it is observed that there is a suppression of the fluorescence. The suppression of maximum fluorescence by the higher concentrations of nitrous acid is evident in Fig. 3. This straight line plot indicates that under the experimental conditions used in this study the limiting fluorescent yield is approximately 90 in the absence of the quenching effect of the nitrous acid. This type of quenching has been referred to by Bowman (10) as an inner filter effect. Apparently the excess nitrous acid absorbs some of the activating energy, or some of the fluorescent

energy, or both, thus reducing the amount of fluorescent energy reaching the measuring unit.

Rate Expression.—It was observed rather early in this work that both hydrogen ions and nitrous acid had an effect on the rate of fluorescent formation. Thus, it is obvious that the observed rate constant, $k_{obs.}$, consists of a number of component parts which can be resolved experimentally. If it is assumed that the first step in the reaction, that is the formation of the intermediate complex, is quite rapid, the rate-determining step is the second step, and the over-all rate of reaction is equal to k_2 multiplied ty the product of the concentration of the intermediate complex and the hydrogen-ion concentration, *i.e.*,

$$-\frac{d(R)}{dt} = k_2[R:HONO]\,[H^+] \quad \text{(Eq. 2)}$$

If $[R_t]$ equals the initial concentration of protonated reserpine present in the reaction mixture and [R:HONO] the concentration of the intermediate complex, then $[R_t] - [R:HONO]$ is equal to the concentration of free protonated reserpine remaining. Thus

$$K_1 = \frac{[R:HONO]}{([R_t] - [R:HONO])\,[HONO]} \quad \text{(Eq. 3)}$$

or

$$[R:HONO] = \frac{K_1\,[R_t]\,[HONO]}{1 + K_1\,[HONO]} \quad \text{(Eq. 4)}$$

By substituting Eq. 4 into Eq. 2 one obtains

$$-\frac{d(R)}{dt} = \frac{k_2\,[H^+]\,K_1[R_t]\,[HONO]}{1 + K_1\,[HONO]} \quad \text{(Eq. 5)}$$

If the right hand side of Eq. 5 is substituted into Eq. 1 the following expression for $k_{obs.}$ is obtained

$$k_{obs.} = k_2[H^+]\,\frac{K_1[HONO]}{1 + K_1[HONO]} \quad \text{(Eq. 6)}$$

If the assumptions made thus far have been correct, then one should obtain by plotting the observed rate constant *versus* the hydrogen-ion concentration a straight line which passes through the origin and whose slope is equal to k_2K_1 [HONO]/1 + K_1 [HONO]. Such a linear relationship is obtained as shown in Fig. 4. It is possible to mathematically manipulate Eq. 6 to give the following expression

$$\frac{1}{k_{obs.}} = \frac{1}{k_2K_1[H^+]}\left(\frac{1}{[HONO]}\right) + \frac{1}{k_2[H^+]} \quad \text{(Eq. 7)}$$

The above expression states that, if the original contentions are correct, a plot of the reciprocal nitrous acid concentration *versus* the reciprocal observed rate constant should yield a straight line whose intercept is equal to $1/k_2$ $[H^+]$ and whose slope is equal to $1/k_2K_1[H^+]$. Figure 5 is such a plot, and it is indeed linear. Using the above plot one is able to determine that the equilibrium constant K_1 is equal to 690 L. mole^{-1} and k_2 is equal to 2.2 L. mole^{-1} min.$^{-1}$ at a hydrogen-ion concentration of 0.24 mole L.$^{-1}$ and 25°.

The data presented above support the proposed general mechanism. However, the authors are at present unable to delineate in any great detail the nature of the nitrous acid–reserpine addition product. It apparently forms with great ease and is always in equilibrium with the unreacted alkaloid. Establishment of its chemical structure would be most interesting.

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Zeta Potential in the Development of Pharmaceutical Suspensions

By ROBERT A. NASH and BRUCE E. HAEGER

A discussion of a microelectrophoretic mobility apparatus (Zeta-Meter) is presented in connection with some prerequisite studies with a model dispersed system. The limitations of the fundamental Helmholtz-Smoluchowski formula for calculating zeta potential as it applies to a study of real pharmaceutical systems is presented. A description of the apparatus, including a discussion of the advantages of the dark-field stereoscopic microscope, molybdenum-platinum electrode system, cylindrical, tube-type, plastic electrophoresis cell, and the automatic sample-transfer device is also presented. The usefulness of zeta potential in developing pharmaceutically stable, flocculated suspensions is illustrated with three different examples: (*a*) an adrenocorticoid in which flocculation was directly achieved by varying the concentration of electrolyte added; (*b*) a system in which flocculation was initially induced in the absence of an electrolyte; (*c*) the development of a diagnostic system for blood-cell agglutination testing based upon the principles of zeta potential.

ALTHOUGH the zeta potential (ZP) concept as it relates to particles in suspension has been known to colloid chemists for more than 40 years (1–3), only recently, with the introduction of new techniques for measuring electrophoretic mobility (EM), have industrial and pharmaceutical applications of its principles been possible (4–6, 15). Haines and Martin (7), studying the electrokinetic behavior of insoluble drug particles in dispersion, were able to correlate the magnitude of ZP to the caking tendencies of pharmaceutical suspensions. Since then little has been reported in the pharmaceutical literature concerning ZP. Even though the debate over the physicochemical meaning of ZP continues in the general literature (8,9), the electrokinetic behavior of dispersed systems can still be investigated in terms of an observable quantity, the EM value.

The processes involved in the formation of pharmaceutical suspensions and the concept of ZP as it applies to a study of such systems have been reviewed recently (10).

The ZP of a particle in suspension can be calculated approximately from EM with the aid of the simple Helmholtz-Smoluchowski Eq. 1. Where ZP represents the zeta potential of the suspensoid in millivolts, 4π is the Smoluchowski factor, the factor 9×10^3 converts electrostatic units to practical electrical units and microns to centimeters, η is the viscosity of the suspending liquid in poise, ϵ represents the dielectric constant of the suspending liquid, and EM represents the electrophoretic mobility of the suspensoid in μ/sec./v./cm.

$$ZP = 4\pi \cdot 9 \times 10^3 \cdot \frac{\eta}{\epsilon} \cdot EM \qquad \text{(Eq. 1)}$$

For suspensions in water at 25° Eq. 1 is reduced to

$$ZP = 12.869 \cdot EM \qquad \text{(Eq. 2)}$$

Calculation of so-called "true ZP values" in terms of the physical meaning of the system under study involves refinement of the 4π multiplication factor (9). Its magnitude, which is related to the thickness of the electrical double layer surrounding the particle, is dependent upon particle size and shape, the type and concentration of electrolyte in the bulk liquid, the distortion of the electric field by the suspended particle (electrophoretic retardation effect), the surface conductance of the electrical double layer, and ZP itself.

A series of multiplication factors for systems containing a typical 1:1 electrolyte (potassium chloride), which takes into account the influence of some of these systematic variables, have been calculated and summarized in Table I. An inspection of these values reveals that when the size of the electrical double layer is small with respect to the particle, *i.e.*, in suspensions with moderate to high concentrations of electrolyte, the Smoluchowski 4π factor can be employed with sufficient accuracy.

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TABLE I.—FACTORS FOR A 1:1 ELECTROLYTE TO BE USED IN THE HELMHOLTZ-SMOLUCHOWSKI FORMULA IN PLACE OF THE 4π FACTOR

KCl Concn., % w/v	Specific Conductance, μmhos./cm.	κa [a]	ZP, mv. 0	25	50	70
		a, Particle Radius = 1 μ				
1.0×10^{0}	1.775×10^{4}	1203.	4.00π	4.00π	4.00π	4.00π
1.0×10^{-1}	1.820×10^{3}	380.	4.04π	4.08π	4.08π	4.12π
1.0×10^{-2}	1.905×10^{2}	120.3	4.16π	4.20π	4.24π	4.32π
1.0×10^{-3}	2.04×10^{1}	38.	4.40π	4.48π	4.64π	4.88π
1.0×10^{-4}	2.2×10^{0}	12.	4.84π	5.00π	5.36π	6.12π
		a, Particle Radius, μ	0	25	50	70
1.0×10^{-3}		1.0	4.40π	4.48π	4.64π	4.88π
1.0×10^{-3}		2.0	4.20π	4.24π	4.39π	4.52π
1.0×10^{-3}		4.0	4.12π	4.16π	4.16π	4.28π

[a] κa represents the ratio between the radius of the particle and the extension of the electrical double layer.

DISCUSSION

However, when the electrical double layer is extended, *i.e.*, in suspensions which contain little or no electrolyte, then the factor approaches Henry's 6π value (11). It is also well to point out that the concentration of ions (charge density) in the electrical double layer bears an inverse relationship to the size of the double layer itself.

The use of the Helmholtz-Smoluchowski formula is further complicated by the fact that the viscosity term in Eq. 1 exerts a more pronounced effect upon ZP than does the dielectric constant. For example, in calculating the ZP for particles dispersed in 50% syrup (sucrose in water) where the viscosity of the suspending vehicle at 25° is 0.125 poise and the dielectric constant is 64.2, the EM of the particle would have to be reduced by a factor of $^{1}/_{17}$ to give a ZP equivalent to that in water at the same temperature. The reason is that the viscosity of syrup is 14 times greater than that of water, while the ϵ of syrup is only $^{4}/_{5}$ of the value for water. With water systems thickened with a small amount of a hydrophilic colloid such as carboxymethylcellulose U.S.P. or methylcellulose U.S.P. (where the ϵ is essentially equal to that of water) the viscosity effect would be even greater. Therefore, it would seem plausible that the η term in Eq. 1, which in essence represents the shear stress between the electrical double layer and the bulk of the liquid, is only applicable to simple liquids. The use of non-Newtonian liquids and vehicles with viscosities greater than several centipoise units will seriously limit the usefulness of the basic Helmholtz-Smoluchowski expression.

EM is equal to the particle velocity per unit field strength and is expressed in μ/sec./v./cm. Particle velocity is determined microscopically by multiplying the divisions traveled per μ per eyepiece division and dividing the result by the time traveled in seconds. If a circular tube-type electrophoresis cell is employed, then the circle of zero endosmotic flow is situated at a depth which is 14.7% of the tube diameter. Since the over-all flow of the suspending liquid is zero at this point, the net motion is that of the particles themselves.[1] EM can be calculated from either the effective length of the electrophoresis cell, *l*, in cm.

[1] The electrophoresis cell of the apparatus is so designed as to make this critical depth coincide with the counting line on the eyepiece of the stereoscopic microscope.

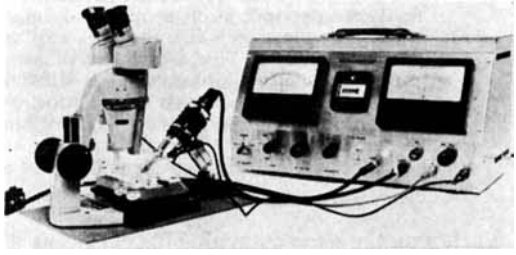

Fig. 1.—The instrument for measuring electrophoretic mobility of drug particles in suspension. Detailed instruction for its operation and calibration are provided with the equipment.

$$\text{EM} = \frac{v \cdot l}{E} \qquad \text{(Eq. 3)}$$

or from the cross-sectional area of the cell, *q*, in cm.^{2},

$$\text{EM} = \frac{v \cdot q \cdot L}{I} \qquad \text{(Eq. 4)}$$

where *v* equals particle velocity in μ/sec., *E* is the applied voltage in practical volts, *L* is the specific conductance of the cell, and *I* is the current flow through the cell in amperes. In the authors' work both Eqs. 3 and 4 were used to calculate EM.

APPARATUS

A new device[2] measures the microelectrophoretic mobility of particles in aqueous suspension. The term "Zeta-Meter" is a misnomer since the instrument neither measures nor determines directly the ZP or effective surface charge of dispersed particles. The equipment is shown in Fig. 1.

The apparatus offers the following advantages.

(*a*) Availability of a ready-to-use complete research system for determining the EM values of colloids and small dispersed particles in essentially aqueous suspension.[3]

(*b*) Since the anode chamber of the Zeta-Meter electrophoresis cell is closed and the cathode chamber is open, when electric current passes through

[2] Zeta-Meter. Marketed by Zeta-Meter, Inc., New York, N. Y.

[3] The authors are unable to comment about the capability of the instrument with respect to its use in connection with essentially nonaqueous systems because of their lack of familiarity with this particular field of study.

systems with a high concentration of electrolyte, appreciable amounts of oxygen can be produced at the anode, due to electrolysis. Such gas formation will interfere with the performance of the cell by inducing liquid movement and false particle migration. The use of a readily oxidizable molybdenum anode, which forms a tight film of oxide on its surface, prevents gaseous imbalance from creating a condition of liquid flow in the cell. A platinum strip cathode, at which hydrogen is evolved, does not induce false migration as the gas passes freely into the atmosphere. However, at low electrolyte concentrations, electrolysis is not a problem and a pair of platinum electrodes can be used. The solid molybdenum-platinum electrodes, which are provided with the unit, are more convenient than a solid-liquid, copper-saturate copper sulfate coupling, with porous plaster of paris plugs which have been previously employed to prevent gasification (12).

(*c*) The use of a stereoscopic microscope of sufficient magnification (120 to 180×) with bounced-beam illumination permits indirect viewing of colloids below 1 μ by reflected light emanating from the particle's surface. The technique is commonly referred to as the "dark-field effect." Effective view of particles extends from 0.05 to 0.02 μ. However, the lower limit of view depends upon particle shape.

(*d*) The cylindrical, tube-type plastic electrophoresis cell utilized offers several obvious advantages over the conventional Briggs flat thin-glass cell which for years has been considered a standard for EM measurement (13). The over-all depth of a Briggs cell (0.6 to 1.0 mm.) limits its ability to handle particle flocs greater than 0.1 mm. Such suspensions can be measured quite easily with the Zeta-Meter cell. In addition, the rugged construction of the plastic cell is in sharp contrast to the fragility of the glass Briggs cell.

(*e*) A new automatic sample-transfer device, also available with the basic unit, is shown in Fig. 2. This device rapidly clears the cell reintroducing fresh sample between measurements, and thereby eliminates the problem of particle drift due to the convection currents by maintaining a condition of isothermality in the cell. The problem of particle drift and thermal overturn is especially acute in flocculated or partially flocculated systems where EM is extremely slow and electrolyte concentration is high. In addition, the use of this auxiliary equipment permits an increase in the number of EM measurements that can be made with the basic unit, thereby enabling the equipment to be used as an EM or ZP titrimeter for particle adsorption and desorption study.

(*f*) In addition to these basic features, the electrophoresis cell can function as a conductivity cell once it is calibrated in the usual manner with a standard potassium chloride solution. Specific conductance values thus obtained are of sufficient accuracy and can be determined during the course of an EM measurement.

However, Schmut (14) lists several disadvantages for the apparatus in a paper concerning ZP applications in the paper industry. The most serious drawback he found is its sensitivity to vibration. The problem is especially acute when the electrophoresis cell is connected to the automatic sample-transfer device. Nevertheless, the condition can be alleviated somewhat by the use of coarse nylon screens in the inlet and outlet tubes leading to the cell. The screen apparently disrupts eddy currents that are set in motion by the oscillation of the automatic pinch-clamp.

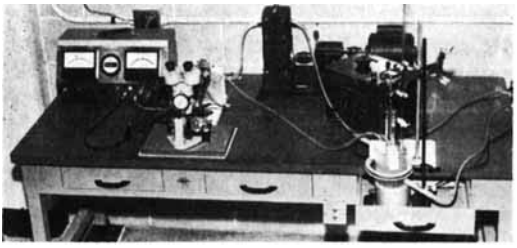

Fig. 2.—Instrumentation required to perform EM or ZP titrations, including the automatic sample-transfer device, Beckman model G pH-meter, and Zeta-Meter.

The authors have found that poor reproducibility of experimental results and meaningless EM values can be attributed to the limitation of the ZP theory to explain the significance of such data rather than instrument failure itself. Furthermore, problems of particle settling during measurement and false migration in difficult systems can be greatly diminished by the use of the automatic sample-transfer device.

STUDIES WITH PURIFIED SILICA

Purified silica[4] was selected as a model dispersed system for study. Such studies are important both as a prerequisite for work with more difficult pharmaceutical systems and to enable the investigator to become more familiar with the equipment and techniques involved in EM measurement. The material consists of high-purity, microcrystalline silica containing 99.9% silicon dioxide. Silica approaches an ideal solid for study since the material is relatively inert, suspensions of it are essentially neutral, and complications associated with surface ion-exchange are negligible (15). An additional advantage is that the material is easily wetted and simple aqueous dispersions can be conveniently prepared with low-shear equipment.

Dispersing Silica with Surfactants.—With a standard 0.01% suspension of purified silica in fresh nitrogen-sparged distilled water, a series of dispersion curves were developed with three typical surface-active agents (more commonly referred to as surfactants). They included an anionic surfactant (dioctyl sodium sulfosuccinate N.F.), a cationic surfactant (benzalkonium chloride U.S.P.), and a nonionic surfactant (polysorbate 80 U.S.P.). Surfactants are primarily used as wetting agents for hydrophobic particles, which act in this capacity by lowering the interfacial tension between suspensoid and vehicle. They can, however, also function as dispersing agents, *e.g.*, by being adsorbed on the surface of silica particles. Adsorption of a negatively charged, polymeric anionic surfactant increases the negative ZP of dispersed suspensoid particles, while conversely, adsorption of a positively charged,

[4] Purified crystalline silica was supplied by the Pennsylvania Glass Sand Corp. under the trademark 5-μ Min-U-Sil; 98% of this material has a diameter less than 5 μ. The surface area of the sample is 20,600 cm.^{2}/Gm. with an average particle size of 1.1 μ.

cationic specie reduces the existing negative ZP on silica particles. Addition of more cationic surfactant eventually produces particles that are positively charged. Since in both instances these agents are ionic in nature, there are enough dissociated counterions in the bulk liquid to induce adsorption.

Adsorption curves showing the influence of an anionic surfactant (dioctyl sodium sulfosuccinate N.F.) and a cationic surfactant (benzalkonium chloride U.S.P.) on the ZP of a 0.01% purified silica dispersion are given in Fig. 3. ZP of the suspensoid apparently increases linearly as increasing amounts of dispersant (surfactant) are added to the system. A point of maximum particle dispersion is reached which corresponds to the point of maximum ZP, either negative or positive depending upon the surfactant under study. In an adsorption process (which will be briefly discussed later) this also corresponds to the formation of a monolayer of adsorbed surfactant. Further addition of surfactant beyond this critical point produces no additional change in ZP which is reflected in no further adsorption of dispersant. The region of maximum ZP for these dispersants is also apparently very close to the critical micelle concentration (CMC) of the surfactants themselves.[5] Since the shapes of interfacial tension curves for determining the CMC of surfactants are also quite similar to the dispersion curves shown in Fig. 3, and since both parameters are manifestations of a more basic adsorption process, it is conceivable that a ZP technique, such as the one described above, can also be used to determine the CMC of surfactants.

With nonionic surfactants (Fig. 4), since the polymer supplies no appreciable ionic species to induce adsorption, the addition of an electrolyte, such as potassium chloride, is required. In essence, the presence of an electrolyte alone is all that is required to disperse silica particles. The addition of a nonionic surfactant merely decreases the concentration of electrolyte required for dispersion. However, the presence of a nonionic surfactant such as polysorbate 80 U.S.P. also apparently decreases the ZP required for complete dispersion and thus interferes with the establishment of a strong electrical double layer, probably through steric hindrance of adsorbed, bulky, surfactant molecules. Therefore, nonionic surfactants are probably less efficient dispersing agents for silica and other suspensoids than are their ionic counterparts.

Influence of Various Polyvalent Anions on Purified Silica Dispersions.—As was previously pointed out, electrolytes alone can be used to disperse silica particles in suspension. A series of dispersion curves, in which the addition of various sodium salts (chloride, sulfate, citrate, and pyrophosphate) were used to disperse a standard 0.01% silica suspension, are shown in Fig. 5. The salts show an increasing order of dispersing efficiency as polyvalent anions. On a molar basis, approximately 3 times more citrate ion, approximately 6.6 times more sulfate ion, and 10.6 times more chloride ion is required to achieve the same degree of dispersion as with the quadrivalent pyrophosphate ion.

The magnitude of the ZP of dispersed silica particles is also increased when polyvalent ions are used to aid dispersion. In all cases, however, the addition of excess electrolyte well beyond the point of maximum dispersion causes a reduction in ZP which has been described as the "bulk stress effect" (17). The phenomenon is probably caused by the collapse of the electrical double layer by the proximity of large numbers of counterions in the vicinity of the particle. With particles that are coated with adsorbed colloids or polyelectrolytes, bulk stress may induce a "salting out" of colloid, which alone is sufficient to decrease ZP.

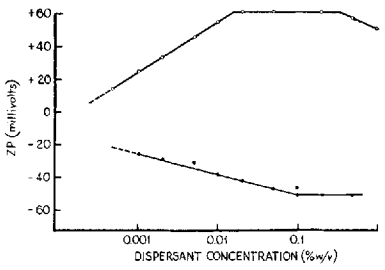

Fig. 3.—The influence of two ionic surfactants on the ZP of a 0.01% purified silica suspension. Key: top curve, benzalkonium chloride U.S.P. (cationic type); bottom curve, dioctyl sodium sulfosuccinate N.F. (anionic type).

Quantitative treatment of the efficiency with which polyvalent electrolytes disperse silica is complicated by the ionization, in solution, of the various polyprotic acids (sulfuric, citric, and pyrophosphoric) which produce these salts. No attempt was made in this study to work at a constant pH value, nor was there any attempt to consider the contribution of lower valence anions in the dispersion process.

In addition to their ability to increase the efficiency of particle dispersion, the size and type of both anions and cations are also important in the flocculation of suspended particles (18).

Dispersion as an Adsorption Process.—Another basic study of silica was concerned with determining the amount of dispersant required for maximum dispersion. Dispersion curves were constructed for a series of concentrated silica suspensions. Sodium hexametaphosphate was selected as a dispersing agent.[6] Increasing amounts of the dispersant were added to each suspension in order to determine, graphically, the amount of reagent required to reach the point of maximum ZP. This particular value is associated with the adsorption of a monolayer of dispersant on the silica particles. The amount of dispersant required in the bulk liquid to initiate adsorption was readily determined by preparing a very dilute 0.001% silica suspension. Since the concentration of silica was extremely small (10 p.p.m.) EM measurements were made directly on the suspension. In determining the EM values for the more concentrated silica slurries (5, 10, 20, and 50%), after a calculated amount of dispersant was added during each step in the construction of the dispersion curve, a sample of the slurry was filtered through a medium porosity, cellulose nitrate-membrane filter to obtain a clear filtrate of equilibrated bulk liquid. A small amount of concentrated residue was added to permit maximum visibility of silica particles for measurement. Samples prepared

[5] Determined experimentally by the surface tension method (16).

[6] The sodium hexametaphosphate used in this study was a sample supplied by the Olin Mathieson Chemical Corp. under the product designation Sodium Polyphos Ground.

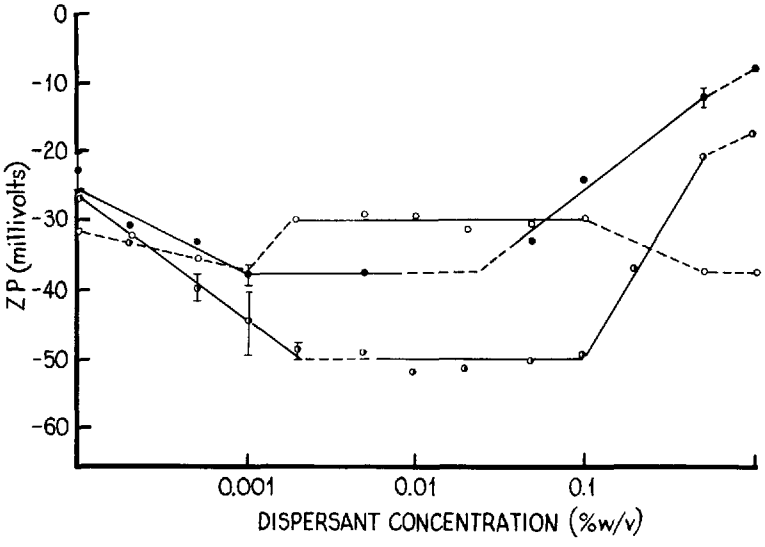

Fig. 4.—The influence of a nonionic surfactant on the ZP of a 0.01% purified silica suspension. Key: ○, polysorbate 80 (nonionic type); ●, polysorbate 80 + KCl; ◑, KCl (neutral electrolyte).

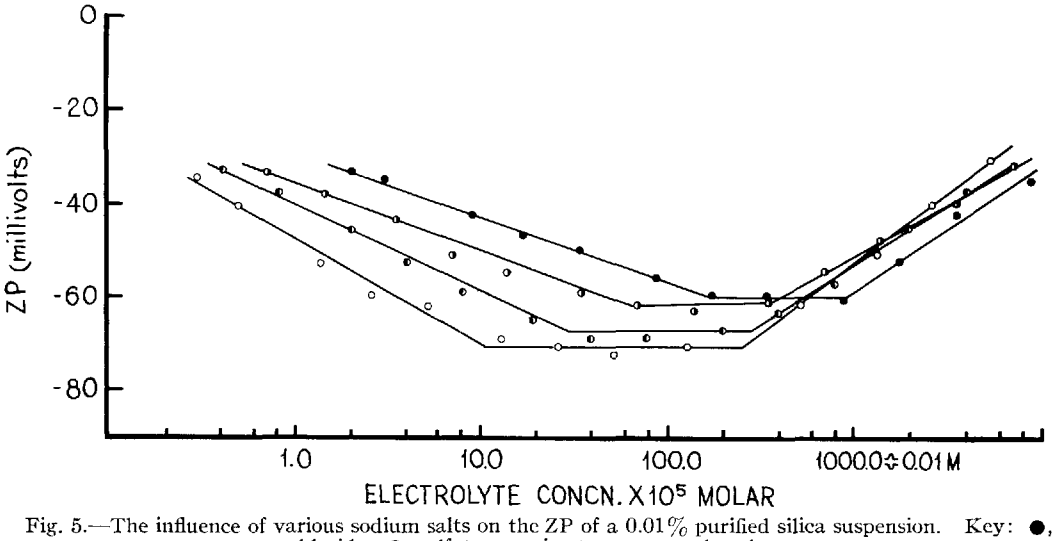

Fig. 5.—The influence of various sodium salts on the ZP of a 0.01% purified silica suspension. Key: ●, chloride; ◑, sulfate; ◐, citrate; ○, pyrophosphate.

in this manner gave EM values that were representative of the concentrated slurry from which they were prepared. In this manner, a series of dispersion curves were constructed for highly concentrated slurries of silica which could not be measured in the electrophoresis cell directly. The resulting dispersion curves are presented in Fig. 6.

The relationship between the amount of dispersant adsorbed and its concentration in the suspending vehicle may be represented by the adsorption equation of Langmuir (19), which is based upon a supposition that dispersion is fundamentally an adsorption process. A plot of concentration of dispersant per ZP *versus* concentration of dispersant yields a straight line whose slope is equal to some conversion constant multiplied by the Langmuir factor β/α and an intercept equal to $1/\alpha$. The Langmuir equation differs from other adsorption isotherms in that adsorption approaches a finite limit (the point of maximum ZP) as the concentration of dispersant added is increased. However, beyond the point of maximum dispersion no further adsorption takes place (Figs. 3–6). The relative constancy in ZP beyond the point of maximum dispersion is further proof that these particular dispersions conform to a Langmuir adsorption process.

In Fig. 6, the average amount of sodium hexametaphosphate adsorbed per gram of silica was found to be 0.655 mg./Gm. and the amount of dispersant in the bulk liquid required to initiate adsorption was 0.052 mg./ml. Since sodium hexametaphosphate is a water-soluble, glassy, condensed polymeric phosphate of indefinite composition, no attempt was made to determine the surface area of dispersant for monolayer adsorption. Nevertheless, the amount of dispersant required for complete disper-

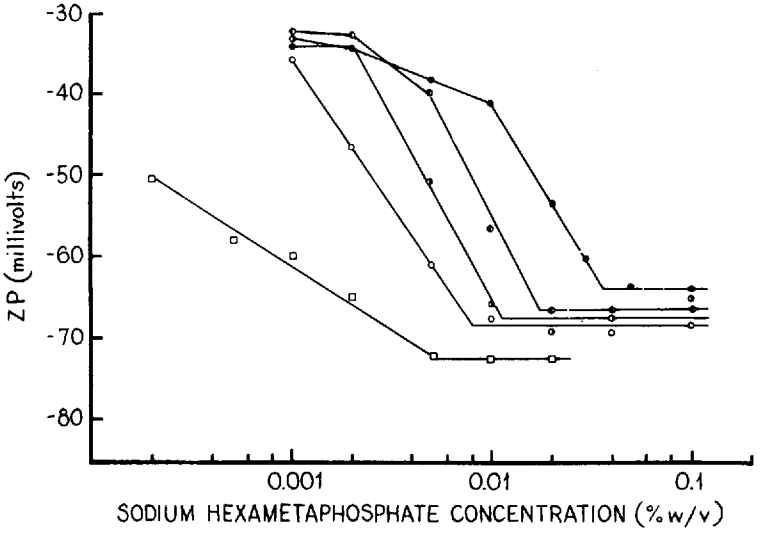

Fig. 6.—Dispersion curves for dilute and concentrated silica suspensions prepared with sodium hexametaphosphate. Key: □, 0.001% silica; ○, 5%; ◑, 10%; ◐, 20%; ●, 50%.

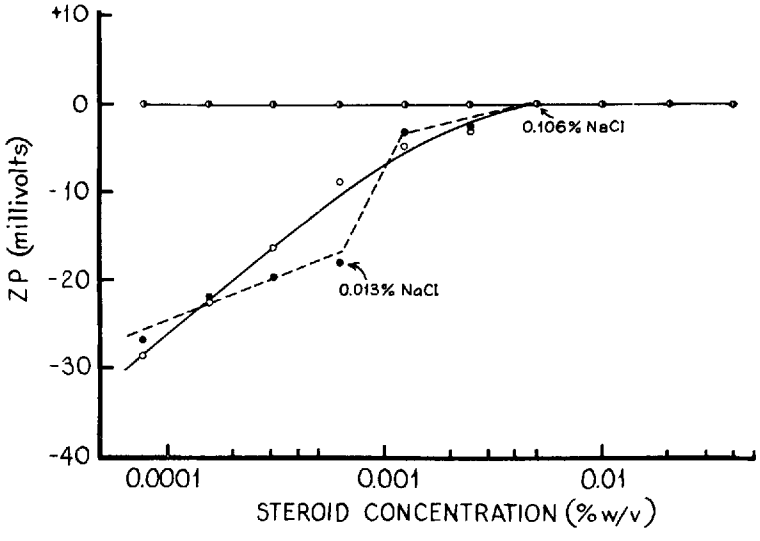

Fig. 7.—Desorption curves of a flocculated adrenocorticoid suspended in various partial vehicles. Key: ◑, partial vehicles containing NaCl; ●---●, diluted with salt-free vehicle. Curve shows differential desorption; ○—○, diluted with water.

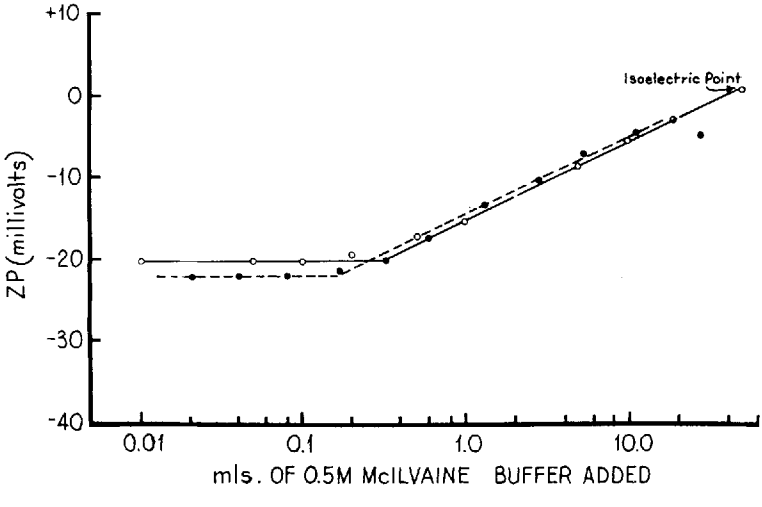

Fig. 8.—Flocculation curve for a drug suspension which was initially partially flocculated with nonelectrolytes. Key: - - - - -, desorption curve; ——, adsorption curve.

sion for any given slurry concentration of silica can be calculated according to the method of Riddick (20) from the two values reported above.

Attempts to determine the amount of an anionic or cationic surfactant required to completely disperse silica or any other suspensoid, are complicated by the fact that a portion of surfactant added to such systems usually is involved in micelle formation, which apparently is a competitive physical phenomenon.

APPLICATION OF ZETA POTENTIAL IN PHARMACEUTICAL PRODUCT DEVELOPMENT

The following three examples were chosen to illustrate how ZP can be used to develop pharmaceutically stable, flocculated suspensions.

Influence of Electrolyte in Altering ZP.—A formulation for a parenteral suspension of an adrenocorticoid was developed empirically without the use of the apparatus. The system consisted of 4% micronized steroid suspended in an aqueous vehicle composed of a wetting agent (polysorbate 80 U.S.P.) to disperse the drug, a preservative (benzyl alcohol), an electrolyte (sodium chloride) to adjust tonicity, and a small concentration of an adjuvant polymer (polyethylene glycol 4000 U.S.P.) to provide a pharmaceutically stable, "noncaking"

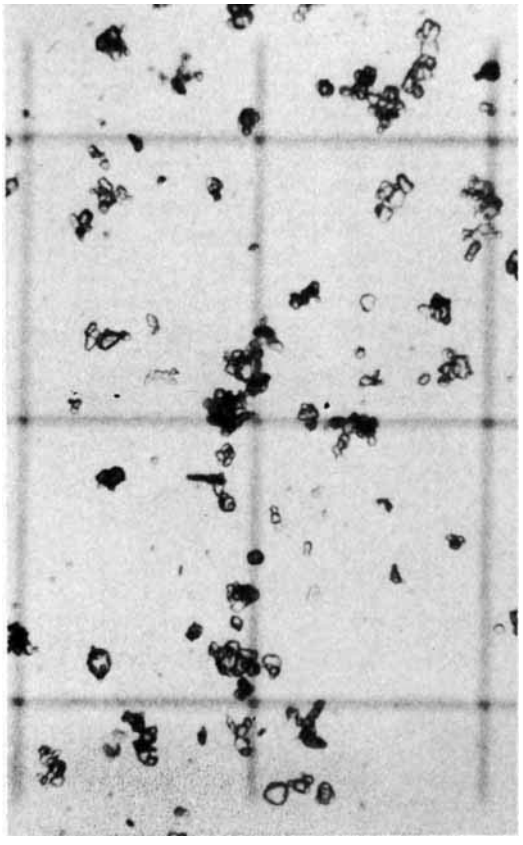

Fig. 9.—Photomicrograph of a deflocculated suspension prior to the addition of a critical concentration of a nonelectrolyte. The width of each scale division equals 96 μ.

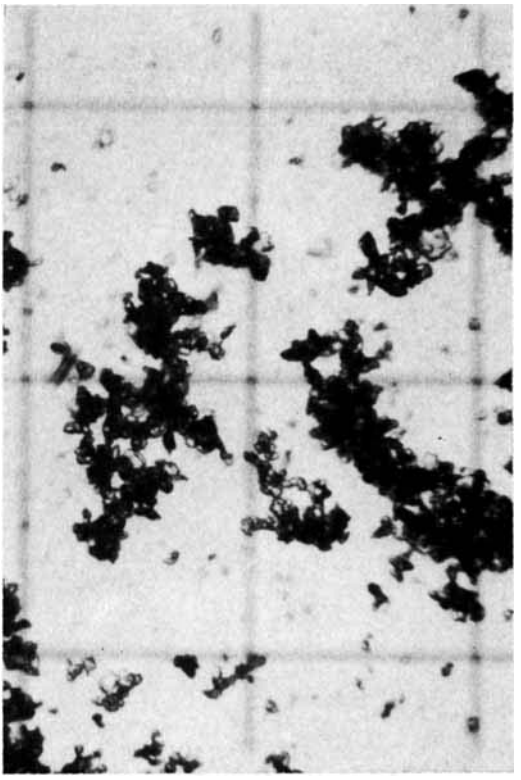

Fig. 10.—Photomicrograph of a partially flocculated suspension after the addition of the nonelectrolyte. The width of each scale division equals 96 μ.

suspension. It was assumed that "stable floc" formation was brought about by the presence of a critical amount of nonionic surfactant acting in combination with the polymer and electrolyte. In succeeding batches of the formula, the degree of overflocculation increased resulting in a suspension with poor drainage characteristics. The basic suspension was examined with the apparatus in order to determine the agent or agents actually responsible for flocculation.

A series of desorption curves were run using a $^1/_{50}$ dilution of the 4% suspension in additional suspending vehicle. Further dilutions ($^1/_2$, $^1/_4$, $^1/_8$, $^1/_{16}$, etc.) were made by replacing half of the total volume in each step with an equal volume of either distilled water or a series of different partial vehicles. The partial vehicles consisted of the complete vehicle formula minus one of the components, such as sodium chloride or polysorbate 80. The results are summarized graphically in Fig. 7.

Suspensions diluted with partial vehicles that contained sodium chloride did not deflocculate. These systems were all fully flocculated and maintained a zero ZP throughout the range of dilution. Dilution with water produced a smooth deflocculation or desorption curve after the third dilution step. Dilution of the suspension with a salt-free vehicle containing all vehicle components minus sodium chloride also produced a desorption curve. In this system, deflocculation was also initiated after the third dilution step when the salt concentration was

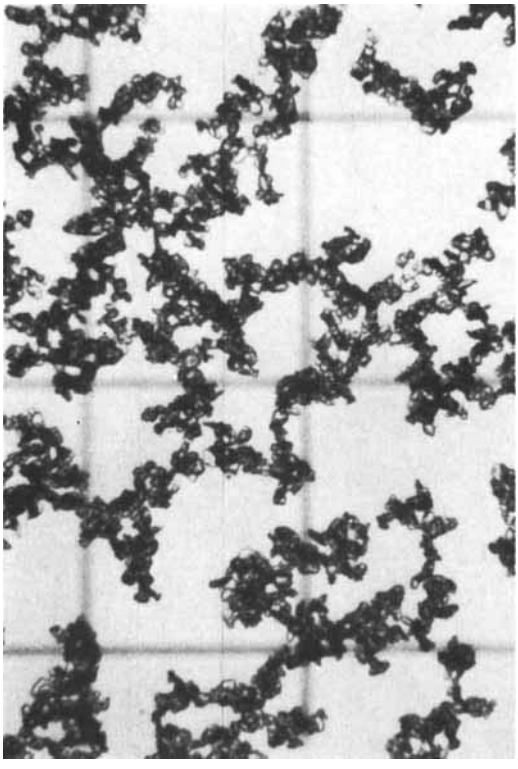

Fig. 11.—Photomicrograph of the suspension completely flocculated with the required concentration of McIlvaine buffer. The width of each scale division equals 96 μ.

reduced below 0.106%. However, the sigmoidal shape of the deflocculation curve in the region between 0.016 and 0.013% sodium chloride was apparently the result of a differential desorption.

In differential desorption, as the concentration of electrolyte and surfactant polymer is reduced, some suspensoid particles may begin to develop their own integral electrical double layer, a manifestation of which is the increase in ZP while other particles remain either partially or fully flocculated. The difference in the rate of desorption between smaller ions and larger polymer molecules apparently produces this effect.

In analyzing the desorption curves in Fig. 7, it is clear that the presence of a small concentration of electrolyte provided the necessary driving force to produce particle flocculation. However, it is not readily apparent in this study of desorption whether this is accomplished by destroying the integrity of the particles coated with adsorbed surfactant-polymer through a salting out process with electrolyte, or forcing interparticle bridging with polymer-surfactant and electrolyte. In any case, reducing the concentration of electrolyte in the formula improved drainage and the physical stability of the over-all suspension. These results also showed that an excess of salt was not required to produce "stable floc" formation.

Flocculation in the Absence of an Electrolyte.—In another example, primary flocculation was initiated in the absence of an electrolyte. Nonionic surfactants, such as polysorbate 80, acting in combination with certain other nonelectrolytes at a critical ratio of surfactant to nonelectrolyte, have the ability to produce partially or fully flocculated suspensions. However, with respect to ZP, since these systems are hydrophobic drug particles, some source of ions is required in the environment of the particle to establish a surface charge and/or electrical double layer. Therefore calculated ZP values of minus 10, 20, or 30 mv. have little or no practical significance for systems composed of neutral drug particles, nonelectrolytes, and nonionic surfactants. However, with the introduction of electrolyte, a degree of stability with respect to the ZP of the suspensoid is realized and a trend toward either deflocculation or flocculation can be ascertained.

Figure 8 shows complete adsorption and desorption curves for a drug suspension that was partially flocculated with a combination of a nonionic surfactant and a nonelectrolyte. "Stable-floc" formation was initiated with the addition of 0.03 ml. of 0.5 *M* McIlvaine buffer and completed when a total of 40 or 50 ml. of the same buffer had been added. Photomicrographs of suspensions are presented in Figs. 9–11, respectively, that show (*a*) the condition of deflocculation before the critical concentration of nonelectrolyte was added, (*b*) partial flocculation after the addition of the nonelectrolyte, and (*c*) complete flocculation when the buffer was added. The addition of electrolyte to the system in the absence of the required nonelectrolyte produces a dispersed or deflocculated system. Visual and photomicro-

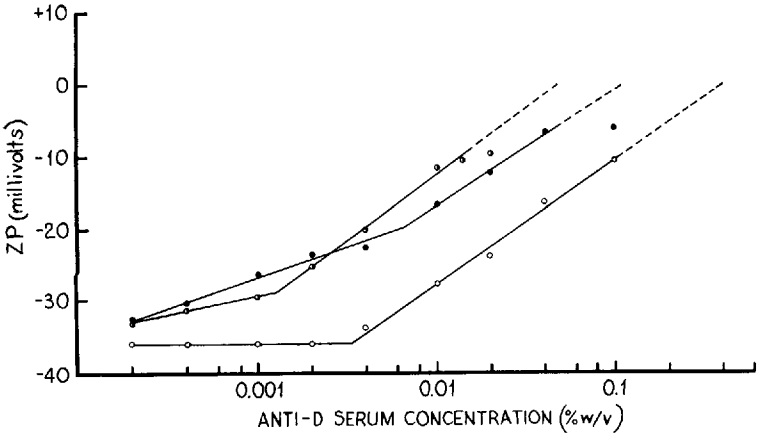

Fig. 12.—ZP titration curves for 0.01% purified silica dispersed in 0.45% NaCl and flocculated with various anti-D sera. Key: ◑, improved formula *A*; ●, control system *B*; ○, original formula *C*.

scopic inspection of these three suspensions were used to confirm the authors' findings with the apparatus.

Therefore, a physically stable suspension of the drug was developed using a calculated amount of buffer to maintain pH control and insure complete floc formation.

Flocculation with Anti-D Sera Using Purified Silica.—The final example is drawn from experiences with a biological application in determining the flocculating efficiency of several anti-D sera, available as diagnostic reagents for Rh blood typing. It was felt that a suitable product could be developed with the proper ZP for agglutination testing. Purified silica was chosen as a substitute for red blood cells (RBC's) because of the difficulties involved in working with blood dispersions (21).

Approximately 500 ml. of a 0.01% purified silica dispersion in 0.45% sodium chloride solution was prepared. The ZP of such a system (approximately −30 mv.) is comparable to the value for human blood dispersions in saline solution (22). Increments of a $^1/_{100}$ dilution of anti-D serum in 0.45% sodium chloride solution were added to each purified silica dispersion. The EM of the system was determined after each addition of reagent. After a total of 0.02% serum reagent had been added, undiluted portions of serum were used to complete each ZP titration.

The results of this study are presented in Fig. 12. The flocculating efficiency of the improved formula *A* was found to be greater than a control system *B* or the original formula *C*. Recommendations for product improvement were made on the basis of a previous study of the original formula *C*. The amount of anti-D serum required to obtain complete flocculation was based upon an extrapolation of linear portions of each test curve to the isoelectric point (zero ZP). The length of the concentration-induction period before flocculation began was also related to the efficiency of the diagnostic reagent. The ranking of the three anti-D sera according to flocculating efficiency for purified silica correlated very well with results of blood-test agglutination with human RBC's.

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Notes

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By V. F. COTTY and H. M. EDERMA

A procedure was developed for directly determining acetylsalicylic acid in human blood specimens. The method instantly stops enzymatic hydrolysis, removes salicylic acid and conjugates of salicylic acid by reaction with ceric ammonium nitrate, automatically hydrolyzes acetylsalicylic acid, and determines the resulting salicylic acid fluorometrically. It sensitively measures acetylsalicylic acid in the presence of salicylic acid, salicylamide, salicyluric acid and salicylic acid ether glucuronide (*O*-carboxyphenyl glucuronide).

SEVERAL recent publications have reported acetylsalicylic acid (ASA) concentrations in the blood of humans following the ingestion of ASA-containing proprietary preparations (1–3). In each case, ASA was calculated as the difference between the level of salicylic acid (SA) in the hydrolyzed and unhydrolyzed serum (1) or in the blood extracts (2, 3). "Difference" procedures can be characterized by

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scopic inspection of these three suspensions were used to confirm the authors' findings with the apparatus.

Therefore, a physically stable suspension of the drug was developed using a calculated amount of buffer to maintain pH control and insure complete floc formation.

Flocculation with Anti-D Sera Using Purified Silica.—The final example is drawn from experiences with a biological application in determining the flocculating efficiency of several anti-D sera, available as diagnostic reagents for Rh blood typing. It was felt that a suitable product could be developed with the proper ZP for agglutination testing. Purified silica was chosen as a substitute for red blood cells (RBC's) because of the difficulties involved in working with blood dispersions (21).

Approximately 500 ml. of a 0.01% purified silica dispersion in 0.45% sodium chloride solution was prepared. The ZP of such a system (approximately −30 mv.) is comparable to the value for human blood dispersions in saline solution (22). Increments of a $^1/_{100}$ dilution of anti-D serum in 0.45% sodium chloride solution were added to each purified silica dispersion. The EM of the system was determined after each addition of reagent. After a total of 0.02% serum reagent had been added, undiluted portions of serum were used to complete each ZP titration.

The results of this study are presented in Fig. 12. The flocculating efficiency of the improved formula *A* was found to be greater than a control system *B* or the original formula *C*. Recommendations for product improvement were made on the basis of a previous study of the original formula *C*. The amount of anti-D serum required to obtain complete flocculation was based upon an extrapolation of linear portions of each test curve to the isoelectric point (zero ZP). The length of the concentration-induction period before flocculation began was also related to the efficiency of the diagnostic reagent. The ranking of the three anti-D sera according to flocculating efficiency for purified silica correlated very well with results of blood-test agglutination with human RBC's.

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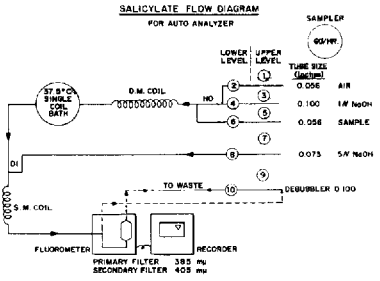

Fig. 1.—System for the analysis of acetylsalicylic acid in extracts of blood.

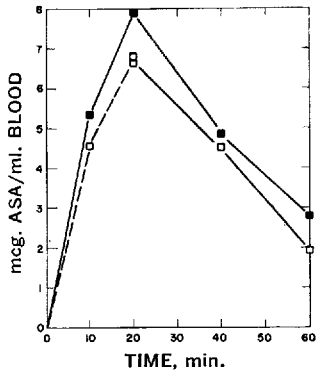

Fig. 2.—Blood levels of ASA obtained by the difference method (□) and by the direct method (■) following the ingestion of ASA. In each of the three studies, analytical blood specimens were drawn immediately before and at the indicated times after each subject ingested 2 tablets (10 gr.) of buffered aspirin. Analyses were performed by either the difference or the direct procedure.

TABLE I.—RECOVERY OF ASA[a]

ASA Added	ASA Found	Other Compd. Added
0.5	0.5	...
0.8	0.7	...
1.3	1.4	30 SA
1.3	1.0	50 SA
1.8	2.1	...
2.1	2.5	...
2.6	3.4	20 SA
2.6	3.6	40 SA
3.9	3.9	10 SA
4.4	4.3	...
6.0	5.7	...
7.0	7.0	...
9.1	9.1	...
9.1	10.0	70 SA
9.6	9.5	60 SA
11.7	11.6	...
0	0	100 SA
0	0	100 SA
0	0	100 SA
0	0	100 SA
0	0.1	100 SA
0	0.1	100 SA
0	0.0	70.7 Salicyluric acid
0	0.0	70.7 Salicyluric acid
0	0.1	141.3 Salicyluric acid
0	0.0	141.3 Salicyluric acid
0	0.0	49.7 Salicylamide
0	0.0	49.7 Salicylamide
0	0.3	99.3 Salicylamide
0	0.0	99.3 Salicylamide
1.3	1.4	141.3 Salicyluric acid
2.6	2.4	141.3 Salicyluric acid
5.2	5.1	141.3 Salicyluric acid
10.4	10.3	141.3 Salicyluric acid
1.3	1.6	99.3 Salicylamide
2.6	2.5	99.3 Salicylamide
5.2	5.5	99.3 Salicylamide
10.4	10.6	99.3 Salicylamide
0	0	227.5 Salicylic acid ether glucuronide
0	0	227.5 Salicylic acid ether glucuronide
1.3	1.6	227.5 Salicylic acid ether glucuronide
2.6	2.5	227.5 Salicylic acid ether glucuronide
5.2	5.4	227.5 Salicylic acid ether glucuronide
10.4	10.3	227.5 Salicylic acid ether glucuronide

[a] ASA and related compounds were added to whole pooled human blood and ASA was then determined. Concentrations in mcg/ml.

accumulated errors which may become particularly serious when a small difference exists between large analytical values. Thus, it is difficult to measure low concentrations of ASA in the presence of relatively high concentrations of SA by these methods. A second problem is that the difference method may not distinguish between ASA and conjugates of SA. The purpose of the present communication is to present a semiautomated direct procedure for the determination of ASA in human blood which is not interfered with by SA, salicylamide, salicyluric acid, or salicylic acid ether glucuronide (*O*-carboxyphenyl glucuronide).

EXPERIMENTAL

Determination of ASA.—Blood specimens are initially inactivated and extracted as described by Cotty *et al.* (3) in the following manner. Five milliliters of whole blood, immediately upon withdrawal, are measured into a bottle containing 0.5 ml. of 6 *N* HCl and 50 ml. of ethylene dichloride (EDC). The bottle is capped with a polyethylene snap cap and then immediately shaken vigorously by hand until the blood becomes a brown color. The bottle is then shaken on a mechanical shaker for 10 min. The EDC is separated from the precipitated blood by filtering through dry filter paper (Whatman No. 42). The EDC extract is placed in a bottle containing 0.1 ml. of an aqueous solution of 40% ceric ammonium nitrate and 5.9 ml. of distilled water. The bottle is then capped and shaken on a mechanical shaker for 1 hr. The EDC is separated from the aqueous layer in a separator and filtered through a dry Whatman No. 42 filter paper. Thirty milliliters of the filtered EDC is transferred to a bottle containing 4 ml. of 1% $NaHCO_3$. This bottle is capped and shaken vigorously on a mechanical shaker for 10 min. Upon separation, the aqueous layer is transferred into a polystyrene sample cup which is placed in the sampler of a system[1] consisting of a special salicylate manifold, proportioning pump, heating bath, fluorometer, and recorder. (See Fig. 1.) As it traverses this system, the sample is mixed

[1] AutoAnalyzer, Technicon Instruments Corp., Chauncey, N. Y.

TABLE II.—COMPARISON OF DIFFERENCE AND DIRECT METHODS OF ANALYSIS WHEN EMPLOYED IN CLINICAL STUDIES[a]

Time, min.	Blood Levels, mcg. ASA/ml. — Difference Method						Direct Method		
	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n	$\bar{x}$	S.E.	n
10	4.6	0.51	38				5.3	0.77	39
20	6.8	0.44	38	6.3	1.24	39	7.9	0.66	40
40				4.6	1.28	38	4.8	0.27	40
60				1.9	1.40	39	2.7	0.17	40

[a] Each subject ingested 2 tablets of proprietory buffered ASA (10 gr.) after the initial (blank) blood specimen was drawn. Other specimens were drawn for analysis at the time intervals indicated. The results of two studies employing the difference method and one the direct procedure are shown in the table.

with air and 1 N NaOH in a double mixing coil. The sample then passes through a heating bath at 37.5° where aspirin hydrolysis is completed. The analytical sample is then mixed with 5 N NaOH and passed through the fluorometer flow cell. The range selector of the fluorometer is set at 30X with a No. 4 light shield slit and interference filters of 385 and 405 mμ. The results are recorded on a linear recorder. A reagent baseline is established at about 15% transmission with a 1% $NaHCO_3$ solution in the place of the sample. The sampler is set at 60 samples per hour and samples are placed in alternate holes on the turntable. Ethylene dichloride (EDC) used for the extractions was specially prepared by the Fisher Scientific Corp., Fairlawn, N. J. Ceric Ammonium Nitrate was manufactured by the G. F. Smith Co., Columbus, Ohio. Pure salicyluric acid (4) and salicylic acid ether glucuronide (*O*-carboxyphenyl glucuronide) (5, 6) were synthesized.[2] These compounds yielded satisfactory elemental analyses and produced single spots when subjected to thin-layer chromatography. Salicylamide and aspirin were U.S.P., and all other chemicals were of reagent grade. Standard curves are prepared by adding 1 to 10 mcg. of aspirin per ml. of freshly drawn blood and then carrying out the procedure as described above. ASA concentrations are plotted against peak heights.

Results.—Table I lists the amount of ASA found when analyses were performed upon blood to which known amounts of ASA had been added. The addition of related substances (salicylic acid, salicylamide, salicyluric acid, and salicylic acid ether glucuronide) does not affect precision because the discrepancies between added and found amounts of ASA are small and not increased over those obtained when ASA alone is present.

A practical comparison of the difference and direct methods was made in the following manner. Data collected from two absorption studies in which the difference method (3) was used were compared with a study in which the direct method was employed.

Each subject received two tablets (total 10 gr. ASA) of proprietary aspirin[3] and blood specimens were collected immediately before and at 10, 20, 40, and 60 min. after administration.

One study employing the "difference" method measured ASA blood levels at 10 and 20 min. has been reported previously (3). The second measured ASA blood levels at 20, 40, and 60 min. The single study employing the direct procedure measured ASA levels at 10, 20, 40, and 60 min. Thirty-eight to 40 persons were employed in each study. In most cases the subjects were the same for all studies. The analytical data of all studies are plotted in Fig. 2. The arithmetic means and standard errors are tabulated in Table II. Note the general agreement between results obtained with the two different analytical procedures. There are no significant differences between corresponding values.

DISCUSSION

Previous attempts to develop chemical procedures for the direct determination of ASA have been frustrated by its lack of a suitable characteristic group which easily reacts with appropriate reagents. The present methodology makes use of this situation, for it employs a reagent, ceric ammonium nitrate, which reacts with compounds possessing the phenolic or aliphatic hydroxyl group, as do the metabolites of ASA. The use of this reagent resulted from a screening of a number of reagents which were known to react with phenols. The formation of complexes with phenols and their subsequent oxidation by ceric salts has been described by Duke and Smith (8) and Sharma and Mehrotra (7). Thus, acetylation "protects" the phenolic group in ASA and this substance remains in solution in the organic solvent.

The high sensitivity and specificity of the procedure described above should make it a useful tool in investigations into the nature of the pharmacological responses produced by the ingestion of ASA as well as other salicylates. Much present day research on salicylates involves the relative roles of ASA and SA in the analgesic, antipyretic, and anti-inflammatory responses to salicylate therapy. These can be tested more reliably with the use of the procedure described above.

SUMMARY

A direct, semiautomated method for the determination of ASA in human blood has been described which is sensitive, reproducible, and specific. Results obtained during absorption studies of ASA in which this method was employed yielded results similar to those obtained in studies employing a "difference" method.

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[2] The assistance of Mr. Leonard Weintraub and Mr. Stanley Oles in performing the syntheses is acknowledged.
[3] Marketed as Bufferin by Bristol-Myers Products, Hillside, N. J.

Some Factors Affecting the Release of a Water-Soluble Drug from a Compressed Hydrophilic Matrix

By HERBERT LAPIDUS* and NICHOLAS G. LORDI

A number of factors controlling the rate of drug release from a hydrophilic matrix were investigated. The release of chlorpheniramine maleate from a hydrated methylcellulose matrix was found to be controlled largely by drug diffusivity rather than dissolution of polymer and water penetrability. The release pattern obtained appears to follow the theoretical relationships previously proposed for solid drugs dispersed in solid matrices.

THIS STUDY has been undertaken in an effort to quantitatively describe the mechanism of drug release from a prolonged action tablet previously patented (1), more recently described in the literature (2), and currently providing the basis for several pharmaceuticals. This tablet utilizes a hydrophilic polymer as a matrix in order to inhibit the release of water-soluble and slightly water-soluble active ingredients so as to afford continuous release of said medication.

The release pattern obtained from such a tablet, when measured under idealized conditions, appears to follow the theoretical relationships proposed by Higuchi in his equation dealing with the rate of release of solid drugs suspended in ointment bases (3) and subsequently applied to solid drugs dispersed in solid matrices (4). The equation describing drug release from the single face of a tablet is

$$Q = \left[\frac{D\epsilon C_s}{\tau}(2A - \epsilon C_s)t\right]^{1/2} \qquad \text{(Eq. 1)}$$

where Q is the amount of drug released per unit surface after time, t, D is the diffusion coefficient of the drug in the release medium, ϵ is the porosity of the tablet, τ is the tortuosity of the tablet, C_s is the solubility of the drug in the release medium, and A is the concentration of the drug in the tablet.

Desai *et al.* (5) have recently reported an experimental study of factors influencing drug release from tableted insoluble plastic matrices to which Eq. 1 was applied. This report deals with release from a matrix that reacts with the release medium, a case which was excluded in the derivation of Eq. 1.

EXPERIMENTAL

The tablets used in this study were prepared by mixing chlorpheniramine maleate U.S.P. with a hydroxypropyl methylcellulose ether[1] and granulating with U.S.P. ethanol. The granulation was dried and screened through a 20 mesh sieve. A 7/16 in. flat face tablet was made from this granulation using a Carver press at a force of 3000 lb. Each granulation was assayed by dispersing it in water to effect solution of the drug and measuring the absorbance of the resulting solution at 262 mμ.

Chlorpheniramine maleate was selected as an example of a soluble drug whose ultraviolet absorption characteristics enabled its measurement without interference from other components of the tablet. The methylcellulose was selected as an example of a polymer whose effectiveness in delaying drug release had been demonstrated both *in vivo* and *in vitro* (1). It rapidly hydrated on contact with water, yet was not readily water dispersible.

The tablet was forced into a cylindrical tube composed of a fluorocarbon polymer[2] which had been machined so that a precise fit was obtained. Consequently, drug release could be measured from a single face of the tablet. No water was observed to penetrate between the tablet and the walls of the cylinder, thus eliminating the need to embed the tablet in wax as has previously been required with glass (5).

In order to obtain an accurate picture of the initial stages of drug release, a modification of the continuous flow technique described by Sjogren and Ervik (6) was utilized. A sketch of the apparatus is shown in Fig. 1.

The tablet in its holder was placed into a specially designed flow cell so that a constant flow of solvent could be maintained past the tablet face with no undue turbulence. The total volume of fluid circulating through the apparatus was 155 ml. The entire flow cell was immersed in a water bath so as to maintain the temperature at 37°. The flow of solvent through the cell was accomplished by means of a model T-8 Sigma-Motor pump set at a speed of 75 ml./min. A continuous flow cell, Beckman No. 92522, was positioned in a Beckman DK-2 recording spectrophotometer. The instrument was operated at the required fixed wavelength (262 mμ for all release media studied) and absorbance recorded as a function of time, usually for a period of 115 min. An absorbance value of 1.00 measured under these conditions was equivalent to the release of 10.3 mg. of drug in pure water.

Figure 2 shows the absorbance-time curve characterizing the release of chlorpheniramine maleate in pure water using a 300-mg. tablet containing 50 mg. of drug. This result was duplicated on numerous occasions to within ±1% deviation. The lag time in the apparatus at 75 ml./min. was 1.1 min. At lower flow rates an oscillation in the absorbance curve (the dotted line in Fig. 2), whose amplitude increased with diminishing flow rate, was observed during the first 15 min. of the experiment. The oscillation was effectively eliminated at 75 ml./min. flow rate.

In some experiments drug release from the whole tablet was measured, using the U.S.P. disintegration test apparatus (7) and a large beaker in place of the specially designed flow cell. A single tablet was confined in one of the tubes of the apparatus by a coarse wire mesh screen so that the tablet would not float to the surface. The release pattern was found to be independent of the rate of flow of solvent main-

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[1] Marketed as Methocel 90 HG 15,000 by Dow Chemical Co., Midland, Mich.
[2] Marketed as Kel-F by the M. W. Kellog Co., Jersey City, N. J.

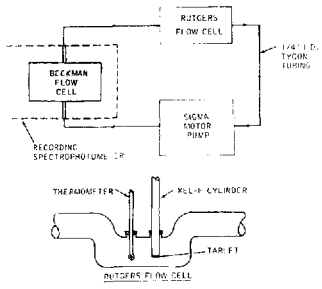

Fig. 1.—Apparatus used to study release rates.

tained through the spectrophotometer. The total volume of solvent was 1 L.

RESULTS AND DISCUSSION

Figure 3 shows plots of amount of drug released as a function of $\sqrt{t}$ demonstrating the apparent application of Eq. 1, which predicts a linear relationship, to this system. These data refer to 300-mg. tablets containing between 25 and 150 mg. of chlorpheniramine maleate, the remainder of the tablet being polymer. Furthermore, Fig. 4 indicates that a plot of the slopes of these lines (determined by a least mean squares fitting of the data) as a function of the dose of drug in the tablet is also linear up to 100 mg.

This is in agreement with a further extension of Higuchi's theory (4) which considers the effect the dissolution of the active material may have on the release rate. In this case the leaching type of release mechanism depends not only on diffusion outward but also on the change in porosity due to the elimination of this material from the tablet matrix. Equation 1 can be modified by introducing a linear relation between drug concentration and porosity, assuming that the initial porosity of the matrix is negligible. Therefore,

$$\frac{Wr}{t^{1/2}} = W_0\left(\frac{S}{V}\right)\sqrt{\frac{DKC_s}{\tau}(2 - KC_s)} \quad \text{(Eq. 2)}$$

should quantitatively describe Fig. 4. Here Wr is the amount of drug released in time, t, W_0 is the dose, S is the effective diffusional area, V is the effective volume of the matrix, and K is introduced to convert A ($= W_0/V$) to its corresponding volume fraction.

If one visualized water penetration as a front moving into the tablet, hydrating the polymer matrix, and dissolving the active material, which then diffuses out through this hydrated matrix, the application of Eq. 1 to this system would appear to be valid. Since the polymer swells on hydration, V in Eq. 2 is considerably greater than the volume of the tablet. Furthermore, it was noted that the face of the hydrated polymer film formed at the surface of the tablet was not planar but slightly convex. A slight lateral extension of the film was also observed, making S in Eq. 2 greater than the area of the tablet face. However, no significant change in diffusional area was evident once the film formed. Generally, this required less than 3 min. In an experiment in which the tablet (containing 50 mg. of drug) was depressed 0.15 mm. in its holder, $Wr/\sqrt{t}$ was found to be 12% less than the value observed when the tablet was flush with the surface of the holder. This indicated the magnitude of the area change owing to lateral extension, although a change in the effective diffusional path length may also be involved.

If Eq. 2 was applied to an insoluble matrix, K would be the specific volume of the drug. However, K must be interpreted as the fraction of hydrated polymer volume replaced by a unit weight of drug. Thus, K equals the product of the specific volume of the drug and the ratio of the volume of the hydrated to unhydrated matrix. Consequently, the replacement of polymer by an insoluble as well as a soluble diluent should result in an increase in the rate of drug release (contrary to the case of an inert matrix). The $Wr/\sqrt{t}$ values observed for 300-mg. tablets containing 50 mg. of chlorpheniramine maleate and 75 mg. of diluent were 33.2 and 24.6% greater than that observed in the absence of the diluent for lactose and tricalcium phosphate, respectively.

The tortuosity must also be a function of the extent of hydration and type of gel structure formed as water penetrates the matrix. This factor would also be influenced by air entrapped in the matrix as well as the presence of diluent. The deviation from linearity observed in Fig. 4 at high drug doses (>100 mg.) could be explained by an effective decrease in tortuosity resulting in increased drug release.

Equation 1 was derived assuming that $A \gg \epsilon C_s$. In the case of chlorpheniramine maleate, $C_s > A$. In order for Eq. 2 to apply, ϵ must be $\lesssim 0.1$. This requires that the free volume of solvent (that available to dissolve drug) be less than 10% of the volume of the hydrated matrix. If $\epsilon C_s \gtrsim A$, then the following equation would apply (4):

$$\frac{Wr}{t^{1/2}} = 2W_0\left(\frac{S}{V}\right)\sqrt{\frac{D}{\tau}} \quad \text{(Eq. 3)}$$

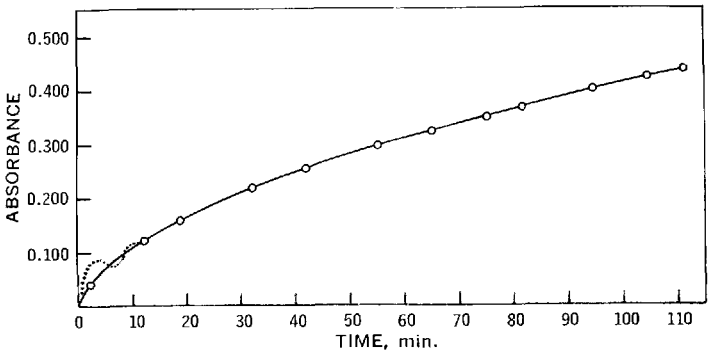

Fig. 2.—Absorbance *vs.* time curve characterizing the release of chlorpheniramine maleate and the effect of flow rates on the actual spectrophotometric recording. Key: ——, 75 ml./min.; . . . . ., 18 ml./min.

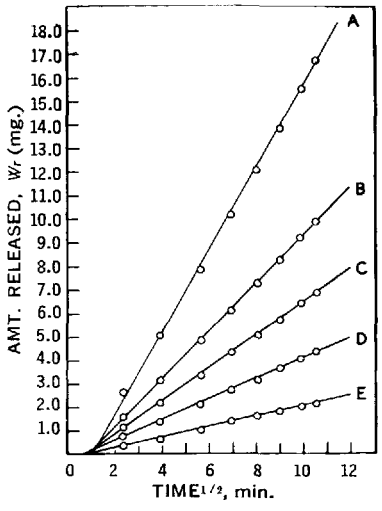

Fig. 3.—Drug release as a function of the square root of time for tablets with varying concentrations of chlorpheniramine maleate. Key: A, 150 mg. per tablet; B, 100 mg. per tablet; C, 75 mg. per tablet; D, 50 mg. per tablet; E, 25 mg. per tablet.

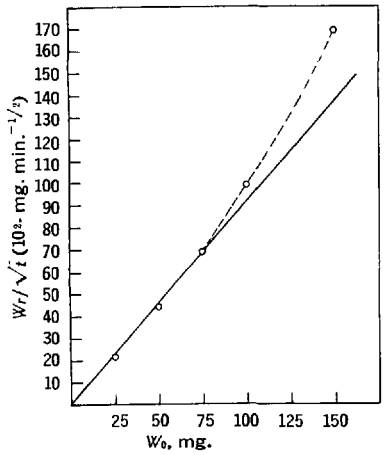

Fig. 4.—Plot of the slopes of the lines represented in Fig. 3 as a function of the dose of drug in the tablet.

In this case, the solvent penetrating the matrix is assumed to completely dissolve the drug on contact. This does not appear to be the case in the specific system reported here.

The term $S/V\tau^{1/2}$ in Eqs. 2 and 3 should measure the effect of the polymer and that of any other parameter which alters the hydration of the polymer in controlling drug release from tablets consisting of only drug and polymer. This presumes no significant complexation between the drug and polymer. The effect of changes in the ionic composition of the solvent should be largely reflected in this term.

Table I lists $Wr/\sqrt{t}$ values obtained in experiments in which the pH or salt content of the solution was varied. In all cases listed, excellent linearity of $Wr/\sqrt{t}$ plots was observed. A specific proton effect was noted. The results indicate that release rates in gastric and intestinal juices will be significantly different if pH is the only factor influencing release. This is consistent with the observed reduction in viscosity of methylcellulose solutions at pH values < 3 (8). This can be attributed to a reduction in hydration owing to protonation of ether linkages. In effect, the tortuosity of the hydrated film is decreased. The decrease in slope observed at pH 7.5 is probably the result of a decrease in the effective solubility of chlorpheniramine maleate owing to a decrease in dissociation.

On the other hand, electrolytes apparently increase the tortuosity of the hydrated film. Since ions reduce the activity of water, hydration of the matrix should be reduced, resulting in a decreased drug release rate. However, when the concentration of sodium sulfate and magnesium sulfate was raised to 0.2 *M*, a very sharp rise in drug release was encountered. This apparent inconsistency may be explained by considering that when the salt content is increased beyond a certain point, the activity of the water becomes so greatly reduced as to prevent uniform hydration of the gum. This then results in a massive discontinuity in the gel structure. In fact, upon removal of the tablet holder from the solvent, a noticeable elongation of the tablet was observed at the 0.2 *M* concentration. It no longer had a flat surface exposed but looked like an inverted "gum drop" with a greatly enlarged surface. Another factor that may be important is that the gel point of the methylcellulose is reduced to less than 37° in these solutions. The effect was not observed when release was measured at 25°.

Table I.—Effect of pH and Salt Concentration on $Wr/\sqrt{t}$ Values Obtained for Tablets Containing 50 mg. Chlorpheniramine Maleate

Solvent	Ionic Strength	pH	$Wr/\sqrt{t}$ 10^2 mg. min.$^{-1/2}$
0.15 *M* sodium acetate			
0.015 *M* boric acid	0.15	7.5	38.6
Water	0	≈5.5	45.5
0.015 *M* sodium acetate			
0.06 *M* acetic acid	0.015	5.2	45.1
0.001 *N* hydrochloric acid	0.001	3.0	47.6
0.1 *N* hydrochloric acid	0.1	1.5	54.7
0.30 *M* sodium chloride	0.30	...	38.6
0.60 *M* sodium chloride	0.60	...	36.0
0.10 *M* sodium sulfate	0.30	...	40.9
0.15 *M* sodium sulfate	0.45	...	37.5
0.175 *M* sodium sulfate	0.525	...	41.6
0.10 *M* magnesium sulfate	0.40	...	44.4
0.15 *M* magnesium sulfate	0.60	...	41.0

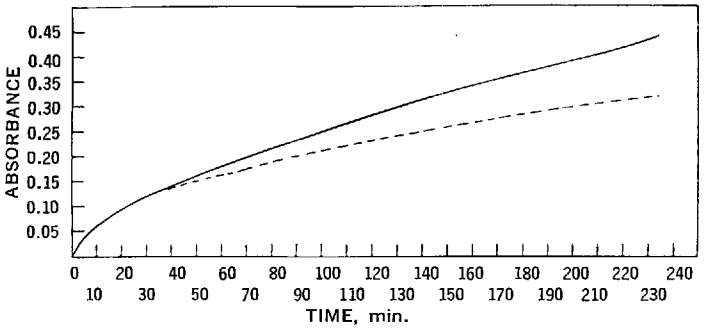

Fig. 5.—Absorbance *vs.* time curve showing release of chlorpheniramine maleate from a whole tablet. The dotted line represents the release pattern that would be necessary in order to maintain linearity if plotted against the square root of time.

Besides diffusion through a hydrated matrix, two other processes could be rate determining in affecting drug release from a compressed hydrophilic matrix. These are the rate of penetration of water through the hydrated layer and convective transport of hydrated polymer away from the tablet. Christenson and Dale (1) have suggested that hydration of the surface of the tablet results in a relatively water impermeable barrier. Drug release is achieved as the barrier is worn away. Fresh surface of the tablet is subsequently exposed, and the barrier is renewed. The tablet is described as having slowly disintegrated rather than dissolved.

Fatt and Goldstick (9) have recently presented a theoretical treatment of water transport in swelling membranes which should apply to a hydrophilic polymer matrix. They have shown that the mass of water absorbed by a thick slab of material should be a function of $\sqrt{t}$, assuming an average transport coefficient. The relative hydration and, consequently, the effective volume of the hydrated film diminish with increasing depth of penetration of the water front. If water penetration was the rate-determining step, then the transport coefficient characterizing the water-matrix system rather than the diffusivity of the drug would be the crucial parameter. The effect of electrolytes, pH, and diluents would be explainable in terms of alteration in the permeability of the swollen matrix to water.

The derivation of Eq. 1 presumes a uniform matrix through which diffusion takes place. If the rate of hydration of the matrix is slow compared to the dissolution of solid drug embedded in the matrix, then drug release would involve a leaching mechanism described by Eq. 1. If the rate of hydration is rapid, as was the case for the hydroxypropyl methylcellulose ether, then dissolution would only occur in a matrix in which hydration was effectively complete. Although the hydrated portion of the matrix through which the drug is diffusing may not be completely uniform, the results enable us to assume uniformity, at least for the time during which release was measured. This was nominally 2 hr. When release rates were observed for a period of 5 hr., the linear relationship described by Eq. 1 continued to apply. An estimate was obtained of the mass of the unhydrated tablet involved in the diffusional process. In the case of a 300-mg. tablet containing 50 mg. of chlorpheniramine maleate, 4.7 mg. of drug was released in water in 115 min. At that time, the tablet was removed from the solution and the hydrated matrix scraped from the face of the holder and assayed for drug which amounted to 2.6 mg. The total, 7.2 mg., indicated that 7.2/50 or about 14.4% of the mass of the tablet had been involved at this point. At the end of 5 hr. only 7.0 mg. of drug had been released.

That convective transport was not the rate-controlling step was confirmed by measurements of release patterns from whole tablets as shown in Fig. 5. Examination of the tablet at the end of a run in which 86% of the dose had been released showed a thick hydrated layer surrounding a small unswollen core. The figure shows that $Wr/\sqrt{t}$ was only constant during the early stages of release. Since Eq. 2 only applies to release from a plane surface, it is difficult to assess the actual factors contributing to the release in this case. Convective transport may still play a role as it must when the concentration of polymer in the tablet is significantly lowered by the addition of diluents. However, the existence of an intact hydrated layer establishes that diffusion is the most important factor contributing to the rate in this system.

The relative contribution of convective transport, water penetrability, and drug diffusivity to drug release from a hydrophilic matrix can only be assessed by independent measurement of the permeability of the hydrated polymer to water and drug in conjunction with a study of release patterns measured under the conditions described in this report. The experimental techniques, while not accurately reflecting *in vivo* conditions, do provide a reproducible means of evaluating different polymer–drug combinations. It should be anticipated that different systems in which the water solubility of the polymer or the drug may be altered would provide additional information to further clarify the mechanism of drug release.

The data reported here are only preliminary. Results of experiments in progress will be reported later.

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4,6-Disubstituted Quinolines and Decahydroquinolines

By ARVIN P. SHROFF*, HATIF JALEEL†, and F. M. MILLER

This paper describes the synthesis of some 4,6-disubstituted quinolines and decahydroquinolines.

IN CONNECTION with a general study of the chemistry of Iboga alkaloids, it was of interest to synthesize some quinolines and decahydroquinolines. A modified Doebner-Miller synthesis (1) on *p*-aminobenzoic acid with methyl vinyl ketone gave the expected 4-methylquinoline-6-carboxylic acid (I). Oxidation of the 4-methyl group using a variety of oxidizing agents did not proceed in satisfactory yields. It was, therefore, converted to the styryl base (II) and oxidized with potassium permanganate (2) to give quinoline-4,6-dicarboxylic acid (III) in 76% yield. Hydrogenation of III gave a mixture of the *dl-cis* and *dl-trans* isomers of decahydroquinoline-4,6-dicarboxylic acids (IV). These were separated by virtue of the fact that the *dl-trans* isomer of the ethyl ester (V*b*) forms a solid hydrochloride more readily than the *dl-cis* isomer. The ratio of *dl-cis* to *dl-trans* was 1:4. Varying the conditions and/or the catalyst failed to increase the yield of the *dl-cis* isomer. In one experiment the *dl-cis* isomer (V*a*), obtained as an oil, was heated on an oil bath (3), and the reaction was followed by observing the change in the infrared spectrum. The appearance of the lactam carbonyl peak at 5.96 μ with retention of absorption due to the ester group (Fig. 1) suggested that the reaction proceeded to form VI as shown in Scheme I. Under similar conditions the *dl-trans* isomer (V*b*), as the free base, failed to react.

The low yield of the *dl-cis* isomer and the failure to obtain the lactam (VI) in a pure state precluded further studies in this area.

EXPERIMENTAL[1]

4 - Methylquinoline - 6 - carboxylic Acid.—A mixture of 42.9 Gm. (0.315 mole) of *p*-aminobenzoic acid, 135.1 Gm. of (0.5 mole) $FeCl_3 \cdot 6H_2O$, 10 Gm. of zinc chloride (anhydrous), and 250 ml. of ethyl alcohol was heated with stirring at 60–65°. To this mixture was added 17.5 Gm. (0.25 mole) of methyl vinyl ketone over a 2-hr. period. The mixture was allowed to reflux for an additional 2 hr. and left overnight at room temperature. Excess ethyl alcohol was removed under vacuum, and the residue was treated with 300 ml. of 20% sodium hydroxide solution. The mixture was filtered, and the filtrate was acidified with glacial acetic acid to pH 5. The light brown precipitate was washed with water and alcohol. Recrystallization from absolute alcohol gave 32 Gm. (54.1%) of 4-methylquinoline-6-carboxylic acid, m.p. 280–282°. $\lambda_{max.}^{KBr}$ 5.87 μ; 6.3 and 6.4 μ.

Anal.—Calcd. for $C_{11}H_9NO_2$: C, 70.58; H, 4.85; N, 7.48. Found: C, 71.00; H, 5.36; N, 7.16.

4-Styrylquinoline-6-carboxylic Acid.—A mixture of 14.0 Gm. of 4-methylquinoline-6-carboxylic acid, 78.7 Gm. of benzaldehyde, and 6.6 Gm. of anhydrous zinc chloride was heated on an oil bath for 5 hr. at a temperature of 180–190°. The reaction mixture was cooled and poured into 300 ml. of 25% sulfuric acid. Ethyl ether (1 L.) was added to the mixture and the styryl quinoline sulfate was filtered off. The precipitate was washed successively with 25% sulfuric acid, alcohol, and ether to give 18.6 Gm. of the salt. Recrystallization from absolute alcohol gave yellow crystals, m.p. 250–252°. The styryl sulfate (18.6 Gm.) was treated with 15% sodium hydroxide solution and filtered. The filtrate was acidified with glacial acetic acid and the buff-colored precipitate was filtered. Recrystallization from absolute alcohol gave 13.4 Gm. (65%) of 4-styrylquinoline-6-carboxylic acid, m.p. 292–293°.

Anal.—Calcd. for $C_{18}H_{13}NO_2$: C, 78.53; H, 4.76; N, 5.09. Found: C, 78.35; H, 5.07; N, 5.27.

Quinoline - 4,6 - dicarboxylic Acid.—To a mixture of 10.0 Gm. of 4-styrylquinoline-6-carboxylic acid in 250 ml. of 50% aqueous pyridine was added 14.0 Gm. of potassium permanganate over a period of 2 hr. The mixture was maintained at 0° throughout the addition. Stirring was continued for 2 hr., and the mixture was allowed to come to room temperature. It was allowed to stand overnight, and then 20 ml. of 20% potassium hydroxide solution was added. The solution was then treated with sodium bisulfate until there was a complete change

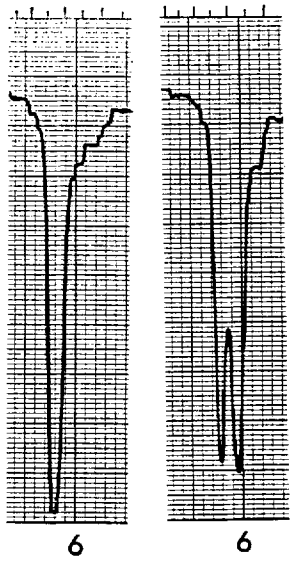

Fig. 1.—Infrared spectrum of *cis*-diethyl decahydroquinoline-4,6-dicarboxylate (V*a*) (left) and the cyclized lactam (VI).

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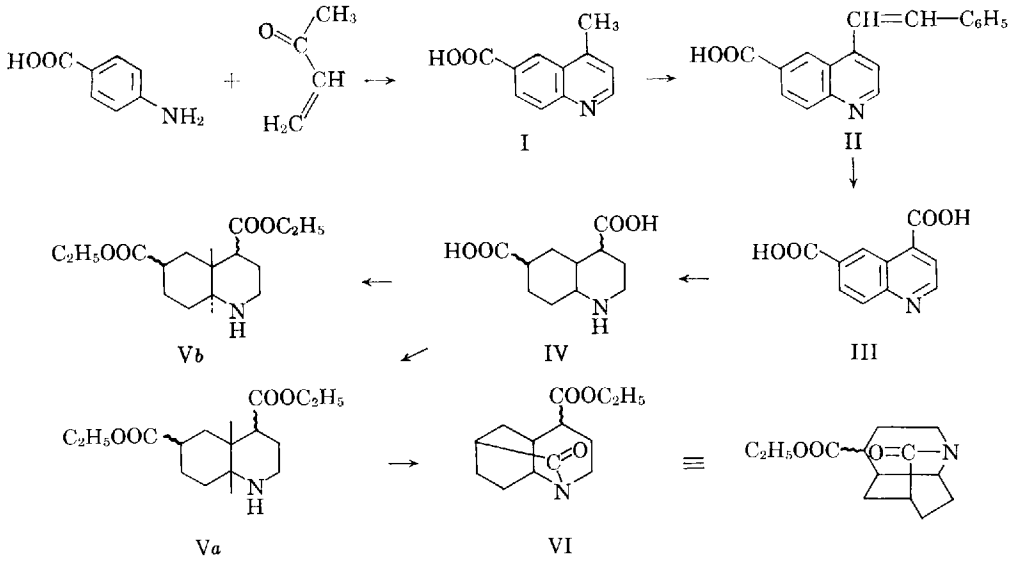
[1] All melting points were taken on a Fisher-Johns melting point apparatus and are uncorrected. The infrared and ultraviolet spectra were determined on a Perkin-Elmer Infracord spectrophotometer and a Beckman DB spectrophotometer, respectively. Elemental analyses were performed by Weiler and Strauss, Oxford, England.



Scheme I

in the color of the solution. It was filtered, and the filtrate was acidified with concentrated hydrochloric acid to pH 5. The precipitate was washed successively with water, alcohol, and then dried to give 6.0 Gm. (76%) of quinoline-4,6-dicarboxylic acid. Recrystallization from alcohol–acetonitrile gave an analytically pure sample, m.p. >300°.

Anal.—Calcd. for $C_{11}H_7NO_4$: C, 60.83; H, 3.25; N, 6.45. Found: C, 60.29; H, 3.45; N, 6.68.

Decahydroquinoline - 4,6 - dicarboxylic Acid Hydrochloride.—Quinoline-4,6-dicarboxylic acid (3.7 Gm.) was treated with 20 ml. each of water, HCl, and acetic acid, and 500 mg. platinum dioxide. The mixture was hydrogenated at 60° for a period of 36 hr. The catalyst was removed by filtration, and the filtrate was evaporated under vacuum to give white crystals of decahydroquinoline-4,6-dicarboxylic acid hydrochloride. Recrystallization from acetonitrile gave an analytically pure sample, m.p. 280–285°. The yield was 4.0 Gm. (90%) λ_{max}^{KBr} 5.85.

Anal.—Calcd. for $C_{11}H_{18}ClNO_4$: C, 50.09; H, 6.87; N, 5.31. Found: C, 50.83; H, 7.30; N, 5.16.

Diethyl Decahydroquinoline-4,6-dicarboxylate Hydrochloride.—Decahydroquinoline - 4,6 - dicarboxylic acid (2.7 Gm.) was added to a mixture of 15 ml. of alcohol, 20 ml. of benzene, and 2.5 ml. of concentrated sulfuric acid. It was refluxed for 20 hr., and the water formed was continuously removed. Excess solvent was removed under vacuum and the residue was treated with ammonium hydroxide until neutral to litmus and extracted with benzene–ether. The extracts were dried over sodium sulfate. Anhydrous hydrogen chloride was passed through the solution, and the solvent was removed leaving an oily residue. Addition of ether to this oily residue gave 2.6 Gm. (80%) of *dl-trans*-decahydroquinoline-4,6-dicarboxylic acid diethylester hydrochloride, m.p. 154–156°. The *dl-cis* isomer remains as an oil.

Anal.—Calcd. for $C_{15}H_{26}ClNO_4$: C, 56.32; H, 8.19; N, 4.37. Found: C, 56.31; H, 8.36; N, 3.81.

Cyclization of dl-cis-Diethyl Decahydroquinoline-4,6-dicarboxylate to VI.—The free ester of the *cis*-isomer was obtained by dissolving the above oil in 1.0 ml. of 5% sodium hydroxide and extracting with 10 ml. of ethyl acetate. The organic layer was washed with water, dried, and evaporated. The diethyl ester, obtained as an oil, was cyclized by heating in a distillation flask to 190–200°. The reaction was followed by observing the change in the infrared spectrum in the lactam region. After 15–20 min. the flask was cooled and the residue was chromatographed on neutral alumina. Elution with benzene gave the desired lactam VI. $\lambda_{max.}^{CHCl_3}$ 5.96 μ.

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Effect of Drugs and Catecholamines on Rat Diaphragm Carbohydrate Metabolism

By LEON L. GERSHBEIN

By the method of paired hemidiaphragms, the mean differences in oxygen and glucose uptake and glycogen content were evaluated for a variety of drugs. Incubation was carried out in a phosphate-saline medium containing 120 mg. per cent glucose. Glycogen was depressed by quinidine sulfate (1.00 mg.), quinine·HCl (1.00 mg.), cinchophen (0.50 mg.), colchicine (0.75 mg.), bishydroxycoumarin (0.25 mg.), dimethyl sulfoxide (1.00 mg.), diphenylhydantoin (0.75 mg.), chlorcyclizine·HCl (0.50 mg.), chlorpromazine·HCl (0.50 mg.), and yohimbine·HCl (1.00 mg.). Glucose utilization was inhibited by the last three agents as well as by tubocurarine chloride (0.50 mg.), picrotoxin (1.00 mg.), atropine sulfate (1.00 mg.), mephenesin carbamate (0.50 mg.), and promethazine·HCl (0.50 mg.). More physiologically significant data resulted with potassium estrone sulfate (10 mcg.) which markedly inhibited glucose utilization and with testosterone (0.52 μm.) and its derivatives, norethandrolone and methandrostenolone, both at 0.33 μm., which depressed glycogen but had little effect on glucose uptake except for testosterone as such. Of the catecholamines, epinephrine at 10 mcg. depressed Q_{O_2}, glycogen, and glucose utilization, the effect persisting at 0.10 mcg. or lower as was also the case with norephinephrine. The latter at 50 mcg. promoted glycogenolysis. Adrenochrome and *dl*-metanephrine·HCl likewise affected glucose uptake at dosages of 10 or 50 mcg., but very high levels of the *O*-methylamine were required for a glycogenolytic response.

ALTHOUGH the isolated rat diaphragm has been used in conjunction with insulin, epinephrine, and several sterols in rather classical muscle metabolic researches, it has been little applied to broader classes of drugs. More recently, findings have been advanced for sulfhydryl compounds (1), thyroid hormones (2), thalidomide (3), and disulfiram (4). This report presents hemidiaphragm oxygen uptake, glucose utilization, and glycogen data for a variety of drugs, therapeutic sterols, and catecholamine derivatives and metabolites screened up to very high levels. The effect of these agents as such or by preincubation on glycogen turnover in the presence of insulin was not ascertained.

EXPERIMENTAL

The drugs were obtained from general commercial sources, mainly Nutritional Biochemicals Corp.[1] The medium of Stadie and Zapp (5) was employed in the incubation of hemidiaphragms, the composition being: 0.04 *M* Na_2HPO_4, 0.005 *M* $MgCl_2 \cdot 5H_2O$, and 0.08 *M* NaCl, pH 6.8–7.0; the glucose content was 120 mg. %.

Male Holtzman rats weighing 135–160 Gm. were starved for 24 hr., sacrificed by swift decapitation, incised, and the hemidiaphragms removed and immediately placed in chilled saline. The tissues were trimmed, blotted between filter paper, weighed, and introduced into the Warburg flasks, one hemidiaphragm being incubated with 1.0 ml. double-strength Stadie and Zapp medium and 1.0 ml. saline as such (control) and its mate, with medium and 1.0 ml. saline–drug solution (treatment). In some instances, as with cinchophen, solution was effected with aqueous NaOH and the mixture then diluted with saline to the desired concentration. A few drugs of low solubility were employed as fine suspensions. With acetylsalicylic acid and the sterols, solutions were prepared in propylene glycol, the desired dosage being delivered in a volume of 0.20 ml.; the respective flasks contained 1.0 ml. of double-strength phosphate–glucose medium, 0.80 ml. of saline, and 0.20 ml. of either glycol or the solution (6). Flasks containing the media but without tissues were also included in each run. The system was gassed with pure oxygen and incubated at 37° for 1 hr., after which time the hemidiaphragms were removed and rinsed 3 times with saline, filter paper blotting being applied between washings. The tissues were digested with alkali and glycogen precipitated and determined by the anthrone reagent (7, 8). The supernatant fluid, after removal of the hemidiaphragm, was deproteinized and analyzed for glucose by the Somogyi method (9, 10). A more detailed description of the pertinent procedures has been advanced earlier (11).

RESULTS

Mean differences in Q_{O_2}, glucose uptake, and glycogen content for hemidiaphragms incubated with drugs, testosterone derivatives, KES, epinephrine, norepinephrine, metanephrine, adrenochrome, and 4-hydroxy-3-methoxymandelic acid together with the requisite Fisher *t* values are presented in Table I. In the calculations involving the bulk data, differences in excess of $\bar{R} \pm 2.5\ R$, where $\bar{R}$ is the average range, were excluded (12). The mean differences were not statistically significant with the following, the values denoting the highest levels screened: acetylsalicylic acid (0.30), acetylcholine (1.00), barbituric acid (0.050), chloral hydrate (0.50), chlorpheniramine maleate (0.50), cyclizine·HCl (0.50), ergotamine tartrate (0.25), sodium heparin (1.00), meperidine (1.00), meprobamate (0.50), meprobamate metabolite (0.50), morphine sulfate (1.00), ouabain (0.50), pentylenetetrazol (1.00), picrotoxin (1.00), pilocarpine·HCl (0.50), procaine·HCl (1.00), reserpine (0.25), strophanthidin (0.25), strychnine sulfate (1.00), and veratrine

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[1] The following originated from the firms as specified: cyclizine·HCl and chlorcyclizine·HCl (Burroughs Wellcome); meprobamate and its metabolite, 2-methyl-2-β-hydroxypropyl-1,3-propanediol dicarbamate (Wallace); dimethyl sulfoxide (DMSO, Baker); phenylbutazone (Butazolidin, Geigy); MER-25 (ethamoxytriphetol, Merrell); norethanedrolone (17α-ethyl-19-nortestosterone, Nilevar Searle); methandrostenolone (1-dehydro-17α-methyltestosterone, Dianabol, Ciba); sodium heparin of 150 U.S.P. units/mg. and epinephrine (Wilson); pentylenetetrazol (Bilhuber, Knoll); mephenesin carbamate (ester of 3-*o*-toloxy-1,2-propanediol, Squibb); potassium estrone sulfate (KES, Penick); chlorpheniramine maleate (Hexagon); and diphenylhydantoin (Dilantin, Parke Davis Co.).

TABLE I.—MEAN DIFFERENCES IN Q_{O_2}, GLUCOSE UPTAKE, AND GLYCOGEN CONTENT OF HEMIDIAPHRAGMS INCUBATED WITH DRUGS AND HORMONES[a,b]

	Compd., mg.	Mean Q_{O_2} Change, μl./mg. Wet Tissue/hr.	t	Mean Glucose Difference,[c] mcg./mg. Wet Tissue/hr.	t	Mean Glycogen Difference, mcg./mg. Wet Tissue/hr.	t
General Agents	Atropine sulfate (1.00)	0.08 ± 0.077 (11)	1.10	1.64 ± 0.387 (11)	4.20[f]	0.13 ± 0.087 (11)	1.40
	Chlorcyclizine·HCl (0.50	0.09 ± 0.039 (12)	2.35[g]	0.31 ± 0.093 (11)	3.34[f]	0.14 ± 0.054 (12)	2.68[g]
	Chlorpromazine·HCl (0.050)	−0.02 ± 0.040 (11)	0.45	0.79 ± 0.281 (12)	2.82[e]	0.07 ± 0.201 (12)	0.36
	Chlorpromazine·HCl (0.50)	−0.02 ± 0.032 (11)	0.64	1.10 ± 0.313 (10)	3.50[f]	0.62 ± 0.201 (11)	3.08[e]
	Cinchophen (0.050)	0.00 ± 0.040 (12)	0.10	...	...	0.00 ± 0.050 (10)	0.07
	Cinchophen (0.50)	0.17 ± 0.070 (11)	2.11	−0.27 ± 0.154 (11)	1.74	0.36 ± 0.161 (12)	5.85[f]
	Colchicine (0.75)	0.04 ± 0.066 (10)	0.59	...	...	0.18 ± 0.068 (10)	2.66[g]
	Bishydroxycoumarin (0.25)	0.00 ± 0.067 (24)	0.02	0.04 ± 0.481 (19)	0.09	0.52 ± 0.141 (23)	3.71[f]
	Digitoxin (0.50)	0.13 ± 0.050 (11)	2.62[g]	0.49 ± 0.655 (11)	0.74	−0.08 ± 0.051 (10)	1.61
	Dimethyl sulfoxide (DMSO; 0.10)	0.03 ± 0.084 (10)	0.39	0.23 ± 0.480 (11)	0.49	0.02 ± 0.122 (10)	0.19
	Dimethyl sulfoxide (1.00)	−0.04 ± 0.041 (32)	0.86	−0.72 ± 0.360 (29)	1.99	0.22 ± 0.091 (29)	2.30[g]
	Diphenylhydantoin (0.75)	−0.01 ± 0.085 (10)	0.13	−0.96 ± 0.580 (11)	1.70	0.73 ± 0.240 (12)	3.02[e]
	Ethyl carbamate (1.00)	0.06 ± 0.056 (18)	1.05	1.15 ± 0.440 (18)	2.60[e]	0.05 ± 0.084 (18)	0.61
	Mephenesin carbamate hemihydrate (0.50)	0.18 ± 0.057 (11)	3.17[f]	1.02 ± 0.148 (12)	6.91[f]	−0.12 ± 0.062 (10)	1.92
	MER-25 (0.10)	0.05 ± 0.030 (11)	1.73	...	...	−0.02 ± 0.020 (9)	0.85
	MER-25 (0.50)	0.14 ± 0.044 (11)	3.12[e]	−0.01 ± 0.316 (11)	0.04	0.17 ± 0.069 (12)	2.48[g]
	Promethazine·HCl (0.50)	−0.05 ± 0.060 (12)	0.86	0.51 ± 0.115 (11)	4.40[f]	−0.05 ± 0.076 (10)	0.68
	Phenylbutazone (0.50)	0.69 ± 0.112 (16)	6.20[f]	0.43 ± 0.524 (9)	0.81	0.30 ± 0.153 (16)	1.94
	Picrotoxin (1.00)	−0.06 ± 0.053 (12)	1.00	0.88 ± 0.301 (12)	2.90[e]	−0.20 ± 0.145 (12)	1.80
	Quinidine sulfate (0.10)	0.13 ± 0.061 (11)	2.16	0.95 ± 0.560 (11)	1.70	−0.02 ± 0.045 (12)	0.40
	Quinidine sulfate (1.00)	−0.23 ± 0.105 (12)	2.20	−0.11 ± 0.435 (12)	0.24	0.87 ± 0.171 (11)	5.10[f]
	Quinine·HCl (0.10)	0.02 ± 0.028 (11)	0.73	...	...	0.31 ± 0.255 (12)	1.20
	Quinine·HCl (1.00)	−0.15 ± 0.096 (11)	1.54	0.52 ± 0.560 (11)	0.93	1.66 ± 0.182 (12)	9.10[f]
	Tubocurarine chloride (0.50)	−0.13 ± 0.080 (12)	1.53	0.92 ± 0.346 (11)	2.65[g]	0.03 ± 0.155 (10)	0.21
	Yohimbine·HCl (1.00)	0.01 ± 0.154 (11)	0.18	1.39 ± 0.370 (12)	3.80[f]	0.44 ± 0.180 (11)	2.40[g]
Sterols	Methandrostenolone (0.10)[d]	0.30 ± 0.123 (15)	2.45[g]	−0.50 ± 0.370 (12)	1.35	0.18 ± 0.066 (15)	2.77[g]
	Norethandrolone (0.10)[d]	0.23 ± 0.051 (21)	4.43[f]	−0.62 ± 0.810 (18)	0.77	0.14 ± 0.048 (20)	3.48[f]
	KES (0.010)	0.05 ± 0.076 (11)	0.63	1.89 ± 0.195 (12)	9.70[f]	0.10 ± 0.211 (12)	0.48
	KES (0.050)	0.03 ± 0.047 (11)	0.60	2.83 ± 0.120 (12)	23.54[f]	0.03 ± 0.061 (12)	0.50
	Testosterone (0.15)[d]	0.20 ± 0.064 (12)	3.13[f]	1.57 ± 0.591 (11)	2.65[g]	0.32 ± 0.096 (11)	3.35[f]
Catecholamines and Derivatives	Epinephrine (1 × 10^{-4})	−0.01 ± 0.036 (14)	0.31	1.98 ± 0.303 (15)	6.55[f]	−0.20 ± 0.152 (12)	1.29
	Epinephrine (0.010)	0.09 ± 0.040 (16)	2.14[g]	1.68 ± 0.412 (17)	4.07[f]	0.16 ± 0.071 (16)	2.22[g]
	Norepinephrine (1 × 10^{-4})	0.10 ± 0.050 (16)	2.06	0.71 ± 0.177 (15)	4.03[f]	0.13 ± 0.161 (11)	0.80
	Norepinephrine (0.050)	0.01 ± 0.033 (22)	0.38	1.14 ± 0.163 (24)	6.96[f]	0.28 ± 0.128 (17)	2.19[g]
	dl-Metanephrine·HCl (0.050)	0.04 ± 0.036 (14)	1.16	1.37 ± 0.248 (15)	5.51[f]	0.13 ± 0.142 (12)	0.88
	dl-Metanephrine·HCl (0.10)	0.03 ± 0.049 (11)	0.69	0.69 ± 0.137 (11)	5.05[f]	0.35 ± 0.243 (11)	1.42
	dl-Metanephrine·HCl (0.50)	0.00 ± 0.048 (17)	0.01	...	...	0.25 ± 0.062 (17)	4.10[f]
	Adrenochrome (0.010)	0.06 ± 0.031 (22)	1.61	1.61 ± 0.236 (23)	6.83[f]	0.17 ± 0.072 (24)	2.40[g]
	Adrenochrome (0.50)	0.08 ± 0.028 (12)	2.95[e]	0.73 ± 0.209 (13)	3.50[f]	0.45 ± 0.105 (13)	4.31[f]
	Adrenochrome semicarbazone (0.015)	0.00 ± 0.070 (12)	0.01	0.97 ± 0.166 (12)	5.86[f]	0.04 ± 0.061 (12)	0.70
	4-Hydroxy-3-methoxy-mandelic acid (1.00)	0.12 ± 0.059 (15)	1.95	0.42 ± 0.358 (15)	1.16	0.18 ± 1.106 (17)	1.71

[a] The means (±S.E.) are deduced from the number of paired hemidiaphragms specified in the parentheses. [b] A negative mean value is indicative of an increase in response in the presence of the drug. [c] Based on the final concentration of the respective media incubated without tissue. [d] In propylene glycol solution. [e] $P < 0.02$. [f] $P < 0.01$. [g] $P < 0.05$.

sulfate (1.00). Acetylsalicylic acid was tested both as a suspension and in propylene glycol mixture.

DISCUSSION

At the concentrations investigated, decreases in diaphragm respiration occurred in the presence of digitoxin, strophanthidin, and phenylbutazone. Of the mean differences, glucose utilization was decreased with promethazine·HCl, picrotoxin, atropine sulfate, and tubocurarine chloride. Chlorpromazine·HCl at 0.50 mg. depressed both muscle glycogen and glucose uptake but when the dosage was lowered to 0.050 mg., only the effect on glucose utilization persisted. Glycogen was depressed on incubation with the higher concentrations of cinchophen (0.50 mg.) as well as with yohimbine·HCl (1.00 mg.), colchicine (0.75 mg.), diphenylhydantoin (1.00 mg.), quinidine sulfate (1.00 mg.), and quinine·HCl (1.00 mg.), the latter two being ineffective at 0.10 mg.; glucose utilization except for yohimbine·HCl was not affected. Glycogen content was decreased but to a small extent with DMSO (1.00 mg.) and MER-25 (0.50 mg.), the last drug also depressing the Q_{O_2}; neither displayed an effect at 0.10 mg.

Both meprobamate and its metabolic product did not significantly alter any of the mean differences. However, mephenesin carbamate (0.50 mg.) depressed glucose utilization as was also observed with ethyl carbamate screened up to 1.00 mg.; Q_{O_2} was decreased in the presence of the former agent. Perhaps at the high levels, carbamate contributes to the diminished glucose uptake and that other structural attributes of the meprobamate molecule might negate the effect. It might be pointed out that the disulfiram metabolite, diethyldithiocarbamate, like the parent compound, was without action on the isolated diaphragm (4).

Regarding the sterols, KES, an interesting water-soluble conjugate of estrone, markedly depressed glucose uptake to the exclusion of any effect on Q_{O_2} and glycogen content at levels down to 10 mcg. (0.027 μm.). Testosterone introduced as a propylene glycol solution elicited inhibitory effects on the three mean differences at 0.15 mg. or 0.52 μm., but the derivatives, norethandrolone and methandrostenolone, each at 0.10 mg. or 0.33 μm., decreased Q_{O_2} and glycogen and produced no effect on glucose uptake. In fact, although not statistically significant, the latter values tended toward increased hexose utilization. In earlier published accounts, rat diaphragm glycogenesis in the presence of insulin and glucose was shown to be reduced by various sterols and the estrogen, stilbestrol (13, 14), and the inhibitory action did not obtain when hemidiaphragms were preincubated with some of these sterols (15). Desoxycorticosterone and an extract of the adrenal cortex were without action on glycogen synthesis in the presence of insulin (16).

The effect of epinephrine on the isolated rat diaphragm has been described by several workers, glucose uptake and glycogen turnover being decreased; the amine antagonizes the action of insulin on glycogenesis (7, 15–18). According to Reisser (16), the substance antagonizing insulin is not epinephrine as such but an oxidized derivative. In the present study, the effects of *dl*-metanephrine·HCl, adrenochrome, and 4-hydroxy-3-methoxymandelic acid (VMA) were ascertained, epinephrine and norepinephrine being included for purposes of comparison under these conditions. Epinephrine at 10 mcg. depressed the glycogen content and glucose utilization was greatly inhibited at dosages below 0.10 mcg. as was also noted with norepinephrine. The latter at 50 mcg. caused a decrease in glycogen. In this regard, both muscle and liver glycogenolysis in the rat is very marked with epinephrine, whereas norepinephrine possesses low activity relative to muscle glycogen but promotes hepatic glycogenolysis (19, 20). Metanephrine·HCl depressed glucose utilization at levels down to 50 mcg., but its glycogenolytic action was noted at higher concentrations (0.50 mg.). The *O*-methylated derivative, like normetanephrine, plays an important role in catecholamine metabolism in the rat and human, and an *O*-methyl transferase system is implicated (21–25). Adrenochrome, the pigment from epinephrine oxidation was similar to the latter amine in causing depressions in both glucose uptake and muscle glycogen at 0.50 mg.; glycogenolysis was definite but of low statistical significance ($p < 0.05$) at 10 mcg. The more stable semicarbazone at a level comparable to the last one (15 mcg.), although markedly inhibiting glucose uptake, was without effect on the glycogen content. However, controls, such as other semicarbazone types or semicarbazide alone, were not instituted, nor were higher concentrations of this derivative screened. The urinary catecholamine metabolite, VMA, was without action on the diaphragm even at a dosage of 1.00 mg.

The data with the above drugs are of great pharmacological interest. Except for the sterols and catecholamines, one is impressed with their relative refractoriness by the metabolic criteria, and where effects could be discerned, these invariably lacked physiological significance in view of the tremendous dosages screened. In contrast to the diaphragm findings, an increase in glucose uptake occurs with rat cerebral cortical slices under the agency of morphine (25); negative data were also obtained earlier with somewhat lower dosages of acetylcholine (16) and with excessive levels of choline (11).

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Evidence for the Presence of an Atropine-Degrading Enzyme in Goat Serum

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Pooled rabbit serum containing atropinesterase, pooled rabbit serum lacking atropinesterase, and pooled goat serum were tested for their effect on some pharmacological parameters of atropine and related compounds. Pooled goat serum was found to contain a previously undescribed enzymatic activity on certain of the effects of these compounds. These activities were compared to those of the rabbit sera. White male mice were used as the test animal.

A SURVEY OF the literature reveals no reference to any protective mechanism of the goat against atropine. Even in the folklore of ancient medicine, where there was widespread belief in the therapeutic powers of different parts of the goat's body, no indication of natural resistance of the goat to atropine, atropine-containing plants, or any atropine-related compounds is found (1).

Metabolic degradation of atropine has been studied in several species, including the mouse (2–4), rat, kitten, guinea pig (5), dog (6), and rabbit. The most extensive studies have been performed on rabbits, where a genetically inherited enzyme, atropinesterase, has been credited with the ability to impart resistance to the pharmacological effects of administered atropine in those rabbits containing the enzyme. Atropinesterase from rabbit serum has recently been purified and characterized by Margolis and Feigelson (7).

This paper presents evidence for the existence of a hitherto unreported atropine-degrading enzyme, present in pooled goat serum, which differs from atropinesterase in that it has no esterase activity.

MATERIALS AND METHODS

Chemicals.—Atropine sulfate (Merck and Co.), hyoscyamine sulfate (Nutritional Biochemicals), tropic acid (Nutritional Biochemicals), and tropine (K & K Laboratories) were all commercial products. Tropine was purified by vacuum distillation in this laboratory.

Sera.—Sterile pooled rabbit serum, no preservative, was obtained from Courtland Laboratories and Baltimore Biological Laboratories. Sterile, pooled, goat serum, no preservative, was obtained from Colorado Serum Co.

Eye Assay.—A modification of the method of Pulewka (8) was used. White, male, Swiss mice were used as the test animal. Initial pupil diameter, under the direct light of an 18-w. microscope maintained 10 cm. from the mouse eye, was obtained using an American Optical binocular microscope fitted with an ocular scale. Animals then received an intraperitoneal injection of 0.5 ml. of serum, followed by 0.2 ml. of atropine or hyoscyamine solution by the same route. Each animal received a total dose of 2 mcg. of the desired alkaloid, with stated exceptions. Pupil diameter was followed at 5-min. intervals until a maximum dilation was obtained. Maximum pupil diameter increase, in arbitrary units, was calculated, and the data subjected to a t test (9) for significance. Control animals received 0.5 ml. of water, followed by 0.2 ml. of alkaloidal solution.

Median Lethal Dose Determinations.—White, male, Swiss mice, 11–12 Gm. in weight, were randomized into five groups of 10 mice each. Each group was subjected to a different dose level, the per cent kills obtained for each group, and the results analyzed by probit analysis after the method of De Beer (10).

Toxicity Studies.—Mice in the test group received 0.8 ml. of serum, followed immediately by 0.2 ml. of the alkaloidal solution, containing a dose of alkaloid at the LD_{50} level. The control group received 0.8 ml. of water, followed immediately by 0.2 ml. of the alkaloidal solution. The total number of kills was obtained for each group, and the results tested for significance, using the χ^2 method of Batson (11). All injections were intraperitoneal.

Esterase Activity.—Warburg respirometer analyses were run on 2.5-ml. reaction mixtures, 0.04 M in phosphate, 0.04 M in bicarbonate, containing 1% of atropine free base and 20% serum, at an initial pH of 7.8. Carbon dioxide production, indicating the hydrolysis of atropine to tropic acid and tropine, was used as the criterion for esterase activity (12).

RESULTS AND DISCUSSION

In a preliminary attempt to compare the effect of esterase-containing and esterase-deficient rabbit serum, and goat serum, on the pharmacological action of atropine, the authors employed two parameters: mydriatic response and gross toxicity.

The presence or absence of esterase activity was determined by Warburg studies. Substantial carbon dioxide production was observed in the case of Courtland rabbit serum, but not with Baltimore rabbit serum or goat serum. On this basis, Courtland serum was judged to contain atropinesterase, and the latter two sera were classified as lacking the enzyme.

LD_{50} studies (Table I) show the relative toxicities of atropine and its components. Atropine, the racemate, and hyoscyamine, the levorotatory isomer, were found to be nearly equal in toxicity, atropine being somewhat more toxic. These results are in agreement with Buckett's observation (13) that, in white mice, the two isomers possess equal

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TABLE I.—MEDIAN LETHAL DOSES FOR THE COMPOUNDS STUDIED[a]

Compd.	LD_{50}, mg./Kg.	95% Confidence Limits of LD_{50}
Atropine sulfate	180	164–198 mg./Kg.
Hyoscyamine sulfate	210	181–244 mg./Kg.
Tropine sulfate	550	490–616 mg./Kg.
Tropic acid (sodium salt)	Nontoxic[b]	...

[a] Median lethal dose and confidence limits were determined by the method of De Beer (10). [b] No deaths were observed at maximum dosages of 1500 mg./Kg.

TABLE II.—PROTECTION OF SERA AGAINST THE LETHALITY OF ATROPINE SULFATE

Serum	Effect	$\chi^{2,a}$
Goat	Protection	16.16
Esterase positive rabbit	Protection	13.00
Esterase negative rabbit	Protection	5.12

[a] Critical values for χ^2: 5% = 3.84, 1% = 6.64, 0.1% = 10.83.

potencies at toxic levels. The authors observed that, upon standing in the laboratory, solutions of hyoscyamine appear to change in toxicity. A solution of hyoscyamine was followed for several hours using a polarimeter, without change in optical rotation. Therefore, racemization does not seem to be a possible explanation for the sometimes markedly different responses that were obtained from hyoscyamine.

Tropine was found to be about one-third as toxic as atropine, and tropic acid was found to be nontoxic. Therefore, the hydrolysis of atropine by those animals containing atropinesterase could serve as a protective mechanism against the lethality of atropine.

Hydrogen-ion concentration was not a factor in the toxicity of the injected drug. Atropine sulfate and hyoscyamine sulfate solutions have a pH of 5.5, and intraperitoneal injections of dilute solutions of sulfuric acid of the same pH caused no deaths or apparent discomforts in injected mice. Tropine solutions were too basic to be used as such, and were adjusted to pH 7.0 with dilute sulfuric acid. Tropic acid, in the acid form, is only very slightly soluble in water. This compound was administered as the sodium salt, pH 7.0, to solubilize enough of the tropic acid anion to permit toxicity studies.

When dose-response curves were run for mydriatic activity, the curves for hyoscyamine and atropine were indistinguishable from one another. Subjection of the data to a t test disclosed no significant difference in the mydriatic action of atropine and hyoscyamine in male, white mice. These findings are in disagreement with Buckett's (13) observation that the levorotatory isomer is 29 times as mydriatically potent as the dextrorotatory isomer. Although Buckett also used the mouse as the test animal, he employed subcutaneous or intravenous injections, while this laboratory employed the intraperitoneal route.

All three sera tested were found to protect against the lethality of atropine (Table II). Since esterase activity was present in only the Courtland serum, it is obvious that protection, while it may be a partial function of atropinesterase in Courtland serum, cannot be a function of esterase activity in the other sera.

Tropine, the alcohol portion of the ester, exhibited lesser toxicity than the parent compound. Goat serum and esterase-lacking rabbit serum were found to protect significantly against its toxicity (Table III). Since there are no ester links in this alkaloid, additional evidence is afforded that protection is due to something other than an esterase. There is an implied association, even in sera that contain atropinesterase, of protective action and ability to degrade or alter the tropine moiety.

The toxic effects of hyoscyamine were unmitigated by all three sera, hence, the protection is stereospecific and involves the dextrorotatory isomer (Table IV). This finding the authors regard as most significant. Atropinesterase is claimed to be specific for the levorotatory isomer (14), and if protection had been a function of hydrolysis, it would have been observed in the case of the pure substrate. This finding also eliminates the possibility that protection is due to serum binding of the administered drug. If this were the mechanism, the enzyme would have bound the natural substrate, the levorotatory isomer, just as much if not more so, as the racemic mixture of isomers.

Alteration of mydriasis showed both rabbit sera to significantly decrease the amount of mydriasis caused by an injected dose of atropine (Table V), again pointing to some activity other than enzymatic hydrolysis. Goat serum had no effect on mydriatic activity.

TABLE III.—PROTECTION OF SERA AGAINST THE LETHALITY OF TROPINE SULFATE

Serum	Effect	$\chi^{2,a}$
Goat	Protection	5.72
Esterase positive rabbit	Not run	...
Esterase negative rabbit	Protection	6.37

[a] Critical values for χ^2: 5% = 3.84, 1% = 6.64.

TABLE IV.—PROTECTION OF SERA AGAINST THE LETHALITY OF HYOSCYAMINE SULFATE

Serum	Effect	$\chi^{2,a}$
Goat	None	1.41
Esterase positive rabbit	None	0.06
Esterase negative rabbit	None	...

[a] Critical values of χ^2: 50% = 0.455, 5% = 3.84.

TABLE V.—PROTECTION OF SERA AGAINST THE MYDRIATIC ACTION OF ATROPINE SULFATE

Serum	Effect	t-Test[a]
Goat	None	1.1
Esterase positive rabbit	Protects	8.02
Esterase negative rabbit	Protects	5.67

[a] $t_{(20)}$ = 3.85 at 0.1%; $t_{(20)}$ = 1.73 at 10%.

CONCLUSION

These studies indicate that the protection rendered by these sera against the pharmacological effects of atropine cannot be ascribed to atropinesterase, or any other esterase. The protection against mydriasis, exhibited by the esterase-lacking rabbit serum, together with the observation that goat serum is ineffective under the same conditions, implies further that the mechanism of protection is of a different nature in the two sera. The evidence indicates that goat serum is specific for the dextrorotatory isomer of atropine, metabolizing it to a compound that is less toxic, but of equal mydriatic activity for the mouse. The rabbit sera data indicate an activity not involving the ester linkage, but metabolizing the compound to a substance of both lesser toxicity and lesser mydriatic activity for the mouse. The data further suggest that decreases in toxicity, with all sera tested, are very likely associated with alterations of the tropine moiety.

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Freezing Point Curve of Dimethyl Sulfoxide–Water Solutions

By RUTH N. HAVEMEYER*

The apparent freezing points have been determined for solutions of dimethyl sulfoxide and water. Several of the samples exhibited increasing viscosity as they were cooled and, at their solidification points, formed amorphous glasses rather than crystalline solids. It is proposed that these glasses form because of a modified lattice structure built of dimethyl sulfoxide and water molecules. The eutectic composition occurs in the region of 0.3 *M* dimethyl sulfoxide.

DIMETHYL SULFOXIDE (DMSO) has been used for many years as a solvent and a reaction medium. It will dissolve many inorganic salts and most classes of organic compounds. As a reaction medium it has been found to increase greatly the rates of many reactions beyond what would be expected (1). It also possesses a number of other unusual properties: a high dielectric constant for an aprotic solvent (2), miscibility with most organic solvents, a high heat of mixing with water (3), and a volume contraction when mixed with water (3). DMSO has also found use as a preservative and freezing medium for biological tissues (4). It has been found in this laboratory that DMSO will greatly lower the freezing point of water. This paper reports on the cryoscopic properties of DMSO–water solutions.

EXPERIMENTAL

Two sets of equipment were employed—one for those samples freezing above −40° and one for those freezing below. For the higher freezing solutions, the apparatus used for measuring the temperature was a copper-constantin thermocouple connected to a Westronics strip chart recorder. The recorder has a working range of −40° to +180° (or −1.46 to +8.24 mv.), with an accuracy of ±0.5°. The cooling chamber was a Dewar flask (12.3 cm. i.d.) containing the dry ice–glycol ether[1] cooling fluid (temperature about −75°).[2] About 2 ml. of a DMSO-water solution was placed in a 7.5 × 1 cm. glass test tube. The thermometric probe was threaded through a cork stopper in the test tube and positioned in the center of the liquid. This assembly was immersed in the cooling bath intermittently, to avoid supercooling, until the contents of the test tube had frozen. The assembly was then suspended from a clamp and allowed to warm at room temperature until the transition from solid to liquid was complete. The freezing point (melting point) of the mixture was taken as the point of inflection on the time-temperature curve.

For those samples with a freezing point below −40°, the equipment used was the Linde BF-3 biological freezer. This is a controlled rate freezer that uses liquid nitrogen as the cooling medium. A differential copper-constantin thermocouple probe is used to monitor the temperature difference between the sample and the cooling chamber. This temperature difference produces a millivolt signal, which is balanced against the controller voltage set to the desired temperature differential. As a result of the continuous comparison of these two voltages, liquid nitrogen is fed into the freezing chamber as needed, through a solenoid valve that is actuated by the controller. Thus, the freezing rate is established and maintained. After the conclusion of a particular freezing operation the sample vials are removed, the

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[1] Marketed as Dowonol 33B by Dow Chemical Co., Midland, Mich.
[2] Determined with a copper-constantin thermocouple and a Brown-Honeywell potentiometer.

CONCLUSION

These studies indicate that the protection rendered by these sera against the pharmacological effects of atropine cannot be ascribed to atropinesterase, or any other esterase. The protection against mydriasis, exhibited by the esterase-lacking rabbit serum, together with the observation that goat serum is ineffective under the same conditions, implies further that the mechanism of protection is of a different nature in the two sera. The evidence indicates that goat serum is specific for the dextrorotatory isomer of atropine, metabolizing it to a compound that is less toxic, but of equal mydriatic activity for the mouse. The rabbit sera data indicate an activity not involving the ester linkage, but metabolizing the compound to a substance of both lesser toxicity and lesser mydriatic activity for the mouse. The data further suggest that decreases in toxicity, with all sera tested, are very likely associated with alterations of the tropine moiety.

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Freezing Point Curve of Dimethyl Sulfoxide–Water Solutions

By RUTH N. HAVEMEYER*

The apparent freezing points have been determined for solutions of dimethyl sulfoxide and water. Several of the samples exhibited increasing viscosity as they were cooled and, at their solidification points, formed amorphous glasses rather than crystalline solids. It is proposed that these glasses form because of a modified lattice structure built of dimethyl sulfoxide and water molecules. The eutectic composition occurs in the region of 0.3 *M* dimethyl sulfoxide.

DIMETHYL SULFOXIDE (DMSO) has been used for many years as a solvent and a reaction medium. It will dissolve many inorganic salts and most classes of organic compounds. As a reaction medium it has been found to increase greatly the rates of many reactions beyond what would be expected (1). It also possesses a number of other unusual properties: a high dielectric constant for an aprotic solvent (2), miscibility with most organic solvents, a high heat of mixing with water (3), and a volume contraction when mixed with water (3). DMSO has also found use as a preservative and freezing medium for biological tissues (4). It has been found in this laboratory that DMSO will greatly lower the freezing point of water. This paper reports on the cryoscopic properties of DMSO–water solutions.

EXPERIMENTAL

Two sets of equipment were employed—one for those samples freezing above −40° and one for those freezing below. For the higher freezing solutions, the apparatus used for measuring the temperature was a copper-constantin thermocouple connected to a Westronics strip chart recorder. The recorder has a working range of −40° to +180° (or −1.46 to +8.24 mv.), with an accuracy of ±0.5°. The cooling chamber was a Dewar flask (12.3 cm. i.d.) containing the dry ice–glycol ether[1] cooling fluid (temperature about −75°).[2] About 2 ml. of a DMSO-water solution was placed in a 7.5 × 1 cm. glass test tube. The thermometric probe was threaded through a cork stopper in the test tube and positioned in the center of the liquid. This assembly was immersed in the cooling bath intermittently, to avoid supercooling, until the contents of the test tube had frozen. The assembly was then suspended from a clamp and allowed to warm at room temperature until the transition from solid to liquid was complete. The freezing point (melting point) of the mixture was taken as the point of inflection on the time-temperature curve.

For those samples with a freezing point below −40°, the equipment used was the Linde BF-3 biological freezer. This is a controlled rate freezer that uses liquid nitrogen as the cooling medium. A differential copper-constantin thermocouple probe is used to monitor the temperature difference between the sample and the cooling chamber. This temperature difference produces a millivolt signal, which is balanced against the controller voltage set to the desired temperature differential. As a result of the continuous comparison of these two voltages, liquid nitrogen is fed into the freezing chamber as needed, through a solenoid valve that is actuated by the controller. Thus, the freezing rate is established and maintained. After the conclusion of a particular freezing operation the sample vials are removed, the

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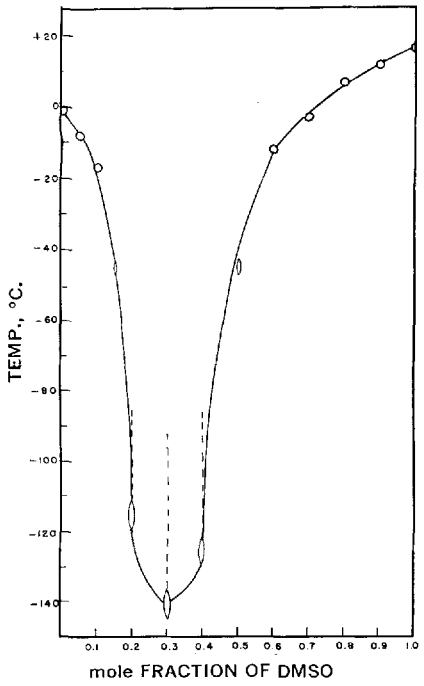

Fig. 1.—Apparent freezing point curve for DMSO–water mixtures. Region of increased viscosity of sample (- - - - -).

TABLE I.—CRYOSCOPIC DATA FOR DMSO–WATER MIXTURES

mole Fraction of DMSO	Freezing Point (°C.)
0.00	−0.5
0.05	−8
0.10	−17
0.15	−45
0.20	About −110[a]
0.30	About −140[a]
0.40	About −125[a]
0.50	−45
0.60	−12
0.70	−3
0.80	+7
0.90	+12
1.0	+17

[a] These mixtures form glassy, amorphous solids.

chamber is reclosed, and a separate warming cycle brings the chamber to room temperature.

Using the Linde freezer 8 ml. of sample solution was placed in a 3-dr. Kimble glass vial, and one leg of the thermocouple probe was immersed in the sample and held in place with a cork stopper. The monitored sample and auxiliary samples were positioned in a carrier and placed in the freezing chamber, and the chamber was tightly sealed. The desired freezing rate was set with the control potentiometers, and the freezing cycle was begun. The time-temperature curves were obtained with a Sargent model SR recorder. Since the temperature region of interest was in the negative millivolt range, the thermocouple leads to the recorder were reversed. This gave a theoretical operating range of +1.45 to −5.38 mv. or about +35° to about −190°. The recorder voltage was calibrated with a Brown-Honeywell potentiometer; the thermocouple-recorder response was checked with chemicals of known freezing points—deionized water, chromatographic grade chloroform, and freshly distilled trichlorofluoromethane.[3] The accuracy of the calibration was about ±2°.

The data obtained by both experimental methods for deionized water, DMSO (pharmaceutical grade, Crown Zellerbach), and mixtures of these two are shown in Fig. 1 and Table I.

RESULTS

The method used for the higher-freezing mixtures did not utilize a controlled rate of freezing or thawing. Nonetheless, there were well-defined inflection points in the time-temperature curves (Fig. 2) to indicate the freezing points (or melting points). The same values were obtained on duplicate samples.

For those solutions with a freezing point below −40°, the samples either would not freeze (0.2, 0.3, 0.4 *M* DMSO) in the dry ice–glycol ether bath, or else showed only poorly defined inflection points (0.15 and 0.5 *M* DMSO). At first, an attempt was made to freeze these samples by intermittent immersion in liquid nitrogen, rather than in the dry ice–glycol ether bath, but the results were unsatisfactory. Stirring the samples with a wire loop and scratching the inner surface of the test tube during cooling were also tried in an attempt to induce crystallization. These methods had no effect. However, it was observed that the 0.2, 0.3, and 0.4 *M* DMSO solutions exhibited a range of viscosity, from glycerin-like liquid to glassy solid, during cooling. The need for a controlled freezing rate was evident, and the equipment employed hereafter was the Linde biological freezer.

Using the Linde freezer, the samples of 0.15 and 0.5 *M* DMSO showed well-defined plateau regions (Fig. 3) even when cooled at the fastest rate—about 5°/min. Samples of 0.2, 0.3, and 0.4 *M* DMSO did not give any indication of their freezing points even when cooled at the slowest rate of about 0.9°/min. However, if these samples were kept in the chamber to the operational limit of the unit, they did solidify as glasses.

These latter three concentrations of DMSO were then re-examined qualitatively. Three or four unmonitored vials were used for each run, in addition to the sample monitored with the thermocouple, so that a vial could be removed from the chamber at different times during the freezing cycle for gross observation. In this way the several stages of viscosity could be seen—slightly viscous, very viscous but flowable, nonflowing but deformable soft glass, nondeformable glass. The dotted lines in Fig. 1 indicate the temperature ranges of increased viscosity. The freezing point of each of these three samples was taken as the temperature at which the surface of the sample could not be indented when pressed with a thin wooden stick. Below this temperature the sample would suddenly show craze or shatter lines. In none of these samples were crystals seen by gross observation.

DISCUSSION

The DMSO–water eutectic forms in the region of 0.3 *M* DMSO (Fig. 1). It is in this same region that

[3] Marketed as Freon-11 by E. I. du Pont de Nemours, Inc., Wilmington, Del.

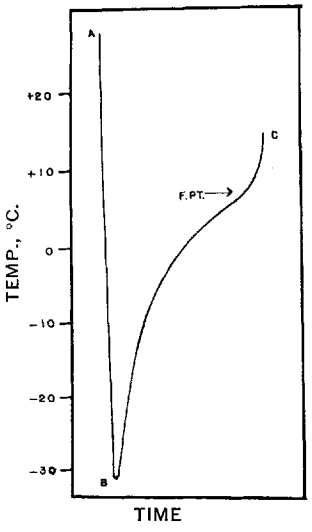

Fig. 2.—Typical freezing point (or melting point) curve of high freezing mixtures. Key: A–B, heat being removed from system; B–C, heat being added to system.

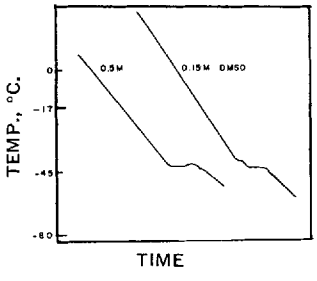

Fig. 3.—Freezing point curves of some lower freezing mixtures.

viscosity and heat of mixing of DMSO–water solutions have maximum values, and there is considerable volume contraction on mixing (3, 5). A 0.33 *M* quantity of DMSO corresponds to a ratio of 2 moles of water to 1 mole of DMSO. It has been proposed that a 2:1 complex does form between water and DMSO through hydrogen bonding and that this is stronger between water and DMSO than between two water molecules (1, 6). It would be expected that a compound with a definite composition would exhibit a maximum, rather than a minimum, in a freezing point curve. It may be that this does occur in this system but that the method of measurement and observation is not sufficiently sensitive to determine it, or supercooling occurs too rapidly to be avoided. The temperature of such a maximum, if it does exist, cannot be estimated. For this reason it is not sketched in on Fig. 1.

In Fig. 3, the "double shoulder" seen in the freezing point curve for 0.15 *M* DMSO was reproducible. These shoulders may be indicative of the beginning of solidification and the transition to the solid solution phase, respectively.

Many organic compounds are known to form glasses rather than crystalline solids when cooled. Among the best known of these are the glycols. For propylene glycol, dipropylene glycol, 2,5-hexane diol, and others Curme and Johnston (7) describe the freezing points as "sets to a glass below −50." The reasons for the formation of this "fourth state of matter" have been discussed for many years. The two major theories are the crystallite and network theories, but the latter seems to have gained wider acceptance (8). According to it, a three-dimensional network develops from a random distribution of loosely linked molecules or particles in space. Because of the haphazard arrangement in the lattice, the forces between units are not of equal strength. As a result, when heat is added to the system the lattice does not collapse all at once at a definite temperature, as in a crystal, but loses its coherence in stages as first the weaker and then the stronger bonds are ruptured (9). Conversely, as heat is removed from the system, the rigidity of the lattice takes place slowly, as evidenced by the stages of increased viscosity, until the amorphous solid is formed. Therefore, a glass has a softening region rather than a sharp melting point; it shows a region of increased viscosity, rather than a well-defined freezing point.

One of the rules of Zachariesan's network theory was that the network formed is built of polyhedra and at least three corners of each polyhedron must be shared. Therefore, the central atom of each polyhedron must have a coordination number of three or more. However, Weyl and Marboe (10) are insistent that they can see no justification for assuming that a tetrahedral structure is essential for glass formation.

It seems reasonable to explain the behavior of the DMSO–water solutions on the basis of a modified lattice theory. If there is definite compound formation between water and DMSO at a ratio of 2:1, then a structure could be formed of subunits of two water molecules tightly bound to one DMSO molecule, with each such subunit more loosely bound to the next subunit. Hence, the over-all structure would be held together by bonds of varying strength or force. At concentrations of water and DMSO other than 2:1, the system would consist of a variety of structural subunits—long and short chains and/or groups of oddly shaped units. Because of the lack of order of the structure and the complexity of the system, crystallization would not occur at the freezing point, but a glass-like solid would form instead.

Those systems that do not crystallize when cooled generally do exhibit temperature-dependent viscosity, and the viscosity reaches a maximum just above the solidification point. If temperature-dependent viscosity is due to asymmetries in the structure of the melt, then this would seem to confirm the existence of a modified lattice structure for DMSO–water solutions.

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Assay of Methenamine Mandelate and Its Pharmaceutical Dosage Forms by Direct Cerimetric Titration

By LESTER CHAFETZ and CHARLES A. GAGLIA, JR.

Avoidance of bisulfate ion and control of acidity permit the direct cerimetric titration of the mandelic acid moiety of methenamine mandelate to a sharp instrumental or indicator end point. Separation and titration procedures for the drug in its dosage forms are described. The procedures compare favorably in speed and selectivity with the U.S.P. assays. The scope and limitations of the analytical reaction are defined.

METHENAMINE mandelate[1] and its tablet and oral suspension dosage forms are recognized in U.S.P. XVII (1) in the urinary antibacterial category. The drug is unique among official salts in that its biological activity derives from both ions. The U.S.P. monograph provides 96% minimum assay values for methenamine mandelate by a procedure selective for methenamine and for mandelic acid by titration with alkali. The net reaction in the U.S.P. mandelic acid assay may be represented:

$$(CH_2)_6N_4H^+ + OH^- = (CH_2)_6N_4 + HOH$$

where the actual acid titrated is the conjugate acid of methenamine; thus the mandelic acid content is inferred rather than determined.

Titration of methenamine mandelate preparations with alkali does not discriminate between methenamine conjugate acid and other organic acids. Difficulty in distinguishing mandelic acid from other organic acids generated during exaggerated stability studies of experimental methenamine mandelate formulations led to the study of conditions for the selective titration of mandelic acid reported here.

Mandelic acid is quantitatively and selectively oxidized by ceric salts according to the equation:

$$C_6H_5CHOHCO_2H + 2Ce(IV) \rightarrow C_6H_5CHO + CO_2 + 2H^+ + 2Ce(III)$$

Helmstaedter (2) applied the Verma and Paul (3) cerimetric titration of mandelic acid to methenamine mandelate. The procedure requires heating mandelic acid with excess ceric sulfate in sulfuric acid solution at reflux and back-titration with a standard reductant. Under these severe conditions the ceric reagent functions as a powerful and general oxidant and formaldehyde liberated from methamine is oxidized also (2). Mathur and Rao (4) reported a residual titration procedure at room temperature with ceric sulfate using visible light catalysis. Chafetz (5) found that ceric oxidation is general for arylglycolic acids and described procedures for their quantitative estimation by ultraviolet spectrometry of the carbonyl reaction product. Because spectrophotometry requires use of a reference standard and the residual titration methods are time consuming and are lacking in selectivity, conditions for the direct titration of mandelic acid in methenamine mandelate and its pharmaceutical dosage forms were investigated.

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[1] Marketed as Mandelamine by Warner-Chilcott Laboratories, Division of Warner-Lambert Pharmaceutical Co., Inc.

Kinetic studies of the Ce(IV) oxidation of mandelic acid (6, 7) showed specific retardation of the reaction rate by bisulfate and hydrogen ions. Using ceric ammonium nitrate in dilute nitric acid, it was found that the oxidation rate of mandelic acid was sufficiently rapid to afford direct titration to an instrumental or color end point. This is the first report to our knowledge of a direct titration of mandelic acid with ceric ion in aqueous system.

EXPERIMENTAL

Titrant.—An approximate 0.05 *N* solution (2.45%) of ceric ammonium nitrate in *N* nitric acid was employed. The titrant was filtered through paper and standardized against primary standard grade ferrous ethylenediammonium sulfate (G. F. Smith Co.), using about 400 mg. of the ferrous salt, accurately weighed, in 100 ml. of distilled water. One drop of nitroferroin indicator solution was used, prepared by dissolving 150 mg. of 5-nitro-1,10-phenanthroline in 15 ml. of freshly made 1.4% aqueous ferrous sulfate. The color change, pink to colorless, coincided with the potentiometric end point in mandelic acid titrations.

Other Reagents and Supplies.—Chemicals used in the study were the best available commercial grades, used without further purification. These included mandelic acid, benzilic acid, phenylacetic acid, atropine sulfate, homatropine hydrobromide, and methenamine mandelate. Methenamine mandelate dosage forms described in this report were samples of regular production lots of Warner-Chilcott Laboratories. Dowex 1-X2 (50–100 mesh), a strong-base anion exchange resin, and a 5% potassium nitrate solution in 0.05 *N* nitric acid were employed in the tablet assay procedures.

Potentiometric titrations were carried out using various commercial platinum billet indicator and saturated calomel reference electrodes with a Sargent model D automatic titrator (used at the "slow" speed) or a Beckman zeromatic pH meter.

Preliminary Observations.—Oxidation of mandelic acid with 0.1 *N* ceric sulfate (U.S.P. XVII, p. 1082) was found to be too slow to afford direct titration at room temperature, even when the benzaldehyde is extracted into a hydrocarbon solvent phase, an expedient suggested by previous work (5). Direct titration proceeded smoothly using a nitric acid system and a sample solution about neutral in pH. Use of *N* nitric acid for the sample as well as titrant led to a severe fall off in reaction rate. Induction of the oxidation can be readily discerned by the increasingly strong odor of benzaldehyde which develops during the titration, a much more selective identity test for mandelic acid than that given in U.S.P.

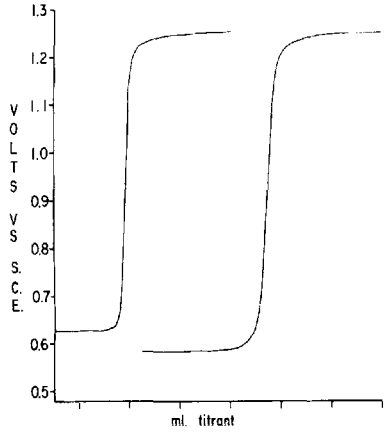

Fig. 1.—Typical titration curves using the recording titrator for methenamine mandelate (left) and mandelic acid by direct cerimetric titration.

Procedure for Mandelic Acid or Methenamine Mandelate U.S.P.—Dissolve approximately 45 mg. mandelic acid or 90 mg. methenamine mandelate, accurately weighed, in 25–50 ml. of water, and titrate the magnetically stirred solution with standard 0.05 *N* ceric ammonium nitrate in *N* nitric acid to the redox indicator or potentiometric end point. The milliequivalent weight of mandelic acid is 76.08 mg.; of methenamine mandelate, 146.17 mg.

$$\text{mg. } C_8H_8O_3 \text{ or } C_6H_{12}N_4 \cdot C_8H_8O_3 = \text{ml.} \times N \times \text{meq. wt.}$$

Assay of Methenamine Mandelate Oral Suspension U.S.P.—Transfer an accurately weighed sample of methenamine mandelate oral suspension, equivalent to about 275 mg. of methenamine mandelate, to a 250-ml. separator. Add 35 ml. of heptane and exactly 100.0 ml. of distilled water. Shake vigorously and allow the layers to separate. Filter about 50 ml. of the aqueous phase through paper, and transfer exactly 25.0 ml. to a 100-ml. beaker. Add 1 drop of nitroferroin indicator solution, and titrate with standard 0.05 *N* ceric reagent from a 25-ml. buret.

$$\text{methenamine mandelate, mg./5 ml.} = \text{ml.} \times N \times 146.17 \text{ mg.} \times 100/25 \times 5 \times \text{density/Gm. sample}$$

Assay of Methenamine Mandelate Tablets U.S.P.—Determine the average weight of 10 tablets and pulverize them. Transfer a portion of the powdered tablets, equivalent to about 1000 mg. of methenamine mandelate, accurately weighed, to a 100-ml. volumetric flask. Add about 60 ml. of distilled water, and shake the mixture mechanically for 10 min. Dilute to the mark with water, mix, and filter through paper. Transfer exactly 5.0 ml. of filtrate to a 5–10 mm. i.d. ion exchange column containing about 1 Gm. of ion exchange resin[2] in the hydroxide cycle. (Prepare the column by equilibrating the resin with about 5 ml. of *N* sodium hydroxide for 10 min., then transferring it to the column and washing with distilled water until the effluent is neutral.) Allow the liquid to drain to the top of the resin bed, then wash the column with about 10 ml. of water, allowing the effluent to drain at the rate of 1–2 ml./min. Stop the flow when the wash level reaches the top of the resin bed, place a 150-ml. beaker as receiving vessel, and elute the mandelic acid with about 50 ml. of 5% potassium nitrate in 0.05 *N* nitric acid at a rate of about 3 ml./min. After collecting about 50 ml., add 1 drop of nitroferroin indicator, and titrate with 0.05 *N* ceric titrant.

$$\text{methenamine mandelate, mg./tablet} = \text{ml.} \times N \times 146.17 \times 100/5 \times \text{mg. av. tablet wt./mg. sample wt.}$$

It may be noted that the procedure may readily be adapted to single unit assay.

Titration of Related Compounds.—Benzilic acid, phenylacetic acid, homatropine hydrobromide, and atropine sulfate were titrated as described above for mandelic acid, using approximately 0.3 mmole of each compound. The ester alkaloids were titrated before and after alkaline saponification.

[2] Marketed as Dowex 1-X2 by the Dow Chemical Co., Midland, Mich.

RESULTS AND DISCUSSION

Potentiometric Data.—Typical titration curves obtained on mandelic acid and methenamine mandelate using an automatic recording titrator are shown in Fig. 1. The oxidation potential of the reaction, Ce(IV) + e = Ce(III), was calculated as +1.464 v. from the titration curve. Half-cell potentials for irreversible oxidation of mandelic acid, methenamine mandelate, and nitroferroin were calculated to be +0.868, +0.832, and +1.044 v., respectively.

Assay of Mandelic Acid and Methenamine Mandelate.—Titrations proceeded smoothly with mandelic acid or its methenamine salt, with no apparent interference from the methenamine. Data found are presented in Table I.

Methenamine Mandelate Oral Suspension.—Samples of suspension in which the vegetable oil vehicle has become rancid cannot be accurately assayed by the U.S.P. method, for titration with alkali does not discriminate between methenamine conjugate acid and fatty acids. The cerimetric method could be successfully applied to assay of methenamine mandelate in rancid vegetable oil without separation of the drug using the potentiometric

Table I.—Cerimetric Titration of Methenamine Mandelate[a]

Trial	mg. Weighed	mg. Found	% Found
1	94.1	93.30	99.2
2	99.9	99.79	99.9
3	109.8	110.30	100.5
4	112.0	111.0	99.1
5	115.5	115.99	100.4
6	100.6	100.76	100.2

[a] Average recovery is 99.9% with relative standard deviation of 0.6%.

Table II.—Replicate Assays of Methenamine Mandelate Oral Suspension

Trial	*1*	*2*	*3*	*4*	*5*	*6*
mg./5 ml. (250 mg. declared)	254.5	257.0	255.0	257.5	256.5	255.0

method by the simple expedient of immersing the electrode tips in the aqueous layer; however, use of the color indicator was preferred because of its simplicity. Observation of the indicator color change in one formulation with a pink color was difficult, for it required discerning a change from pink to colorless in the aqueous layer in the presence of a pink organic layer. Typical results obtained by the extraction procedure using the redox indicator are presented in Table II.

Methenamine Mandelate Tablets.—The ion exchange resin isolation procedure prescribed was employed to circumvent slight interference from sucrose, used as an excipient in the tablet coating. The sugar was slowly oxidized by the titrant. Triplicate assays of methenamine mandelate tablets declaring 500 mg. provided results of 491, 490, and 493 mg./tablet, using a composite sample.

Titration of Related Compounds.—Ceric oxidation of arylglycolic acids appears to be a general reaction (5) which may be represented:

$$\mathrm{Ar{-}\underset{\displaystyle R}{\underset{|}{C}}HOHCO_2H + 2Ce(IV) \rightarrow ArCOR + CO_2 + 2H^+ + 2Ce\,(III)}$$

In this reaction, Ar = aromatic or heteroaromatic, and R = H, an aromatic or alicyclic group. Wholly aliphatic glycolic acid derivatives do not oxidize with the same stoichiometry (6).

Benzilic acid, where Ar = R = C_6H_5—, can be titrated as readily as mandelic acid. Duplicate titrations of benzilic acid provided values 100.1% of theory.

Phenylacetic acid, $C_6H_5CH_2CO_2H$, which may be considered in this discussion a desoxymandelic acid, does not consume titrant. Atropine, the tropine ester of tropic acid, $C_6H_5CH(CH_2OH)CO_2H$, homologous to mandelic acid, did not consume titrant before or after saponification.

The titration could be applied to homatropine hydrobromide, the mandelic acid homolog of atropine, after saponification, but the only indication of reaction in the intact ester was a slight benzaldehyde odor. The saponified ester titrated readily to a value 100.3% of theory.

Interferences.—Although oxidations with ceric salts are selective for mandelic acid and other arylglycolic acids, they are by no means specific. The products obtained depend on reaction conditions employed. Although no interference by methenamine was encountered in the titrations described here, heating methenamine mandelate with excess ceric sulfate results in oxidation of the formaldehyde liberated from methenamine to formic acid (2) as well as oxidation of the mandelic acid to benzaldehyde. In general, some type of separation procedure is necessary for determination of methenamine mandelate in the presence of reducing agents or in colored solutions where the indicator method of end point detection is used.

SUMMARY AND CONCLUSIONS

Mandelic acid is quantitatively and selectively oxidized by ceric salts to benzaldehyde, carbon dioxide, and hydrogen ion. Although residual titration procedures employing excess oxidant have been described, no direct titration has been reported previously. The specific rate retarding effect of bisulfate ion, present in the conventional ceric sulfate titrant, is eliminated by using ceric ammonium nitrate in nitric acid as titrant. This affords a sufficiently rapid reaction rate to permit direct titration to a sharp indicator or instrumental end point, obviates one standard solution, and saves time. The benzaldehyde odor produced during the titration serves as a confirmatory identity test for mandelic acid.

Application of the titration in conjunction with appropriate separation procedures is described for assay of methenamine mandelate and its official preparations. The proposed assays compare favorably with the U.S.P. procedures.

The scope and limitations of the reaction are defined.

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Nuclear Magnetic Resonance Spectra of Amines III. Identification of *N*-Substituted Amino Acids

By W. E. THOMPSON, R. J. WARREN, I. B. EISDORFER, and J. E. ZAREMBO

Spectra of *N*-substituted amino acids examined in alkaline deuterium oxide and in trifluoroacetic acid show chemical shifts and first-order spin-spin splitting patterns that are useful for identification of *N*-methyl, *N*-methylene, *N*-phenyl, and other groups attached to the nitrogen of an amino acid.

STRUCTURE-SPECTRA correlations of amino acids have been a favorite research subject for many years now, so it is not surprising that considerable work has been done correlating amino acid structures with NMR spectra. Among the more interesting studies that have been reported in this field are the correlation of NMR spectra with L- and *m*-structures of cystine (1), and a study of the effect of optical activity of amino acids on their NMR spectra (2). There have also been a significant number of comprehensive analyses of specific amino acid spectra using NMR (3–6). The spectra reported in this paper were needed for the identification of amino acid side chains on a drug metabolite. The authors are following the technique initiated by Silverstein (7, 8), using the simplest compounds available to illustrate the NMR spectrum due to a particular functional group.

The authors have examined NMR spectra of glycine and *N*-substituted glycine in alkaline D_2O and in trifluoroacetic acid as solvents to illustrate the spectral effects of conversion of *N*-substituted amino acid anions into cations.

EXPERIMENTAL

All spectra were obtained with a Varian model A-60 NMR spectrometer using Varian sample tubes. One normal KOD in deuterium oxide was used as the solvent for anion spectra and trifluoroacetic acid as solvent for cation spectra. The probe temperature was 38°. The concentration of amino acid was 50 mg./ml. The sample of glycine was A grade (Calbiochem) purchased from the California Corp. for Biochemical Research. The *N*-substituted glycines were used as purchased from the K & K Laboratories, Inc., Plainview, N. Y.

RESULTS AND DISCUSSION

Figures 1–4 show the effect of conversion of the anions to cations on the NMR spectra of *N*-substituted glycines. The first-order splitting pattern of the methylene or methyl(s) in the cations is related to the number of protons on the nitrogen (9). The number of lines in a multiplet is one more than the number of protons on the nitrogen (cation), a quartet for a primary amine ion, $-NH_3^+$ (Fig. 1, B), a triplet for a secondary amine ion, $=NH_2^+$ (Fig. 2, B) and a doublet for a tertiary amine ion, $\equiv NH^+$ (Fig. 3, B).

The spectrum for *N*-phenylglycine as anion (Fig. 4, A) shows a complex AB_2X_2 pattern for the phenyl protons in the δ = 6.6–7.6 p.p.m. region. When converted to the cation (Fig. 4, B) the AB_2X_2 pattern collapses into a single peak at 7.6 p.p.m. A possible cause of this signal coalescence was postulated in a previous note (10).

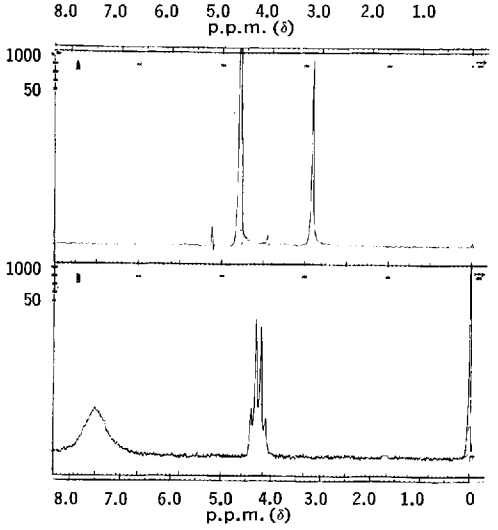

Fig. 1.—NMR spectra for glycine. Key: A, 1 *N* KOD in D_2O as solvent; B, CF_3COOH as solvent.

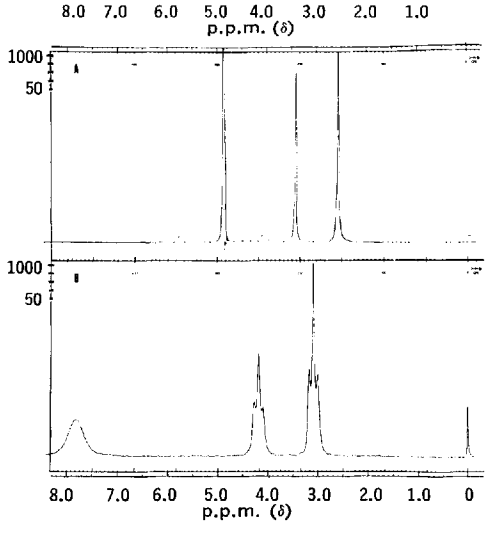

Fig. 2.—NMR spectra for sarcosine (*N*-methylglycine). Key: A, 1 *N* KOD in D_2O as solvent; B, CF_3COOH as solvent.

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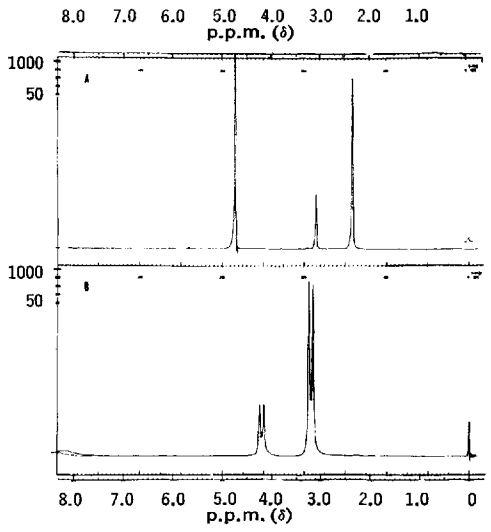

Fig. 3.—NMR spectra for *N,N*-dimethylglycine. Key: A, 1 *N* KOD in D_2O as solvent; B, CF_3COOH as solvent.

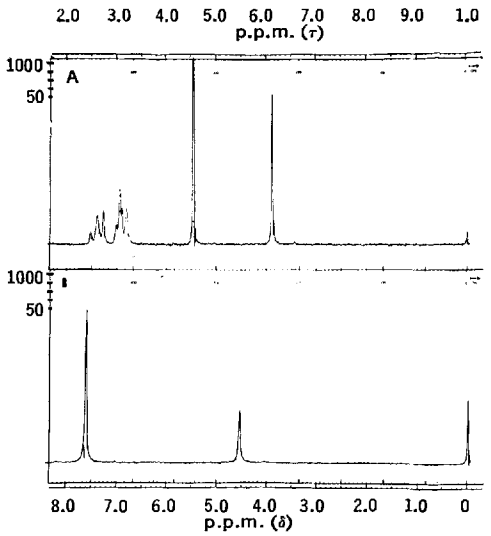

Fig. 4.—NMR spectra for *N*-phenylglycine. Key: A, 1 *N* KOD in D_2O as solvent; B, CF_3COOH as solvent.

TABLE I.—NMR CHEMICAL SHIFTS FOR *N*-SUBSTITUTED GLYCINES

Compd.	Chemical Shifts as Anion in 1 *N* KOD/D_2O	Chemical Shifts as Cation in CF_3COOH
Glycine	$D_2NCH_2COO^-$	$NH_3{}^+CH_2COOH$
	3.18	4.25
	s	*q*
Sarcosine	$CH_3NDCH_2COO^-$	$CH_3NH_2{}^+CH_2COOH$
	2.57 3.40	3.07 4.17
	s *s*	*t* *t*
N,N-Dimethylglycine	$(CH_3)_2NCH_2COO^-$	$(CH_3)_2NH^+CH_2COOH$
	2.35 3.02	3.22 4.23
	s *s*	*d* *d*
N-Phenylglycine	$C_6H_5NDCH_2COO^-$	$C_6H_5NH_2{}^+CH_2COOH$
	6.6–7.6 3.82	7.60 4.53
	AB_2X_2 *s*	*s* *s*

The methylene absorption at 4.53 p.p.m. in Fig. 4, B, remains a single peak in trifluoroacetic acid since this acid is not strong enough to protonate the nitrogen irreversibly. The rapid exchange of protons prevents observation of spin-spin splitting in this solvent at 38°.

Chemical shift data for these *N*-substituted glycines are listed in Table I.

Numbers below hydrogen attached to carbon are chemical shift values in parts per million downfield relative to 3-(trimethylsilyl)-1-propanesulfonate ion for D_2O solutions and relative to tetramethyl silane for trifluoroacetic acid solutions. The abbreviations for splitting patterns are: *s* = single peak, *d* = doublet, *t* = triplet, *q* = quartet, and AB_2X_2 = 5 proton pattern of up to 110 lines.

The spin-spin splitting constant for the doublets, triplets, and quartet was 5 c.p.s.

The chemical shifts for all nonexchangeable protons are downfield on conversion of the anion to cation. These changes in magnetic shielding range from 0.5 p.p.m. downfield for the methyl in sarcosine to 1.21 p.p.m. downfield for the methylene in *N,N*-dimethylglycine. The shielding values for nonexchangeable protons in CF_3COOH solutions seem more consistent than the shielding values in KOH–D_2O. The values for sarcosine in water indicate considerable solvent interaction.

CONCLUSIONS

The spectral changes for an *N*-substituted amino acid observed between alkaline D_2O and CF_3COOH as solvents are useful for locating *N*-methyl, *N*-methylene, *N*-phenyl, and other adjacent structures attached to the nitrogen of an amino acid.

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Modified Medium Containing Phosphomolybdic Acid Useful in the Identification of Specific Yeast-Like Fungi

By JOSEPH E. GRAY

A medium containing phosphomolybdic acid is described which permits growth and differentiation between closely related yeast-like fungi. When properly formulated and packaged, the vials of medium can be stored at room temperature for 6 months or more without loss of stability or effectiveness.

CANDIDA ALBICANS is the most frequent isolate from mycotic vaginitis infections and is considered by many clinicians to be the only pathogenic *Candida* species. However, studies by Haley (1), Kearns and Gray (2), Jillson and Lyle (3), Hurley and Morris (4), and De Sousa and Van Uden (5) have shown that yeast species other than *C. albicans* can initiate or complicate this infection. Taubert and Smith (6) have reported a significant number of cases where a clinical diagnosis of vaginal candidiasis was made and from which *C. albicans* was not cultured.

Various laboratory media have been developed to aid in the identification of *C. albicans*. However, these media are less precise in their ability to distinguish between *Candida* species other than *C. albicans*. Biological methods described by Lodder and Kreger-Van Rij (7), Wickerham (8, 9), and Martin *et al.* (10) are definitive but time consuming. It would appear that a selective medium able to rapidly differentiate between closely related yeast-like fungi would be of value in the diagnosis of mycotic vaginitis.

The use of a medium containing phosphomolybdic acid for *C. albicans* differentiation was first described by MacLaren and Armen (11) in 1958. The cultures were identified by their macrocolony pigmentation and an extracellular reaction seen in the medium.

This original medium had deficiencies which were reported by Holland and Kunz (12), who found a variation in the colony pigmentation obtained within *C. albicans* strains. In unpublished studies in this laboratory it was found that the medium often showed closely related *Candida* species with similar colony pigmentation, a result which hindered positive identification.

The modification described in this report results in a formulation that significantly improves the differentiation of yeast-like fungi encountered in cases of mycotic vaginitis. The distinct colony pigmentation or growth characteristics obtained with the various species, on this medium, makes possible a relatively rapid identification.

PROCEDURE

Essentially the medium consists of two portions: the basal medium and the stock solutions. These are prepared and sterilized before use.

Materials.—Agar (Difco), sucrose (Difco), proteose peptone (Difco), phosphomolybdic acid (20 $MoO_3 \cdot 2\ H_3PO_4 \cdot 48\ H_2O$, Merck), polysorbate 21 (polyoxyethylene sorbitan monolaurate)[1] yeast nitrogen base (Difco), and neomycin sulfate.

Basal Medium.—Distilled water is added to 1.5 Gm. of agar to give a total volume of 75 ml. in a 300-ml. flask. This size flask allows the medium to be properly agitated during formulation. The agar is dissolved by heating in water then auto-

TABLE I.—STOCK SOLUTIONS

Ingredient	Concn. w/v	Amt./75 ml. Basal Medium
Sucrose	40%	10 ml.
Proteose peptone	10%	10 ml.
Phosphomolybdic acid	1.66%	5 ml.
Polyoxyethylene sorbitan monolaurate	11.4%	1 ml.
Yeast nitrogen base	6.7%	3 ml.
Neomycin sulfate	7 mg. neomycin base/ml.	3.8 ml.
	Total vol.	107.8 ml.

TABLE II.—COLONY COLOR AND GROWTH CHARACTERISTICS

Organism	Results
C. albicans	A distinctive olive colony—the reverse of the colony is also olive.
C. tropicalis	A deep blue or blue-green colony—the reverse is also blue. Blue pigment is usually present in the medium.
C. guilliermondi	A brown or dark gray colony with a definite white border.
C. krusei	A small dull white colony.
Torulopsis glabrata	A small, flat, white shiny colony that may be hard to distinguish from *C. krusei*.
C. albicans var. stell.	A small light tan colony.
C. parakrusei	A dull brown slow growing colony, larger and more irregular than the smooth *C. albicans* colony.
Saccharomyces cerevisiae	A bright blue colony, lighter in color and larger than *C. tropicalis*.
Cryptococcus neoformans	A white raised moist colony, larger than *T. glabrata*. Later may show mucoid growth with a tan cast.
Rhodotorula sp.	A pink to pinkish - brown yeast-like colony.

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The author is indebted to Mrs. June Horton and Mr. Ralph Bush for their technical assistance and to Mr. Peter Alotta, Pharmaceutical Research Division, for supplying the packaging data and material.

[1] Marketed as Tween 21 by the Atlas Powder Co., Wilmington, Del.

TABLE III.—MOST FREQUENTLY ISOLATED SPECIES FROM MYCOTIC VAGINITIS INFECTIONS, RESULTS OF CULTURE ON TEST MEDIUM

Organism	Strains Tested, Total No.	Typical Reactions, No.	Atypical Reactions, No.	Explanation
C. albicans	171	162	9	2 = Olive green colony 3 = Light tan colony 1 = Poor growth 3 = No growth
T. glabrata	17	16	1	= No growth
C. tropicalis	8	8		
C. krusei	7	5	2	= No growth
C. guilliermondi	5	3	2	= No growth
C. parakrusei	2	1	1	= Olive white colony
Totals	210	195	15	

claved at 120° for 15 min. The flask is cooled to 50° in a water bath; if necessary the volume is readjusted to 75 ml. with sterile distilled water. The requisite amounts of stock solution are then added.

Stock Solutions.—All solutions are prepared using distilled water and are sterilized by Millipore filtration using type HA filter pads.

The stock solutions must be added to the basal medium in the order shown (Table I). After the addition of the phosphomolybdic acid, the flask is shaken vigorously to insure homogeneity of the heavy precipitate that results. The addition of polyoxyethylene sorbitan monolaurate reduces foam formation and permits the medium to be tubed satisfactorily. It is not necessary to adjust the pH of the basal medium before sterilizing. The pH of the complete medium is about 5.3.

Packaging.—Sufficient medium is slanted in clear glass vials to permit visual examination of colony growth. A screw cap with a moisture proof liner is necessary for storage. The vials can then be stored at room temperature for periods of 6 months or more without loss of sensitivity.

Inoculation.—The medium can be inoculated with a sterile cotton swab or a sterile loop. Better results are obtained with a light inoculum. Mixed yeast infections are more readily detected when discrete colonies develop. Often the presence of excess body exudate may hinder colony growth. If necessary, streak plates on Sabouraud's dextrose agar can be made, then discrete colonies can be transferred to the medium for growth and identification.

RESULTS AND DISCUSSION

Table II shows the colony pigmentation and growth characteristics for those yeast or yeast-like fungi most frequently isolated from mycotic vaginitis infections. These species samples were individually identified by the biological methods mentioned previously (7–10). In addition, some species are listed that appear infrequently in vaginitis infections: *Saccharomyces cerevisiae, Cryptococcus Neoformans, and Rhodotorula species* (2, 5, 13). For purposes of comparison, duplicate cultures representing the various species were obtained from the American Type Culture Collection.

Table III lists the clinical isolates, the number of each species studied, and the results obtained on the test medium. The majority of cultures showed characteristics similar to those described in Table II for the particular species. Of the 15 cultures considered atypical, eight failed to grow on the test medium while showing good growth on Sabouraud's medium.

In a separate experiment, a number of strains were inoculated on freshly prepared media, and media stored at room temperature for 6 months. There was no difference between the two lots of media.

Although the medium described in this report is concerned with the differentiation of fungi associated with mycotic vaginitis, it has also been tested for possible use in the isolation and identification of dermatophytic organisms. With the exception of *Trichophyton rubrum*, most dermatophytes grow rapidly on the medium, but there is no color differential as seen with the yeast-like fungi.

The concentration of phosphomolybdic acid is critical for the production of specific pigments among the closely related *Candida* species. Neomycin sulfate is not necessary for the development of the differential characteristics but does inhibit the utilization of phosphomolybdic acid by bacterial contaminants often found in clinical samples. Those bacteria that do succeed in growing usually show a low, moist blue, confluent streak of growth easily distinguished from that of *C. tropicalis*. To date, no bacterial species that grows similar to *C. albicans* on the medium has been encountered in the clinical samples from cases of mycotic vaginitis.

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Detection of Trace Amounts of Lysergic Acid Diethylamide in Sugar Cubes

By CZESLAWA RADECKA and ISHWAR C. NIGAM

A method for the detection of trace amounts of lysergic acid diethylamide in sugar cubes is described. The free base is extracted from aqueous sodium bicarbonate solution with methylene chloride and hydrogenated with Adam's catalyst. The product is examined by gas chromatography and its identity confirmed by thin-layer chromatography of the eluate which yields two characteristic spots—one blue, the other yellow—on spraying with *p*-dimethylaminobenzaldehyde. The yellow spot exhibits strong blue fluorescence when viewed under ultraviolet light prior to application of the reagent.

THE NEED for sensitive procedures to detect lysergic acid diethylamide (LSD) in narcotic seizures was illustrated in a previous communication from this laboratory (1). The recent discovery of the illegal sale of sugar cubes impregnated with the psychotomimetic drug focused anew the authors' attention on existing analytical methodologies and prompted them to develop an additional microphysicochemical technique for its detection and identification. Exploratory experiments aimed at direct application of gas chromatography to the analysis of LSD were unsuccessful. Due to low volatility, the compound could not be recovered even when using columns of low packing ratio and operating at high column temperatures. Consideration was, therefore, given to hydrogenation of the drug prior to gas chromatography as a means of eliminating its unsaturation (C_9-C_{10} bond) and thereby increasing its stability.

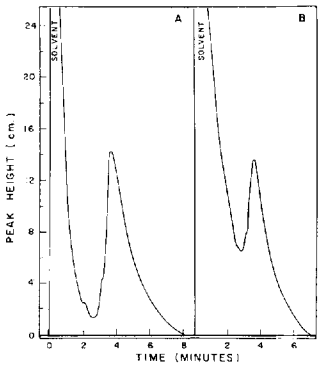

Fig. 1.—Gas chromatograms of hydrogenated LSD. Key: A, reference sample; B, sugar cube extract (exhibit J568). Column temperature: 270°; injector temperature: 290°; nitrogen flow: 22 ml./min.; sample volume: 1 μl.

TABLE I.—THIN-LAYER CHROMATOGRAPHY OF HYDROGENATED LSD

Sample	Spot	Chromatographic Data: Color Observed: Under U.V. Light	After Spraying	*Rf* Value	Relative *Rf* Value[a]
Hydrogenated LSD	1	Blue fluorescence	Faint yellow	0.81	1.04
	2	...	Blue	0.78	1.00
Gas chromatographic eluate, 3–4 min.	1	Blue fluorescence	Faint yellow	0.81	1.04
	2	...	Blue	0.78	1.00
Hydrogenated LSD soln. stored for 2 wk.	1	Blue fluorescence	Faint yellow	0.81	1.04
	2	...	Blue	0.78	1.00
	3	...	Blue	0.54	0.69

[a] Reference: spot 2.

The present communication describes the successful application of this approach to the detection of the psychotomimetic drug in sugar cubes and provides additional identity tests based on thin-layer chromatography of the effluent gas chromatographic peak.

EXPERIMENTAL

Materials

Lysergic Acid Diethylamide Tartrate.—LSD 25 (courtesy Sandoz Ltd., Dorval, Quebec, Canada).

Sugar Cubes.—(*a*) Specimens seized by officers of the Royal Canadian Mounted Police (exhibit J568) and (*b*) specimens prepared by impregnation with aqueous solutions containing 1 mg. of LSD/ml., and drying in a stream of warm air (50°) for about 5 min.

Methods

Extraction of LSD.—The drug was isolated in accordance with the following procedure (2). The sugar cube was dissolved in 10 ml. of distilled water, 0.5 Gm. of sodium bicarbonate was added, and the free base was extracted with four 5-ml. portions of methylene chloride. The lower layer was withdrawn through a piece of cotton wool placed in the stem of the separator, care being taken to minimize exposure of the isolate to light and heat. The extract was evaporated to dryness in a gentle stream of nitrogen.

Hydrogenation of LSD.—The sample (reference compound or material extracted from a sugar cube) was dissolved in 1 ml. of methanol and hydrogenated at atmospheric pressure for 3 hr. using 10 mg. of Adam's catalyst. After filtration, the solution was gently concentrated to 0.1 ml. in a slow stream of nitrogen.

Gas Chromatography.—An Aerograph Hi-Fi,

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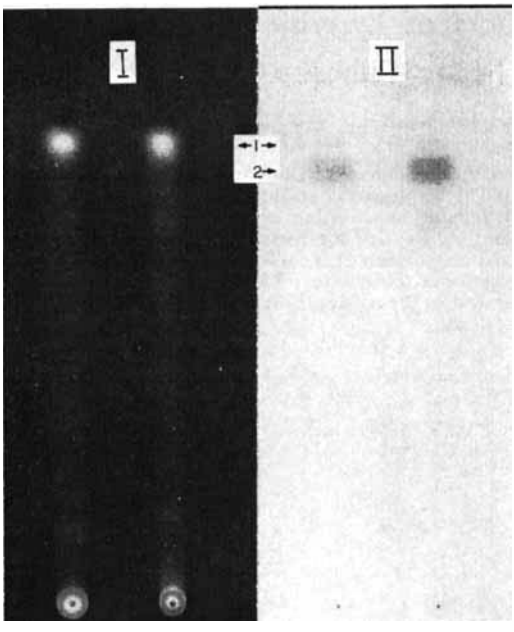

Fig. 2.—Thin-layer chromatograms of hydrogenated LSD. Key: I, observed under U.V. light; II, observed after spraying with *p*-dimethylaminobenzaldehyde; A, reference sample: product from hydrogenation of 1.8 mg. LSD in 0.1 ml. methanol. Volume applied: 2 μl.; B, sugar cube (exhibit J568). Product from hydrogenation of sugar cube extract in 0.05 ml. of methanol. Volume applied: 4 μl.

model A-600-C, equipped with hydrogen flame ionization detector, was employed. A glass tube was inserted into the injection port to minimize degradation of the sample at the metal surface of the preheater. The column, a stainless steel tube (5 ft. × 1/8 in.) was packed with micro glass beads (60 mesh) coated with 0.2% of silicone rubber SE-30. By means of a stream splitter, eluates were collected in capillaries packed lightly with methanol moistened glass wool.

Thin-Layer Chromatography.—*Thin-Layer Plates.*—Glass plates (20 × 20 cm.) coated with aluminum oxide G (Merck). Layer thickness 250 μ.

Solvent System.—Chloroform–ethanol (96:4) (3).

Detection.—Following migration of the solvent front (15 cm.), plates were observed under ultraviolet light (3660 Å.) and subsequently sprayed with a solution of *p*-dimethylaminobenzaldehyde (2 Gm.) in concentrated HCl (20 ml.) and ethanol (80 ml.) (4).

RESULTS AND DISCUSSION

The product obtained by catalytic hydrogenation of LSD could easily be subjected to gas chromatography. A main peak (retention time: 3.6 min.) and two slight shoulders (retention times: 3.1 and 2.1 min., respectively) were observed (Fig. 1). In order to obtain more reproducible retention time data, ergonovine maleate hydrogenated in accordance with the procedure described was used as internal reference. Relative retention time of hydrogenated LSD: 1.64.

Thin-layer chromatographic analysis of the effluent emerging between 3 and 4 min. served to confirm the gas chromatographic identification. The thin-layer chromatogram of hydrogenated LSD exhibited a blue fluorescent spot when viewed under ultraviolet light. After spraying with the reagent, this spot acquired a yellow coloration, while another intense blue spot appeared at a slightly lower R_f value (Table I and Fig. 2). These same spots were observed when the gas chromatographic eluate was similarly examined. Solutions which had been kept for several days showed a number of additional but faint spots, among which one at R_f 0.54 was most prominent.

The thin-layer chromatographic technique was found to be superior to the gas chromatographic technique in that it allowed the characterization of LSD concentrates which failed to produce gas chromatographic peaks. Thus, when a sugar cube impregnated with 50 mcg. of LSD was processed as described, only a shoulder appeared on the descending portion of the solvent peak in the gas chromatogram at 3.6 min., while the corresponding eluate yielded a convincing thin-layer chromatogram.

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REVIEWS

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The stated intent of the Editors is to present not the customary review, but rather critical surveys which will enable the reader who cannot keep up with the explosive growth in the field to maintain an over-all view of the progress in chromatography. As so frequently happens in a compilation of this type, there is a marked difference in the extent to which the individual authors attain this objective.

The single chapter of the volume relating directly to pharmaceutical chemistry (Qualitative and Quantitative Aspects of the Separation of Steroids, by E. C. Horning and W. J. A. Vandenheuvel) thoroughly fulfills the promise of its title. This is one of five chapters which succeeds in maintaining the balance of depth and breadth of coverage needed to achieve the goal of the Editors. Of the other five chapters, two are rigorous theoretical presentations of quite narrow topics, while a third covers a broad field at a rather elementary level. The remaining two fall between these.

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This volume will be a worthwhile addition to the library of the pharmaceutical chemist.

Reviewed by Joseph Levine
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Food and Drug Administration
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The book presents descriptions, with illustrations, of the various types of apparatus available for carrying out hydrogenations as well as more detailed operating instructions for the more commonly used pieces of equipment. A chapter is devoted to the catalyst, reaction conditions, and the effects of variables such as temperature and pressure on the course of a hydrogenation. The catalysts which are discussed are those used most often in catalytic hydrogenation procedures and include nickel, platinum, palladium, ruthenium, copper–chromium oxide, and rhenium. Specific directions for preparing a number of these catalysts are given in an appendix.

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Review Article

Stereochemical Factors and Receptor Interactions Associated with Narcotic Analgesics

By PHILIP S. PORTOGHESE

STEREOCHEMICAL studies on narcotic analgesics can be considered an outgrowth of earlier chemical investigations which were concerned primarily with the elucidation of structural moieties necessary for analgesic activity. During this period, which spans about 50 years, considerable attention had been focused on the design and synthesis of analgesics. The role of steric factors, however, was not well understood and was largely unexplored.

The major advances in configurational and conformational analysis which occurred approximately 15 years ago set the stage for various stereochemical studies on narcotic analgesics which were soon to follow. These studies have drawn attention to the importance of steric factors in analgesia and have provided greater insight into the nature of the analgesic–receptor interaction.

This review is organized into three main sections: (*a*) absolute configurational studies, (*b*) conformational factors, and (*c*) concepts on analgesic–receptor interactions. No attempt has been made to give an exhaustive coverage of the chemical and pharmacological literature on this subject, but rather, discussion has been restricted to key developments and recent research related to the steric aspects of analgetically active compounds.

ABSOLUTE CONFIGURATIONAL STUDIES

One of the best approaches to delineating the steric requirements for analgesia involves the correlation of enantiomeric potency with absolute spatial geometry. In most cases one enantiomer possesses greater analgesic activity than its mirror image form. Since the lipid solubility and dissociation constant of enantiomeric bases are identical, differences in distribution are minimized and the variation in analgesic activity may be ascribed more confidently to events at the receptor level. The difference in potency between enantiomorphs is very likely due to the asymmetric topography of the receptors. In such a dissymmetric environment, (+)- and (−)-antipodes behave differently. Thus, one enantiomer may be more potent by virtue of its greater affinity and/or intrinsic activity (1, 2).

Inasmuch as optical rotation of structurally diverse compounds possessing a common asymmetric center is not necessarily indicative of absolute stereochemistry, correlations of optical rotation with analgesic activity are devoid of

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The author acknowledges the support of this project by grants NB 05192 and GM 09402 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

any meaning other than being an expression of differences in the stereoselectivity of analgesic receptors for (+)- and (−)-enantiomers. In order to correlate molecular spatial geometry with analgesic potency, the configuration of the enantiomers must be established. Most of the methods employed for this purpose have involved chemically relating one of the optical antipodes to a compound of known configuration. The analgesics which have received greatest attention are those which possess an asymmetric center in common with methadone and with isomethadone.

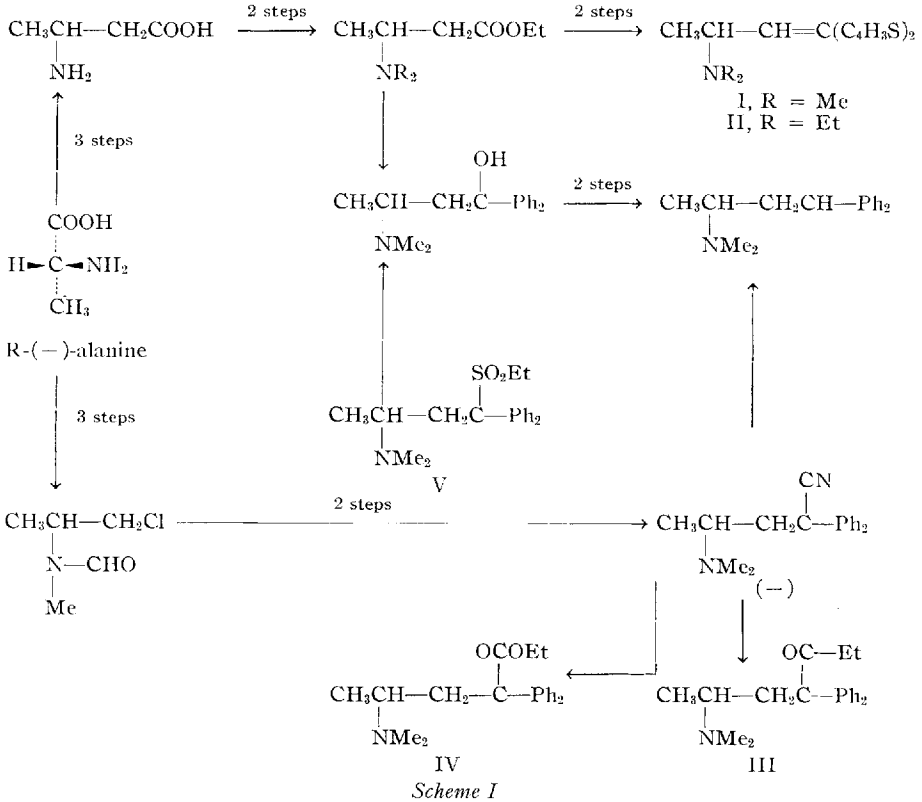
Structures Possessing an Asymmetric Center in Common with Methadone.—The first reported investigation on the configuration of synthetic narcotic analgesics of the methadone type was carried out by Beckett and Casy (3) who related the (+)-thiambutenes (I and II: *Scheme I*) (4) and the (−)-enantiomers of methadone (III: *Scheme I*) (5–8) and analogous structures (IV and V: *Scheme I*) (8, 9) to R(−)-alanine by the route outlined in *Scheme I*. The key reaction in this sequence was the transformation of R-alanine to β-aminobutyric acid *via* the Wolf rearrangement. Since this reaction was generally known to proceed with retention of configuration, the butyric acid intermediate was assumed to be stereochemically related to its precursor. Aminobutyric acid was then converted in several steps to (+)-thiambutene (I: *Scheme I*) and its diethyl homolog (II: *Scheme I*). The configuration of (−)-methadone (III: *Scheme I*) was established by converting the (−)-nitrile, which is a precursor of III: *Scheme I*, to 1,1-diphenyl-3-dimethylaminobutane derived from R-alanine. Since all of these stereochemical interrelationships were based on the assumption that the Wolf rearrangement had proceeded in the usual way, the configuration of III: *Scheme I* was subsequently confirmed unequivocally by converting R-alanine to the (−)-nitrile without involving the asymmetric center in question. The (−)-nitrile was also transformed to the (−)-carbethoxy analog of methadone (IV: *Scheme I*). The (−)-sulfone analog (V: *Scheme I*) was hydrolyzed to the same aminobutanol derivative which was derived from R-alanine.

From molecular rotation studies (−)-phenadoxone (Table III, *2*) (10) was also determined to be configurationally related to R-(−)-methadone.

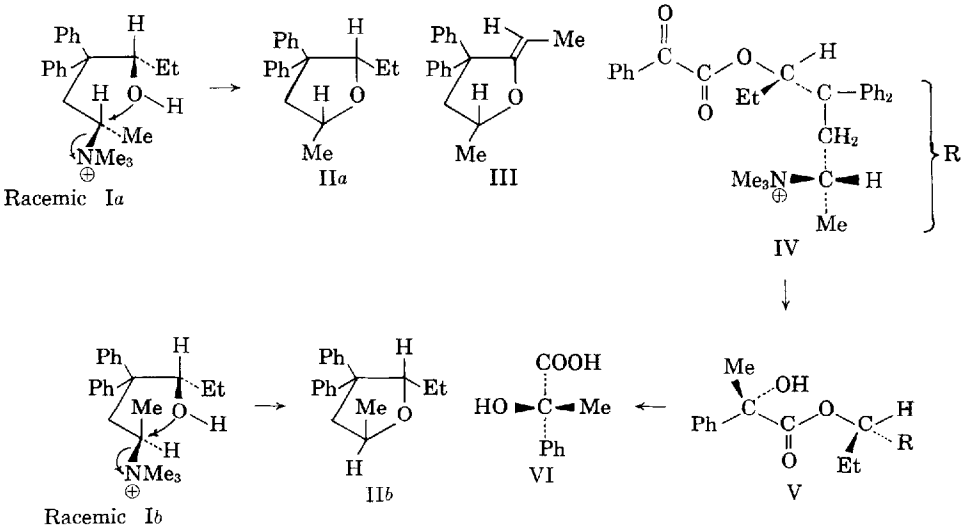
It has been pointed out by Beckett and Casy (11) that the (+)-enantiomers of I and II: *Scheme I* and the (−)-enantiomers of III, V: *Scheme I* and Table III, *2*, are all more active and configurationally related to R-alanine. Equally significant, however, is the fact that the more active enantiomer of IV: *Scheme I* is in the (S)- rather than in the (R)-series.

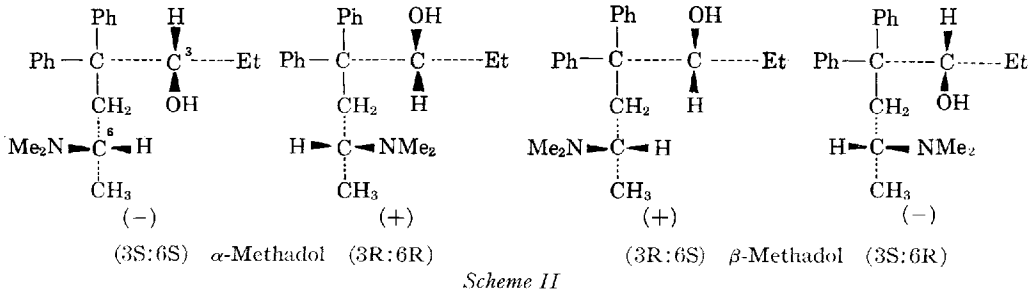
Pohland (8) and Eddy (12) have described the synthesis of optically active diastereomers of methadol (Table I, *1*) and acetylmethadol (Table I, *2*). Catalytic hydrogenation of methadone afforded only α-methadol (Table I, *α-1*), whereas sodium and propanol reduction yielded a mixture of diastereomers in which the β-isomer (Table I, *β-1*) predominated. The absolute stereochemistry at C-6 is now known because the diastereomers were derived from enantiomers of methadone, whose configuration (3) was subsequently determined. The stereochemistry of the C-3 hydroxyl group had not been reported, however. The absolute stereochemistry of the methadols is of particular interest because of the inversion of configurational selectivity[1] of analgesic receptors which occurs on transforming methadone to α-methadol (Table I). Interestingly, no such inversion occurs in the case of Table I, *β-1*. Upon acetylation of Table I, *α-1*, to form Table I, *α-2*, the potency ratio becomes inverted, so that the (6R)-series is once again more active. Portoghese and Williams (13) have investigated the stereochemistry of the methadols and have assigned the configuration at C-3 as designated in *Scheme II*. These assignments were based on the ability of racemic α- and β-methadol methiodide (I*a* and I*b*: *Scheme II*, respectively) to undergo stereospecific ring closure to isomeric tetrahydrofurans II*a* and II*b*: *Scheme II*. It was found that catalytic hydrogenation of the ethylidene compound (III: *Scheme II*) gave rise to II*a*: *Scheme II* as the preponderant isomer. Molecular models indicated that adsorption by the catalyst would occur on the less hindered top face of III: *Scheme II* to afford the *cis* isomer (II*a*: *Scheme II*). Since ring closure occurs with inversion at the C-6 center of I, the relative stereochemistry of the α- and β-isomers was deduced. Inasmuch as the configuration at C-6 is known (3), the absolute stereochemistry at C-3, therefore, was established. Additional proof was obtained from the dissociation constants of α- and β-methadol. The α-isomer was found to be a stronger base. This indicated less steric hindrance of the α-compound to intramolecular hydrogen bond formation be-

[1] The term "selectivity" rather than "specificity" is employed throughout. The former signifies that pharmacological activity is found predominantly in one isomer, though not exclusively, while the latter implies that activity resides in only one isomer. This definition is adapted from Eliel, E. L., "Stereochemistry of Carbon Compounds," McGraw-Hill Book Co., Inc., New York, N. Y., 1962, p. 436.



Scheme I



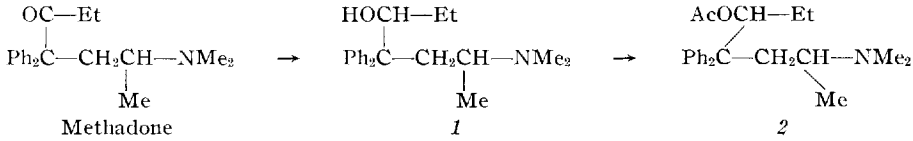


Scheme II

tween the protonated amine function and the oxygen atom of the hydroxyl group (14). Molecular models of α-methadol show that it is less hindered in the internally bonded form than is the β-isomer. Asymmetric induction studies utilizing Prelog's rule confirmed the above stereochemical assignment. Thus, treatment of (3S:6S)-methadol benzoylformate methiodide (IV: *Scheme II*) with methylmagnesium iodide gave the atrolactate ester (V: *Scheme II*) which on basic hydrolysis afforded S-atrolactic acid (VI: *Scheme II*) in 8% optical purity. A knowledge of the stereochemistry of the methadols made possible the complete configurational assignment of the acetate esters (Table I, *2*) and other related compounds. (See also Table III.)

The configuration of several basic anilide analgesics (15, 16), which have an asymmetric center in common with methadone, has been investigated by Portoghese and co-workers (17, 18). The (−)-enantiomers of these compounds were related to R-alanine by the route outlined in *Scheme III*. The (+)-diamine (V: *Scheme III*) was converted to the (−)-antipodes of I through IV: *Scheme III*. The stereochemistry of (+)-V: *Scheme III*, was determined by transforming it to (−)-VI: *Scheme III*, whose configuration was established by synthesis from R-alanine. Significantly, the more

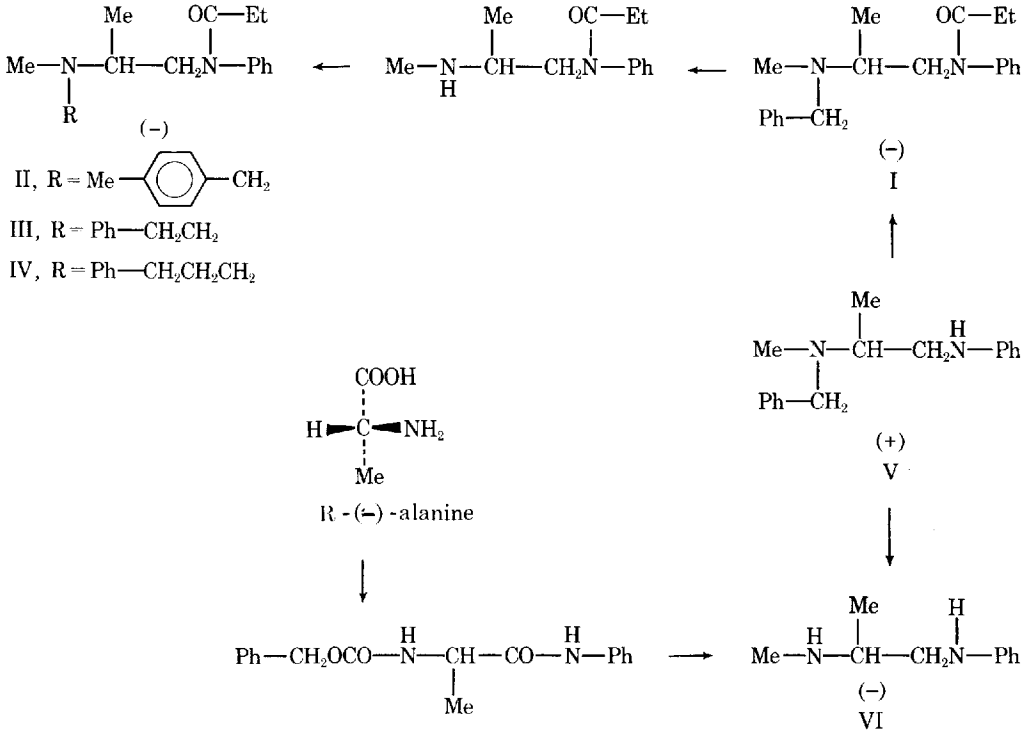
Table I.—Analgesic Potency of Isomeric Methadols and Acetylmethadols[a]



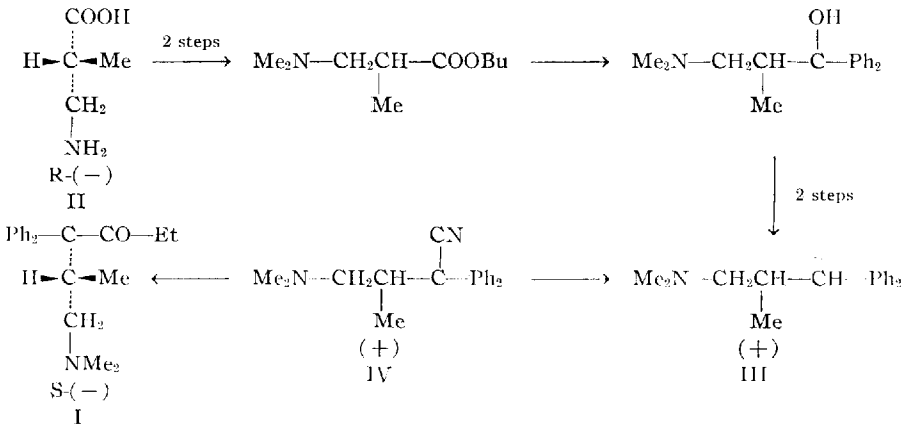
Methadone *1* *2*

Configuration	ED_{50}, mg./Kg.[b]	Isomer	ED_{50}, mg./Kg.[b]	ED_{50}, mg./Kg.[b]
S-(+)	25.7	(−)-α	3.5	1.8
		(+)-β	63.7	4.1
R-(−)	0.8	(+)-α	24.7	0.3
		(−)-β	7.6	0.4

[a] Data from *Reference 12*. [b] Administered subcutaneously in mice.

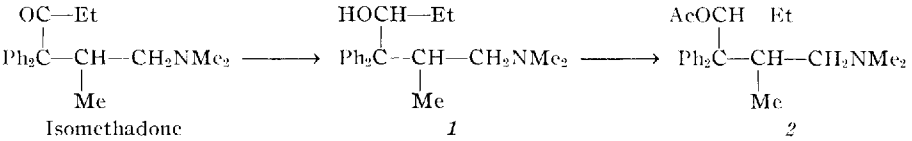


Scheme III



Scheme IV

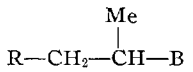
TABLE II.—ANALGESIC POTENCY OF ISOMERIC ISOMETHADOLS AND ACETYLISOMETHADOLS[a]

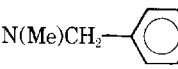
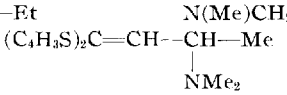
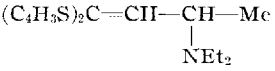


Configuration	ED_{50},[b] mg./Kg.	Isomer	ED_{50}[b], mg./Kg.	Isomer	ED_{50}[b], mg./Kg.
R-(+)	49.8	(+)-α	60.7	(−)-α	62.7
		(−)-β	58.7	(+)-β	70.6
S-(−)	1.2	(−)-α	91.7	(+)-α	2.7
		(+)-β	6.2	(−)-β	10.9

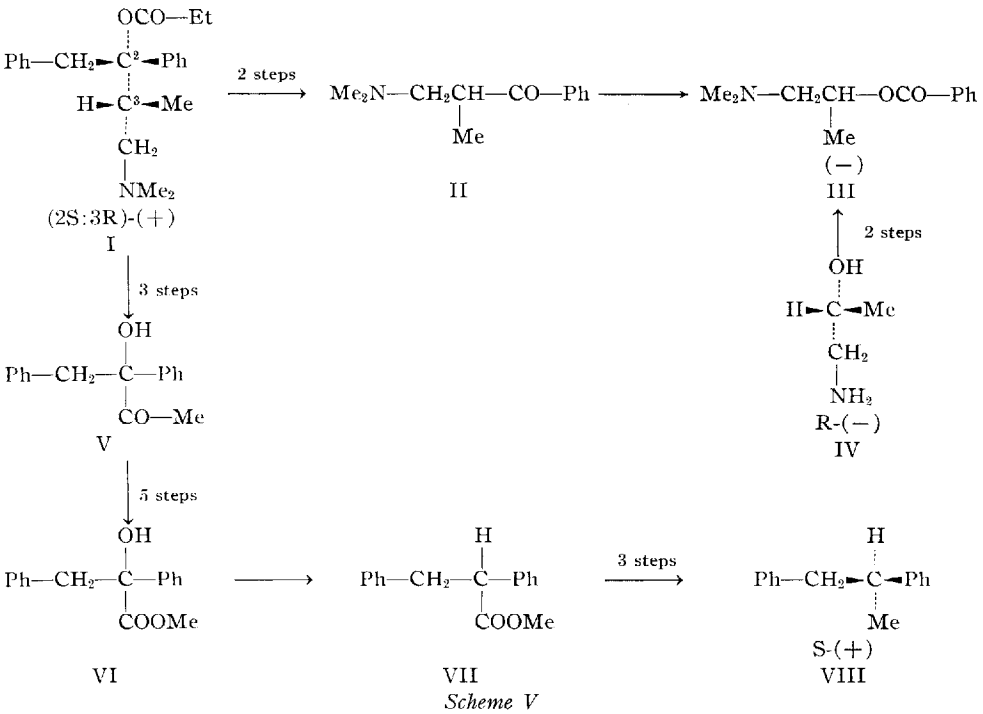
[a] Data from *Reference 19*. [b] Administered subcutaneously in mice.

TABLE III.—CONFIGURATIONAL SELECTIVITY OF ANALGESIC RECEPTORS TOWARD COMPOUNDS HAVING AN ASYMMETRIC CENTER IN COMMON WITH METHADONE

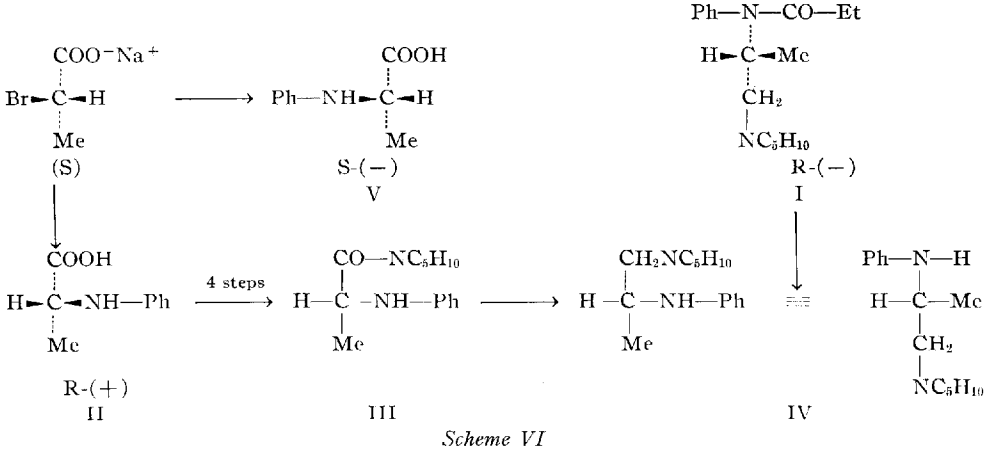


	R	B	Configuration[a]	Ref.
1	Ph_2C—CO—Et	NMe_2	(R)	(3, 6–8)
2	Ph_2C—CO—Et	NC_4H_8O	(R)	(10)
3	Ph_2C—SO_2Et	NMe_2	(R)	(3, 9)
4	Ph_2C—COOEt	NM_2	(S)	(3, 8)
5	Ph_2C—CH(OH)Et	NMe_2	(3S : 6S)	(8, 12, 13)
6	Ph_2C—CH(OH)Et	NMe_2	(3S :6R)	(12, 13)
7	Ph_2C—CH(OAc)Et	NMe_2	(3R:6R)	(8, 12, 13)
8	Ph_2C—CH(OAc)Et	NMe_2	(3S :6R)	(12, 13)
9	Ph_2C—CH(OAc)Et	NHMe	(3R:6R)	(13, 81)
10	Ph—N—CO—Et	$N(Me)CH_2Ph$	(S)	(15–18)
11	Ph—N—CO—Et		(S)	(15–18)
12	Ph—N—CO—Et	$N(Me)CH_2CH_2Ph$	(S)	(15–18)
13	Ph—N—CO—Et	$N(Me)CH_2CH_2CH_2Ph$	(S)[b]	(15–18)
14			(R)	(3, 4)
15			(R)	(3, 4)

[a] Configuration of the more active enantiomer. [b] The S-enantiomer is slightly more potent, although this may not be statistically significant.



Scheme V



Scheme VI

active (+)-enantiomers of the aforementioned compounds are related to S-alanine (Table III) and hence possess a configuration opposite to that of the active antipodes of methadone. Moreover, the stereoselectivity of the receptors decreases (enantiomeric potency ratio approaches unity) as the length of the aralkyl chain is increased. This phenomenon is discussed further in a subsequent section (Table XII).

Structures Possessing an Asymmetric Center in Common with Isomethadone. — Most of the analgesic activity of isomethadone has been found to reside in the (−)-enantiomer (I: *Scheme IV*) (19). The absolute stereochemistry of this compound was elucidated by Beckett *et al.* (20) by the pathway outlined in *Scheme IV*. R(−)-α-Methyl-β-alanine (II: *Scheme IV*) was transformed to (+)-III: *Scheme IV*, which was also derived from the (−)-isomethadone precursor, (+)-IV: *Scheme IV*. This establishes the stereochemical relationship between II: *Scheme IV* and (−)-isomethadone (I: *Scheme IV*) whose configuration is designated as S-isomethadone.

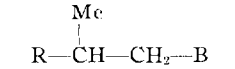
May and Eddy (19) have reduced the carbonyl group of optically active isomethadone to produce isomeric isomethadols. Reduction with lithium aluminum hydride proceeded stereospecifically to give the α-isomer (Table II, *α-1*). Treatment with sodium in propanol

afforded a mixture of Table II, *α-1* and *β-1*, with the latter as the predominant isomer. The analgesic activity of these optical isomers and their stereochemical relationship to (+)- and (−)-isomethadone are shown in Table II. All of the enantiomers in the α- and β-series, which are related to R-isomethadone, have a low order of activity. With isomers derived from S-isomethadone, however, Table II, (+)-*β-1*, is much more potent than Table II, (−)-*α-1*. The reverse is true for the acetate esters, in that Table II, (+)-*α-2*, is more active than Table II, (−)-*β-2*. It can be noted that there is an inversion in the enantiomeric potency ratio of the type seen in the α-methadol compounds (Table I), although in this case, a large diminution of analgesic potency and of stereoselectivity is observed.

(+)-Propoxyphene (I: *Scheme V*) is the only one of four optical isomers which has significant analgesic activity (21). Sullivan, Beck, and Pohland (22) have determined its absolute stereochemistry as (2S:3R) *via* an elegant series of transformations (*Scheme V*). Establishment of the configuration at C-3 was accomplished by converting I to the Mannich base (II: *Scheme V*) and then carrying out a Baeyer-Villiger oxidation to give the (−)-ester (III: *Scheme V*). The identical ester was prepared from R(−)-propanolamine (IV: *Scheme V*). Since the oxidation is known to proceed with retention of configuration, the C-3 center was designated as being in the R-series. Casy and Myers (23) subsequently have confirmed this configurational assignment by a route which did not involve the C-3 asymmetric center. Elucidation of the stereochemistry at C-2 was carried out by transforming (+)-propoxyphene to S(+)-1,2-diphenylpropane (VIII: *Scheme V*). The reaction sequence involved the degradation of I: *Scheme V*, to the methyl ketone (V) which was subsequently converted to VIII *via* intermediates VI and VII. The assignment of the stereochemistry at C-2 is based upon the fact that the steric course of hydrogenolysis of a benzylic hydroxyl group proceeds with high retention of configuration.

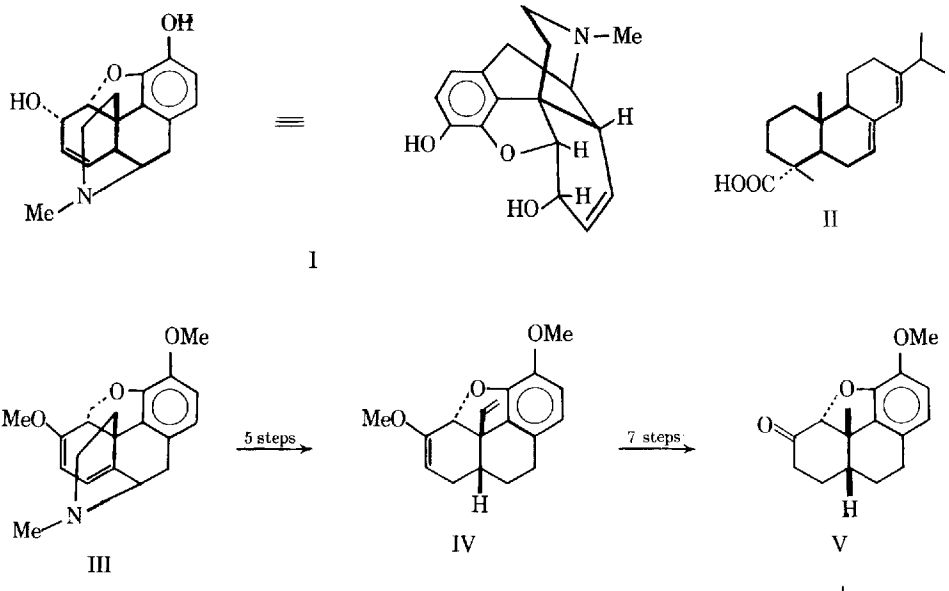
Phenampromide (I: racemic *Scheme VI*) (24), a basic anilide analgesic which can be considered structurally related to isomethadone and propoxyphene, has been resolved and the (−)-enantiomer found to be four times more active than the (+)-isomer. The configuration of I was determined by Portoghese (25) to be related to R-(+)-*N*-phenylalanine (II: *Scheme VI*). The absolute stereochemistry of II: *Scheme VI* was established (26) by studying the steric course of the interaction of aniline with S-2-bromopropionate. When the reaction was carried out in pure or 4 *M* aniline the (+)-amino acid (II: *Scheme VI*) was produced, whereas in a 0.1 *M* aniline solution the (−)-enantiomer (V) was formed. According to the established mechanism (27) of displacement reactions on sodium 2-bromopropionate, the former reaction should take place with net inversion and the latter with net retention of configuration. Since the inverted product (II: *Scheme VI*) is derived from S-2-bromopropionate, II: *Scheme VI*, therefore, possesses the R-configuration. The optically pure amino acid (II: *Scheme VI*), obtained by deracemization, was transformed to the amide (III) and this was reduced to the diamine (IV). The identical diamine was derived from the hydrolysis of (−)-phenampromide (I: *Scheme VI*). It should be pointed out that the more active enantiomers, R-phenampromide, S-isomethadone, and (2S:3R)-propoxyphene, are all stereochemically related at their common asymmetric center (Table IV). This may mean that the aforementioned analgesics are interacting with common receptors, although

TABLE IV.—CONFIGURATIONAL SELECTIVITY OF ANALGESIC RECEPTORS TOWARD COMPOUNDS HAVING AN ASYMMETRIC CENTER IN COMMON WITH ISOMETHADONE



	R	B	Configuration[a]	Ref.
1	Ph_2C—CO—Et	NMe_2	(S)	(19, 20)
2	Ph_2C—CH(OH)Et	NMe_2	α-(6R)[b]	(19)
3	Ph_2C—CH(OH)Et	NMe_2	β-(6S)	(19)
4	Ph_2C—CH(OAc)Et	NMe_2	α-(6S)	(19)
5	Ph_2C—CH(OAc)Et	NMe_2	β-(6S)	(19)
6	Ph—CH_2(Ph)C—OCO—Et	NMe_2	(2S:3R)	(21–23)
7	Ph—N—CO—Et	NC_5H_{10}	(R)	(24, 25)

[a] Configuration of the more active enantiomer. [b] The (6R)-isomer has a very low order of activity and is only 1.5 times more potent than its enantiomer.



Scheme VII

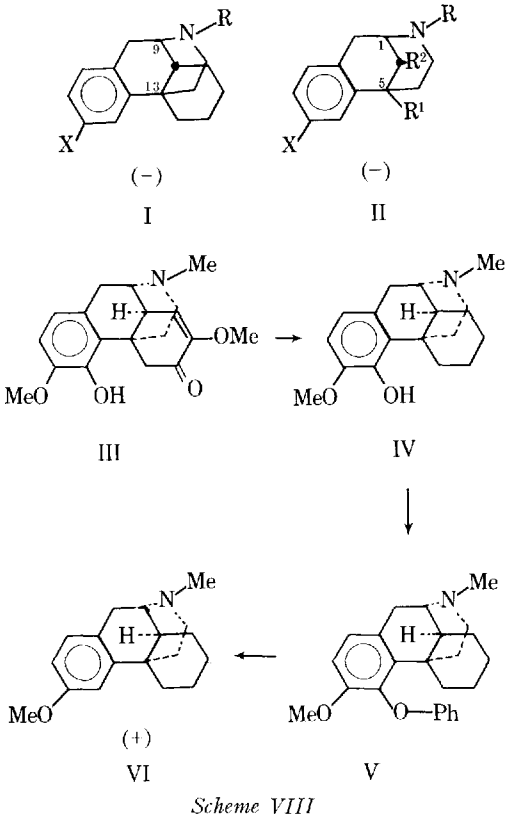
it is also possible that such stereochemical equivalence may be entirely fortuitous.

The relationship between configuration and analgesic activity of compounds having an asymmetric center in common with methadone and with isomethadone are summarized in Tables III and IV, respectively. The data compiled in Table III show an apparent lack of consistency, inasmuch as the more active enantiomers are not all configurationally related. The constitution of the R group appears to play a key role in determining the configurational selectivity of analgesic receptors. Such changes in stereoselectivity recently have been interpreted as being reflective of differing modes of analgesic–receptor interactions (28). (See under *Concepts on Analgesic-Receptor Interactions.*) A much more consistent correlation is shown in Table IV where the more active enantiomers, with one possible exception (Table IV, *2*), are all stereochemically related at a common asymmetric center.

Morphine and Related Structures.—The absolute configuration of (−)-morphine (I: *Scheme VII*) was determined by Jeger and collaborators (29) who converted thebaine (III: *Scheme VII*) to a degradation product (VII) of abietic acid (II) of known absolute stereochemistry (30). The route which was employed is outlined in *Scheme VII*. Hydrogenation of III, followed by exhaustive methylation and degradation, gave the vinyl intermediate (IV). This was transformed, in a series of reactions, to the tetracyclic ketone (V), which was then converted to intermediate (VI). Ozonization of VI afforded the (−)-diacid (VII) which was identical to that obtained from II. MacKay and Hodgkins (31) have come to the same conclusion by means of X-ray crystallography.

It has been determined that unnatural (+)-morphine is inactive as an analgesic (32). Similarly, the activity of optically active morphinans (I: *Scheme VIII*) and benzomorphans (II: *Scheme VIII*) has been found to reside principally in the (−)-enantiomers (33). This strongly suggested that the (−)-isomers of these compounds are configurationally related to (−)-morphine. Chemical evidence in the morphinan series for such a relationship was obtained by degradation of I: *Scheme VIII* (R = Me, X = OH), to VII: *Scheme VII via* a scheme (34) which is similar to the procedure employed in establishing the configuration of morphine. Sawa *et al.* (35) have also related sinomenine (III: *Scheme VIII*), which is enantiomeric to (−)-morphine at the C-9 and C-13 asymmetric centers, to (+)-3-methoxy-*N*-methylmorphinan (VI: *Scheme VIII*). Clemmensen reduction of III: *Scheme VIII*, afforded IV. This compound was converted to

the phenyl ether (V) and subsequently reduced to VI. In accord with the above stereochemical assignment, Beckett (36) has reported that silica gel which has been pretreated with levorphanol (I: *Scheme VIII;* R = Me, X = OH) has greater adsorptive capacity for (−)-morphine than silica gel that has been treated with the corresponding (+)-morphinan.

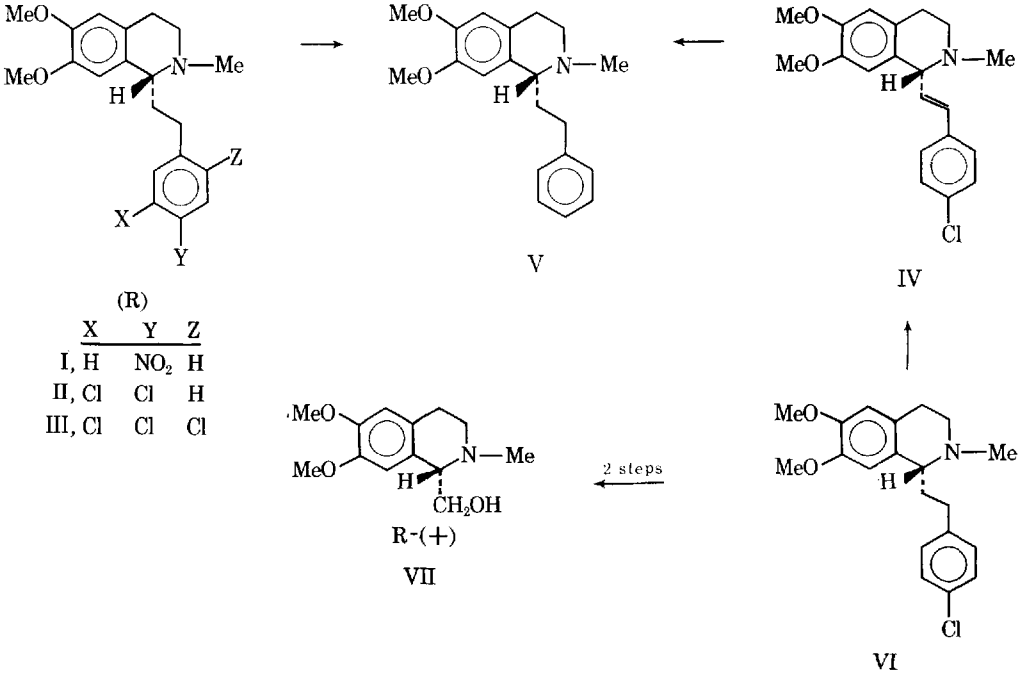


Scheme VIII

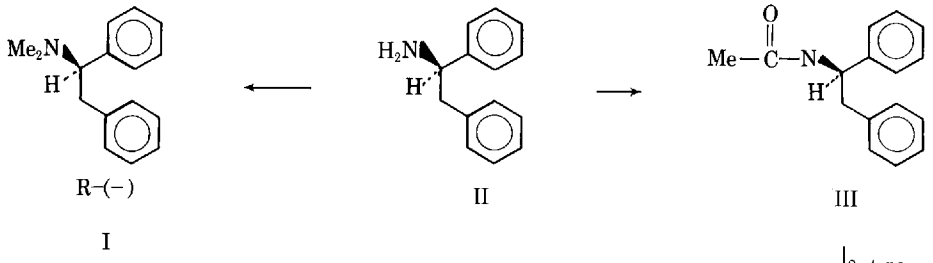
No direct chemical proof of the absolute configuration of benzomorphan derivatives (II: *Scheme VIII*) is available. Beckett (36) has furnished evidence, from stereoselective adsorption studies on silica gel, which suggests that II: *Scheme VIII* (R = R^1 = R^2 = Me, X = OH), is configurationally related to I: *Scheme VIII* (R = Me, X = OH), and hence to (−)-morphine (I: *Scheme VII*). The optical rotatory dispersion (ORD) characteristics of the above compounds have been studied (37), and it has been found that the free bases and salts all exhibit Cotton effects of the same sign. This provides strong evidence that the C-1 and C-5 centers in (−)-II: *Scheme VIII*, are identical to the C-9 and C-13 centers of (−)-morphine.

Miscellaneous Structures.—These compounds are grouped together under this classification because they have no obvious common centers of asymmetry and/or do not bear close structural resemblance to the previously discussed analgesics.

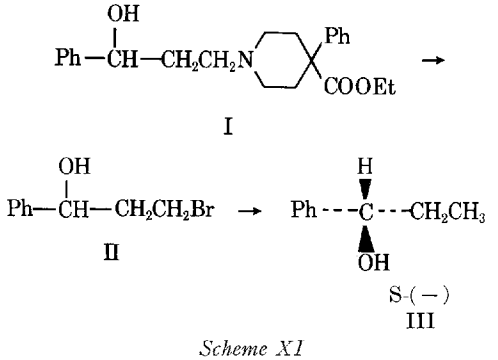
The phenethyltetrahydroquinolines (*Scheme IX*) represent a relatively new class of analgesics having activity in the range of codeine (38). These compounds have been resolved (38, 39)



Scheme IX



Scheme X



Scheme XI

and activity found to reside mainly in one antipode. The absolute stereochemistry of these analgesics has recently been determined by Rheiner and Brossi (39) (*Scheme IX*). The (−)-enantiomers, II and IV: *Scheme IX*, and the (+)-antipode (III) were all converted to the same product (V) by reductive dehalogenation. Reduction of the (−)-nitro compound (I) to the amine followed by deamination also afforded V. Since IV previously (40) had been related to R-(+)-calycotomine (VII) *via* VI, compounds I through IV possess the R-configuration. All of the analgesically more active enantiomers are in the R-series.

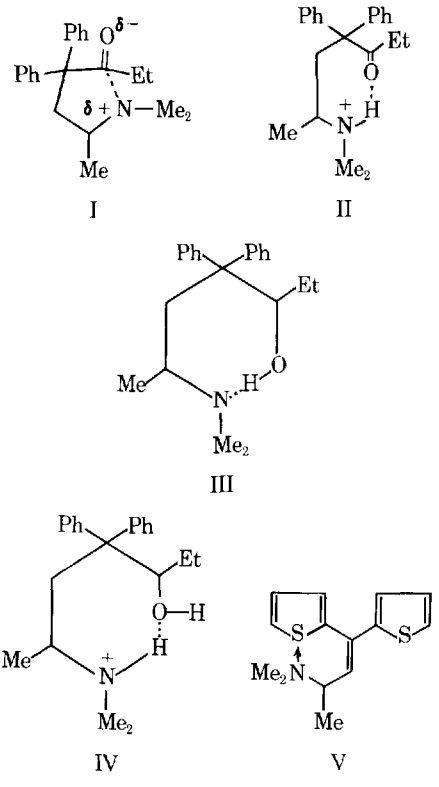
The (−)-enantiomer of *N,N*-dimethyl-1,2-diphenethylamine (I: *Scheme X*) has been reported (41) to be approximately half as active as morphine, while the (+)-antipode is virtually inactive. Nakazaki *et al.* (42) have determined the stereochemistry of I: *Scheme X*, by the procedure shown in *Scheme X*. The amide (III: *Scheme X*), which was derived from the precursor (II) of I, was subjected to exhaustive ozonolysis. The ozonolysis product ultimately was converted to R-aspartic acid (IV). It has been noted (42) that there is a stereochemical resemblance between I and the C-9 asymmetric center of (−)-morphine.

Mazur (43) has resolved the highly potent analgesic, phenoproperidine (I: *Scheme XI*) and found the (−)-isomer to be 4 times more potent than its enantiomer. This suggests that the *N*-aralkyl group is contributing to the pharmacological effect by interacting with a dissymmetric portion of the receptor surface. The configuration of the (−)-isomer was determined by dealkylating with cyanogen bromide to yield the aralkyl bromide (II: *Scheme XI*) and then reducing to the (−)-enantiomer of 1-phenylpropanol (III: *Scheme XI*), whose stereochemistry is known to be in the S-series.

CONFORMATIONAL FACTORS

The relationship between conformational preference and analgesic activity is difficult to assess because differences in conformation *in vivo* can be brought about only by structural variation of the molecule. Since the physical and chemical properties of such compounds may be different enough so that their distribution characteristics are not identical, a knowledge of the relative concentrations of analgesics in the biophase would be needed before differences in potency could be attributed to phenomena related to drug–receptor complex formation. In view of this major drawback, a small difference in potency between diastereomeric compounds, for example, may be difficult to interpret. In spite of the above pitfalls, such correlations appear to be of value, if not for elucidating the optimal conformational requirements for analgesia, then most certainly for determining which conformational species are active.

Open-Chain Analgesics.—It has been postulated (11, 44) that open-chain analgesics such as methadone form ring-like conformations, thereby approximating the over-all geometry



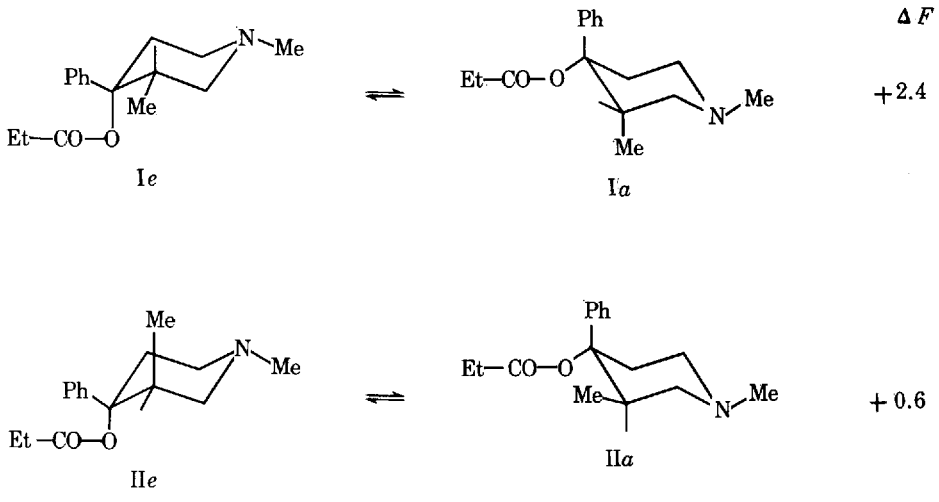
of the piperidine moiety in morphine. Subsequent investigation led Beckett (45) to conclude that such a quasi ring conformation (I) occurs by virtue of an interaction of the basic nitrogen with the carbonyl carbon atom. It has recently been reported by Smith (46) that the NMR spectrum of methadone hydrochloride in chloroform shows magnetically nonequivalent *N*-methyl groups. This has been interpreted as being caused by the molecular asymmetry inherent in the methadone molecule and by intramolecular association between the protonated amine function and the carbonyl oxygen (II). An infrared study of methadol diastereomers has indicated that both the α- and β-isomers (III) are internally hydrogen bonded (13). The protonated forms also are intramolecularly hydrogen-bonded as represented by IV. The α-isomer has been determined to form a stronger hydrogen bond than its diastereomer. (See under *Structures Possessing an Asymmetric Center in Common with Methadone.*) Gero (44) has postulated that thiambutene exists in a conformation (V) which allows intramolecular association between the basic nitrogen and the sulfur atom in the thiophene ring.

Cyclic Analgesics. Cyclic analgesics are restricted to far fewer possible conformations than are open-chain compounds. Moreover, in compounds containing the piperidine ring, the position of equilibria between flip conformational species can be estimated by assuming that the nonbonded interactions are similar to those in cyclohexane.

Among the simplest diastereomeric phenylpiperidine analgesics of known stereochemistry (47–49) are α- and β-prodine (I and II: *Scheme XII*, respectively) (50, 51). β-Prodine is approximately 7 times more potent than the α-isomer. A similar relationship holds for the *N*-phenethyl analogs (52). Estimates of the relative amounts of conformational isomers (I and II: *Scheme XII*) in solution can be obtained if it is assumed that the nonbonded interactions in the solvated free base and cyclohexane are similar. This is a valid assumption in view of the recent report (53) that hydrogen–solvated electron pair and hydrogen–hydrogen interactions differ by only a small factor. It is also reasonably assumed that the *N*-methyl group exists primarily in the equatorial orientation (54) in both flip conformations. The free energy differences between axial- and equatorial-phenyl species have been calculated from average values[2] obtained from the literature (55). It should be emphasized that the ΔF values are approximations, and that small differences ($\Delta\Delta F$) between diastereomers cannot meaningfully be assessed by this method. The ΔF for the conformational equilibrium of the α-isomer (I: *Scheme XII*) has been calculated to be +2.4 Kcal./mole, while that of the β-compound has been estimated to be +0.6 Kcal./mole. This means that the equilibrium for α-prodine is about 98% in the direction of Ie: *Scheme XII*, and that the β-isomer contains approximately 75% of IIe: *Scheme XII*.

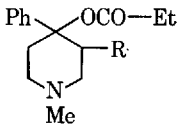
Beckett and Casy (11) have postulated that II: *Scheme XII*, is more active than I: *Scheme XII* by virtue of its greater ability to adopt the axial conformation (IIa: *Scheme XII*) which would be similar to the orientation of the phenylpiperidine moiety in morphine (I: *Scheme VII*). Ziering and co-workers (51) have noted, however, that there is little relationship between stereochemistry and analgesic activity, since other 3-substituted compounds in the prodine series (Table V) do not display parallel activity. The α- and β-isomers of the 3-ethyl compound have about equal activity, and in the 3-allyl analog the α- is more active than the β-isomer. The conformational equilibria of the ethyl and allyl compounds should be comparable to that

[2] The average values employed for ΔF/interaction (in Kcal./mole) are as follows: OCO-Et:H, 0.4; Ph:H, 1.4; Me:H and Me: solvated *N*, 0.9; Me:Me, 3.7; Me:OCO-Et, 2.2.



Scheme XII

TABLE V.—ANALGESIC ACTIVITY OF ISOMERIC PRODINES AND RELATED COMPOUNDS[a]



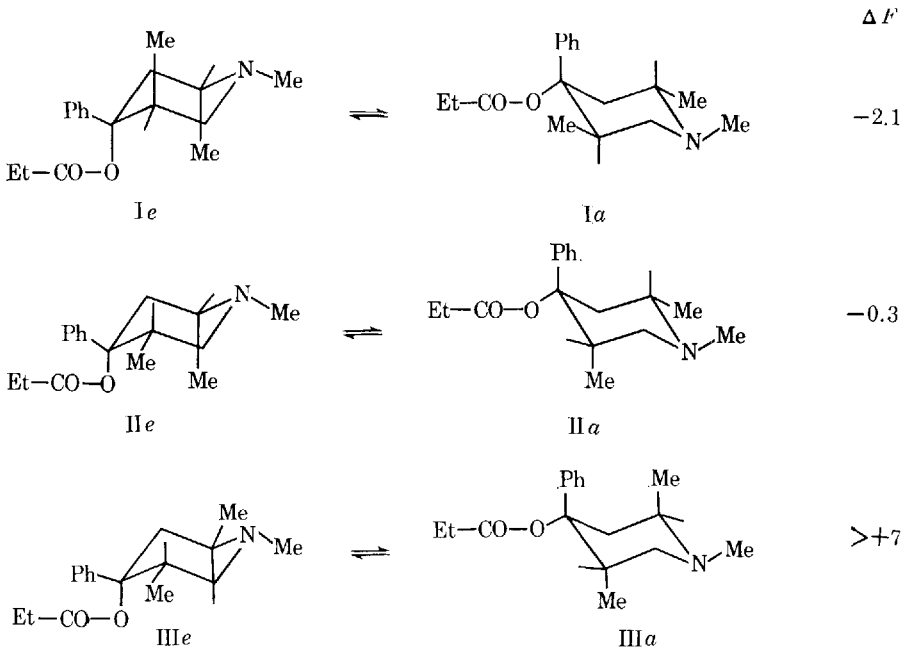
Isomer[b]	R	Relative Activity[c]
α	Me	1.0
β	Me	7.0
α	Et	1.1
β	Et	1.25
α	Allyl	11.0
β	Allyl	3.0

[a] Data from *Reference 51.* [b] α = *trans* Ph:R; β = *cis* Ph:R. [c] Activity relative to meperidine.

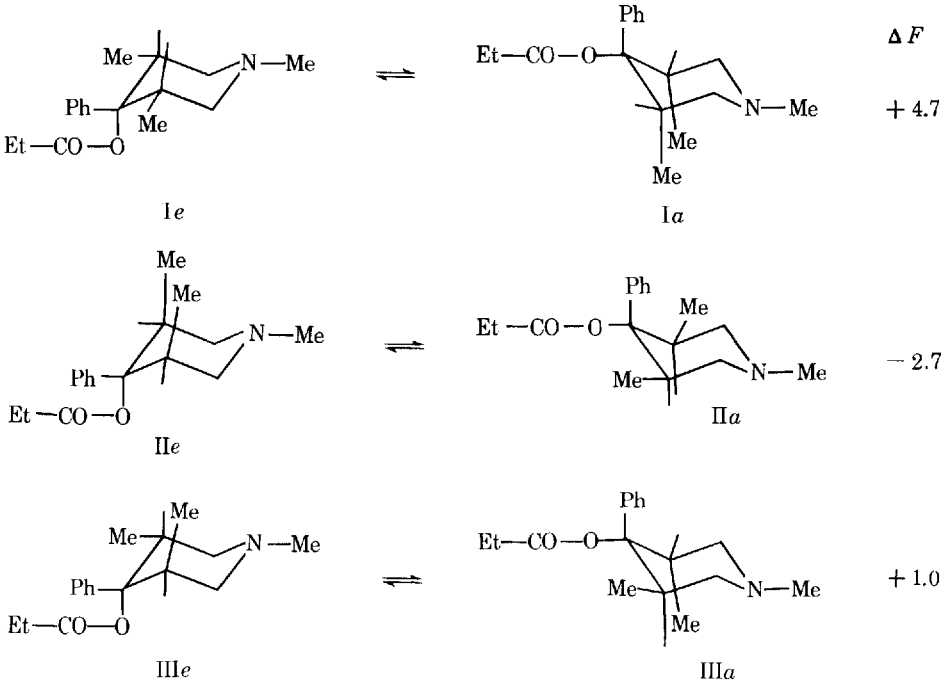
of the 3-methyl diastereomers (I and II: *Scheme XII*), inasmuch as the steric bulk (55) of these groups do not differ substantially. It seems that studies on the distribution and metabolism of these compounds are warranted in order to determine whether the differences in the α/β potency ratio are reflective primarily of events at the receptor level or rather due mainly to concentration differences in the brain. If it is found that differences in activity are related to drug–receptor phenomena, this could also mean that the 3-substituent, rather than the orientation of the phenyl group, exerts a primary influence on drug–receptor association. In any case, it is apparent that an answer to the question of the importance of conformational factors remains highly speculative and awaits experimental clarification.

Nazarov and co-workers (56) have prepared three of the four possible racemates of 1,2,5-trimethyl-4-phenyl-4-propionoxypiperidine. The complete stereochemical assignment of these diastereomeric racemates has been reported (57) to be as illustrated in *Scheme XIII*. [In *Schemes XIII* and *XIV*, ΔF values (Kcal./mole) are approximations only. See *Footnote 2* for information on the calculation of these values.] The γ-isomer (III: *Scheme XIII*), which is known as promedol, exceeds the potency of morphine by about threefold, while the β-racemate (II) is about twice as effective as promedol. The most active compound is the α-isomer (I) which has twice the potency of the β-compound. Calculation of the free energy differences between conformational isomers gives an approximation of the positions of the equilibria. Thus, with the α-, β-, and γ-isomers, the values are −2.1, −0.3, and >+7 Kcal./mole, respectively. This indicates that Ia and IIa: *Scheme XIII* are present to the extent of about 97 and 62%, respectively, while IIIa is virtually absent. It appears therefore that I and II: *Scheme XIII* are exerting their action in both flip conformations and that promedol is acting in the equatorial conformation (IIIe: *Scheme XIII*). The positions of the conformational equilibria correlate with the relative analgesic potency of the diastereomers in that increasing axial character parallels analgesic activity. This is consistent with the ideas expressed by Beckett and Casy (11) in connection with the prodines (I and II: *Scheme XII*), although alternate possibilities for the above correlation, which have already been discussed in relation to the prodines, also should be considered.

The three theoretically possible diastereomers of 1,3,5-trimethyl-4-propionoxy-4-phenylpiperidine (*Scheme XIV*) have been prepared by Sorokin (58). The γ-racemate (III: *Scheme*



Scheme XIII

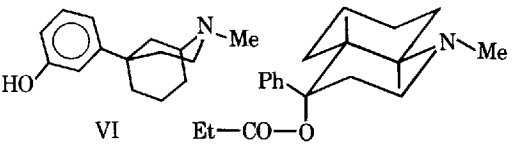


Scheme XIV

XIV) has activity comparable to promedol (III: *Scheme XIII*), whereas the α- and β-isomers are inactive. Conformational analysis indicates no parallelism between potency and the relative amounts of axial-phenyl species in equilibrium with the equatorial form, as has been observed for the prodines (*Scheme XII*) and promedols (*Scheme XIII*). Thus, the inactive β-isomer has a calculated ΔF of −2.7 Kcal./mole, which means that approximately 99% is present as the axial-phenyl conformation (II*a*: *Scheme XIV*). The α-diastereomer, which is also inactive,

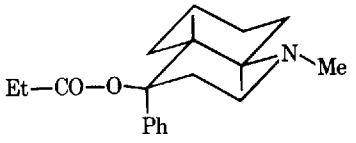
has a value of +4.7 Kcal./mole, which suggests that virtually all of it exists as Ie. On the other hand, the highly potent γ-racemate has a conformational free energy difference (+1 Kcal./mole), which is between the values calculated for I and II: *Scheme XIV*, corresponding to about 15% of IIIa. The preceding analysis suggests that the inactivity of the α- and β-diastereomers (I and II) is related to the 3,5-diequatorial methyl groups which are present in the more stable conformations (Ie and IIa). The active γ-diastereomer (III) is incapable of disposing both the 3- and 5-methyl groups in a similar orientation when in a chair conformation. If the inactivity of I and II are reflective of phenomena at the receptor level, this may be caused by steric hindrance of the 3,5-diequatorial methyl groups to analgesic–receptor association (59).

Although it cannot be stated unequivocally that variation in potency among diastereomeric structures is a direct consequence of the analgesic–receptor interaction, it has been suggested by conformational analysis of flexible phenylpiperidine diastereomers (*Schemes XII–XIV*) that both equatorial- and axial-phenyl conformations have the ability to produce high analgesic activity when other groups on the piperidine ring do not prevent drug–receptor association. Molecules having structural features which prevent conformational inversion can furnish further insight into the nature of the pharmacophoric species. It is well known, for example, that morphine (I: *Scheme VII*), morphinans (I: *Scheme VIII*), and benzomorphans (II: *Scheme VIII*) are all conformationally homogeneous by virtue of the methylene bridge which connects the axial aromatic group to the piperidine ring. The equatorial counterpart to the above compounds is found in the azabicyclononane derivative (VI) (60). The trimethylene bridge prevents conformational inversion and therefore precludes the presence of an axial aromatic ring. This compound has activity comparable to the phenolic benzomorphan structure (II: *Scheme VIII*; R = R^1 = R^2 = Me, X = OH). In a recent study, Smissman and Steinman (61) have prepared two isomeric decahydroquinoline analogs (VII and VIII) of the prodine type analgesics. The *trans* ring juncture in these structures prevents inversion of the piperidine moiety and, thereby, ensures conformational homogeneity. Since both the equatorial (VII) and axial (VIII) isomers were equally potent, it was concluded that no definite conformational requirements of the aromatic ring are necessary for analgesic activity. This is inconsistent with



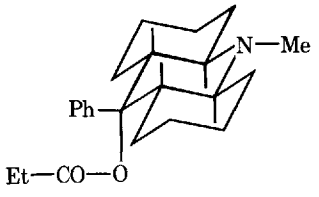
VI

VII



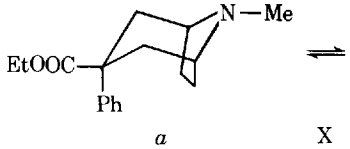
VIII

Beckett's hypothesis (11) which states that analgesics containing an axially oriented aromatic ring should be more potent than those possessing the equatorial conformation. The perhydroacridine analog (IX) was also prepared (62) and found to be inactive. This has been attributed to steric hindrance of the carbocyclic moieties attached to both sides of the piperidine ring, and is consistent with the results obtained from conformational analysis of I and II: *Scheme XIV*.

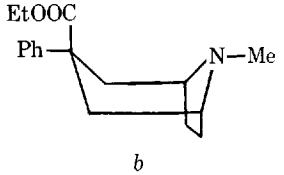


IX

The tropane analog (X) of meperidine was prepared by Bell and Archer (63) and found to be slightly more potent than meperidine (XI). Although inversion of the piperidine moiety cannot occur because of the restriction imposed by the ethylene bridge, spectral evidence (63, 64) suggests that there is a substantial amount of the boat conformation (X*b*) present. This is to be expected in view of the severe diaxial interactions between the phenyl group and the



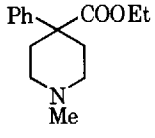
a X



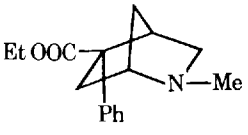
b

ethylene bridge when in the chair conformation (X*a*). Unequivocal evidence demonstrating that

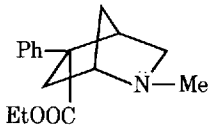
a boat conformation is active has been provided by Portoghese and co-workers (65), who have synthesized and tested bicycloheptane analogs (XII and XIII) of meperidine. In these diastereomers, the piperidine ring is rigidly held in a boat conformation by the C-7 methylene group. The *endo*-phenyl compound (XII), which is about 6 times more potent than the corresponding *exo*-isomer (XIII), is about twice as active as meperidine (XI). The difference in activity between isomers may be due in part to distribution since XIII is a stronger base than XII and hence would not be expected to reach the site of action in the same concentration as its isomer.



XI



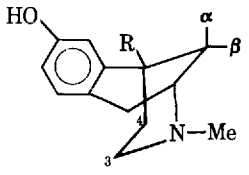
XII



XIII

From all the available data it appears that the conformational requirements for most of the 4-phenylpiperidine type analgesics are minimal. It is rather paradoxical that in certain cases high optical selectivity of the analgesic receptors is observed, whereas a variety of compounds in different conformations are capable of producing analgesia. This paradox can be resolved if it is assumed that differing modes of analgesic–receptor binding (28) occur. (See under *Concepts on Analgesic–Receptor Interactions.*)

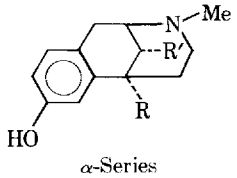
In the preceding discussion the possibility was considered that disposition of groups other than the aromatic ring could also influence analgesic activity. Thus, the activity of prodine-type diastereomers (*Schemes XII–XIV*) could depend in part on the orientation of alkyl groups attached to the piperidine ring. In order to limit the number of variables, it would be informative to examine the effect of a configurational change at a single asymmetric center in analgesics whose geometry is largely restricted to a single conformation. Inasmuch as such compounds are epimeric rather than enantiomeric, it should be mentioned that the observed differences in activity between epimers cannot unequivocally be attributed to events at the receptor level when differences in potency are small. Much of the work on compounds which fall into this category has been carried out by May and co-workers (66–70), who have prepared a variety of epimeric benzomorphans. The conclusions derived from these correlations cannot be extrapolated to the prodines, however, because the modes of interaction of the benzomorphans and the former compounds are most probably different. (See under *Concepts on Analgesic–Receptor Interactions.*) The data presented in Table VI show that compounds in the β-series are consistently more potent than the corresponding α-isomers. Furthermore, it appears that when R = R′ = propyl, the α-compound shows a large decrease in potency while the β-epimer exhibits a relatively small decrease. It can be seen from the three-dimensional representation (XIV) of these isomers



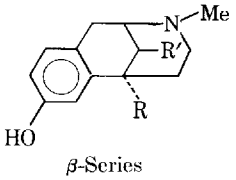
XIV

that the α-series possesses an equatorial C-9 alkyl group, whereas the isomeric β-series has

TABLE VI.– ANALGESIC ACTIVITY OF ISOMERIC BENZOMORPHANS



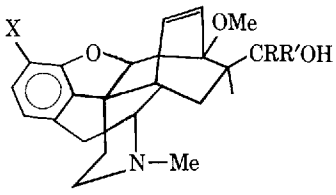
α-Series



β-Series

α-Series ED_{50}	R	R′	β-Series ED_{50}[a]	Ref.
3.0	Me	Me	0.44	(66)
4.9	Et	Me	0.07	(66, 68)
1.5	Me	Et	0.47	(66, 68)
4.2	Et	Et	0.28	(66, 67, 69)
2.9	Pr	Me	0.12	(66, 70)
71.2	Pr	Pr	0.87	(66, 70)

[a] mg./Kg. subcutaneously in mice.



XV

this group oriented in the axial conformation. This suggests that when the C-9 substituent is in the equatorial conformation and beyond a certain size, it may adversely affect drug–receptor association. An equatorial C-9 group would be expected to have little effect on analgesic activity if association with a receptor involved contact only with the C-3,4 hydrocarbon moiety and aromatic ring. Since this is not the case, it appears as if other portions of the molecule are also involved in the receptor interaction. A somewhat related situation exists in certain highly potent analgesics derived from Diels-Alder adducts of thebaine (XV) (71, 72). The —CRR'OH group, which is on the top face of the molecule, can enhance analgesic activity by a factor of up to 7800 times the potency of morphine. Moreover, it has been reported that when the carbinol group is asymmetric (XV; R = Me, R′ = Pr, X = OMe), the activity of one of the isomers is about 90 times that of morphine and approximately 130 times more potent than its epimer. This remarkable difference in potency is probably related, in some way, to the ability of CRR'OH in one of the epimers to enhance receptor binding when in a preferred conformation.

CONCEPTS ON ANALGESIC–RECEPTOR INTERACTIONS

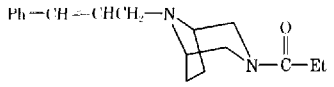
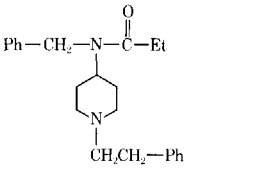
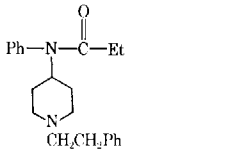
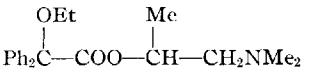
Since isolation and visualization of narcotic analgesic receptors presently is not possible, the medicinal chemist is naturally dependent on the relationship between molecular structure and analgesic activity in order to obtain some insight into the nature and dimensions of such receptors.

The process of determining the pharmacophoric groups necessary for analgesic action evolved slowly and can be traced back to Whalen (73), who in 1902, proposed that the properties of morphine (I: *Scheme VII*) were due to the phenanthrene skeleton. This idea prevailed (74) until 1939, when Eisleb and Schaumann (75) discovered that meperidine (XI) possessed substantial analgesic activity. The structural relationship between morphine and meperidine was realized and it was postulated (76) that the 4-phenylpiperidine moiety was necessary for analgesic activity. With the advent of the methadones (Table X, series 1 and 2) (77), Schaumann (78) modified and generalized his hypothesis by suggesting the structural requirements to be an aromatic ring attached to a quaternary carbon atom two carbons removed from a tertiary amine function. The requirements for analgesic activity, however, were once again outmoded with the appearance of the thiambutenes (Table X, series 5) (4, 79). Gero (44) attempted to rationalize the activity of the thiambutenes and methadones by postulating that these open-chain analgesics formed pseudo-ring conformations.

Beckett and Casy (11) sought to elucidate the receptor requirements for analgesic activity through stereochemical studies of the methadones and thiambutenes. The fact that the more active enantiomers of some of these structures were found to be configurationally related, supported the idea that "fit" at an analgesic receptor is important for activity. A receptor surface was formulated whose dimensions were complementary to certain elements of the phenylpiperidine moiety in morphine. Hence, it was postulated that an analgesic receptor possessed a flat surface, a cavity, and an anionic site which were envisaged to accommodate an aromatic ring, a hydrocarbon moiety, and a protonated basic nitrogen, respectively. These features were depicted as being in a particular sequence, the active enantiomers being capable of three-point contact while the inactive or less active enantiomers were capable of presenting only two of the three essential groups for orientation at the receptor surface. According to this concept, specific orientations of the various pharmacophoric groups in an analgesic molecule are required in order that they may conform to the above receptor dimensions. It was suggested that meperidine and the prodines (*Scheme XII*) were able to associate with this receptor with greater facility when in the axial-phenyl conformation and that the methadones interact by assuming a cyclic conformation. Subsequent studies on the dissociation constants of methadone-type compounds led Beckett (45) to conclude that methadone and related compounds form ring-like conformations by virtue of an interaction of the basic nitrogen with the carbonyl carbon atom. It was concluded (80) from correlations of analgesic activity with the widths of the basic groups, that the anionic site has a width of 7.5–8.5 Å.[3]

[3] This is based on the assumption that the distribution and metabolism of the methadones containing different basic groups are approximately the same. If this is indeed found to be the case by determining the concentration of these compounds in the brain, then their correlation would receive much stronger support.

TABLE VII.—SOME POTENT ANALGESICS OF DIVERSE CONSTITUTION

Compd.	Activity[a, b]
	10 (82)
	2.3 (83)
	~2000 (84)
	5 (85)

[a] Relative to meperidine. [b] References in parentheses.

As more compounds were synthesized and found to possess analgesic activity, it soon became evident that the requirements which were summarized by Braenden, Eddy, and Halbach (60) in 1955 were once again violated (81). It is now known, for example, that there may be as many as five atoms between the aromatic ring and basic nitrogen and still have compounds which are at least as potent as meperidine. Some structurally diverse analgesics, to list only a few, are compiled in Table VII. Other radical departures recently have been reported (86). It is apparent that this presents quite a perplexing problem. Can all of these structures fit a receptor surface having dimensions which have been postulated (11) to be complementary to portions of the morphine molecule while still maintaining high activity? It seems quite probable that this is not the case. Other aspects of the relationship between structure and analgesic activity which are not adequately explained by the Beckett hypothesis are found in Table III, where it can be seen that there is no consistent correlation between the configuration of the more active enantiomers and analgesic activity. It is obvious that the constitution of the R moiety has an important bearing on the configurational selectivity of the receptors. Still another puzzling phenomenon was the ability of identical *N*-substituents to either enhance or diminish analgesic activity when attached to different analgesiophores.[4] For example, replacing the *N*-methyl group in meperidine by the cinnamyl substituent enhances activity by thirty to forty-fold while an identical change in morphine causes a loss of potency (see Table VIII). Along these same lines, it is well known that replacement of the *N*-methyl in morphine by an allyl group results in a compound which has low activity in rodents and morphine antagonist properties. Significantly, similar replacement in meperidine (Table VIII) or in methadone-type analgesics (Table X) results neither in a drastic diminution of potency nor in a compound which has antagonistic properties (87).

Quite recently, Portoghese (28) has introduced a new concept on the mode of interaction of narcotic analgesics with receptors in order to explain all of the above phenomena. It has been postulated that complex formation of different narcotic analgesics with receptors may, in many cases, involve differing modes of interaction rather than a single type of drug–receptor interaction involving binding to the same sites on the receptors. The possibility of induced fit as a factor contributing to receptor binding of diverse analgesics was also recognized (2, 28). Within the framework of this concept the possible modes of interaction were outlined as follows.

Case 1.—Interaction of different analgesics with a single species of receptors; (*a*) identical interaction; (*b*) differing interaction.

Case 2.—Interaction of different analgesics with two or more species of receptors common to the different analgesics; (*a*) identical partitioning on the receptors by different analgesics, (*b*) dissimilar partitioning on the receptors by different analgesics.

Case 3.—Interaction of different analgesics with two or more species of receptors not common to the different analgesics.

Different molecules may interact with identical sites [Case 1(*a*)] or with different sites [Case 1(*b*)] on the same receptor species. Case 1(*b*) is schematically illustrated in Fig. 1. The molecule outlined by the solid line depicts one position of binding, while the dashed line denotes a second position. It is assumed that the steric environment presented to different molecules in different binding positions are not identical.

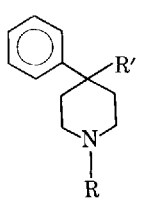
The second case is symbolized by the following equations:

$$\begin{aligned} A + \alpha + \beta &\rightleftharpoons (A\alpha) + (A\beta) \\ B + \alpha + \beta &\rightleftharpoons (B\alpha) + (B\beta) \end{aligned}$$

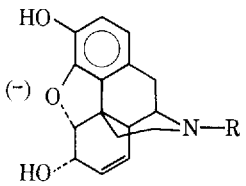
Different analgesics (A and B) and species of receptors (α and β) common to A and B may interact so that the ratios, $(A\alpha):(A\beta)$ and $(B\alpha):$

[4] Analgesiophore is defined as the analgesic molecule less the substituent on the basic nitrogen.

TABLE VIII.—RELATIVE ANALGESIC ACTIVITY OF STRUCTURES CONTAINING THE PHENYLPIPERIDINE MOIETY[a]



1,R′ = CO_2Et
2,R′ = OCOEt
3,R′ = OCOMe



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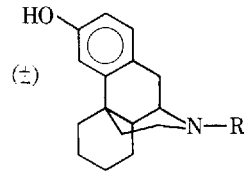

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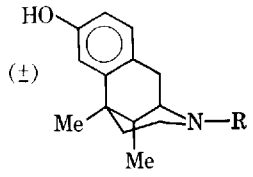


6

R	Relative Activity[b] 1[c,d,e] H.P.[l]	1[c,d,e] R.H.[m]	2[c,f] H.P.[l]	2[c,f] R.H.[m]	3[c,f] H.P.[l]	3[c,f] R.H.[m]	Relative Activity[h] 4[g] R.H.[l]	5[i,j] H.P.[l]	6[j,k] H.P.[l]
Me	1.0	1.0	7.4	26	1.0	2.4	1.0	2	0.7
Et	...	0.5	...	...	...	...	<0.1	0.1	0
n-Pr	...	1.5	...	...	...	...	0	...	0
Allyl	...	0.8[n]	...	...	...	...	<0.1	0	...
n-Bu	...	1.5	...	...	...	...	<0.1	...	0
n-Amyl	...	1.5	...	...	...	...	0.7	~2	~1
$C_6H_5CH_2$	<0.3	<0.5	1.5	1.4	1.0	1.1	<0.1	0	...
	0.15	0.32	3.8	...	...	...	...	...	...
$C_6H_5(CH_2)_2$	2.3	2.6	25	110	12	60	6	~10	~10
	2.7	...	66	69	66	72	...	...	...
$C_6H_5(CH_2)_3$	23	20	162	572	62	265	...	...	0.15
	27	18	318	637	90	142	...	...	...
$C_6H_5(CH_2)_4$	1.6	2.8	54	108	32	39	...	~0.3	...
$C_6H_5CH{=}CHCH_2$	32	40	261	1100	82	376	<0.1	0	...
	61	39	650	785	...	189	...	...	...

[a] Adapted from *Reference 28*. [b] Analgesic activity relative to meperidine; a value of 10 signifies the compound is 10 times more potent than the reference compound. [c] *Reference 90*. [d] Thorpe, R. H., and Walton, E., *J. Chem. Soc.*, **1948**, 559. [e] Elpern, B., Gardner, L. N., and Grumbach, L., *J. Am. Chem. Soc.*, **79**, 1951(1957). [f] Elpern, B., Wetterau, W., Carbateas, P., and Grumbach, L., *ibid.*, **80**, 4916(1958). [g] Winter, C. A., Orahovats, P. D., and Lehman, E. G., *Arch. Intern. Pharmacodyn.*, **110**, 186(1957). [h] Analgesic activity relative to morphine in the same sense as in *Footnote a*. [i] Eddy, N. B., Besendorf, H., and Pellmont, B., *Bull. Narcotics, U. N. Dept. Social Affairs*, **10**, 23(1958). [j] *Reference 33*, p. 157. [k] Ager, J. H., and May, E. L., *J. Org. Chem.*, **25**, 984(1960). [l] Hot plate method using mice. [m] Rat tail radiant heat method. [n] Administered intraperitoneally.

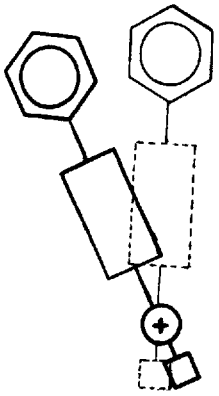


Fig. 1.—A schematic representation of Case 1(*b*). The protonated amine nitrogen is represented by ⊕, the square denotes an *N*-substituent, and the rectangle depicts another portion of the molecule. The different positions of molecular binding are represented by the heavy and dashed lines.

(Bβ), are similar [Case 2(*a*)] or different [Case 2(*b*)].

The third possibility is illustrated by the equations below. In this case α and β are not common to A and B. If different receptor species

$$A + \alpha + \beta \rightleftharpoons (A\alpha)$$
$$B + \alpha + \beta \rightleftharpoons (B\beta)$$

have dissimilar steric requirements in Cases 2(*b*) and 3, then this would be manifested by a difference in the stereoselectivity of the receptors for analgesics A and B.

Combinations of the above cases may also exist, thus creating a much more complex situation. It is probable that Cases 1 and 2 may be the most prevalent types of interaction.

This concept is capable of explaining the lack of correlation between configuration and analgesic activity (Table III). If, for example, methadone (Table III, *1*) and α-methadol (Table III, *5*) are interacting with different patterns of sites on a single species of receptors [Case 1(*b*)] then the steric requirements for the analgesic molecules may not be identical. The fact that the more active enantiomers of the above compounds possess the opposite configuration supports the contention that at least a portion of these analgesic molecules are in different physicochemical environments on the receptors. Figure 2 illustrates schematically how R-methadone and (3S:6S)-methadol, with opposite configuration at C-6, may interact with analgesic receptors. Dipoles conceivably can be sites which are hydrogen bonding donors (X) or acceptors (Y). Interaction of methadone with an analgesic

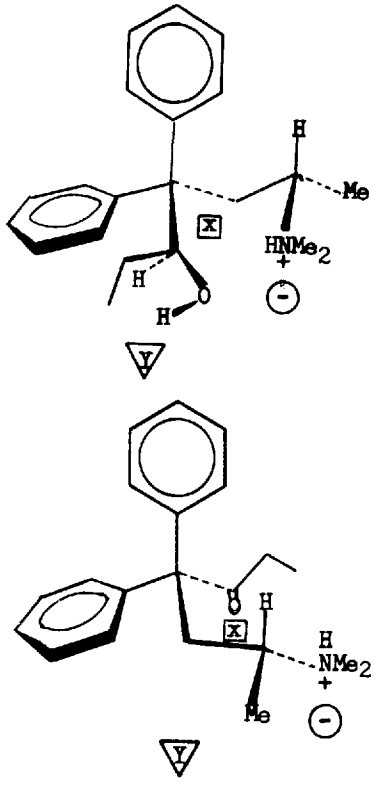


Fig. 2.—One possible mechanism whereby different polar groups in analgesic molecules may cause inversion in the configurational selectivity of analgesic receptors. Hydrogen bonding proton donor and acceptor dipoles are noted by X in square and Y in triangle, respectively. The anionic site is represented by ⊖. Top: (3S:6S)-methadol; bottom: R-methadone.

receptor may involve hydrogen bonding of the ketonic carbonyl group by X, whereas with α-methadol, OH...Y could occur. According to the above interpretation, alteration of a polar group in an analgesic molecule may afford a compound which can interact with dipolar sites and hydrophobic areas that differ from those involved in the binding of the unaltered structure. An alternate explanation can be found in Case 2(*b*). In this case, methadone and α-methadol would interact in different ratios with two or more species of receptors having dissimilar steric requirements. This too could bring about an inversion in configurational selectivity if the steric requirements of the different receptor species common to both analgesics are dissimilar.

The well-known ability of the basic group to influence analgesic activity has been utilized as a means of detecting similarities or differences in the mode of binding to receptors (28, 88). If the mode of interaction between various analgesics and receptors is similar [Cases 1(*a*) and 2(*a*)], then the *N*-substituent should be positioned in a similar physicochemical environment on the receptors and, therefore, contribute to the analgesic effect quantitatively in the same way. Thus, if identical changes of the *N*-substituent in two or more series of compounds produce parallel changes in potency, the mode of binding of the different analgesiophores should be similar. Conversely, dissimilar modes of interaction [Cases 1(*b*), 2(*b*), and 3] should produce nonparallel changes in potency. This is exemplified in Table VIII where parallel relationships can be seen between the meperidines (series 1) and the acyloxy analogs (series 2 and 3). Parallelism is also exhibited among the compounds in the morphine (series 4), morphinan (series 5), and benzomorphan (series 6) series. Comparison of the former (series 1, 2, and 3) with the latter (series 4, 5, and 6), however, shows that there is no parallelism. The correlations in Table VIII indicate that the analgesiophores in series 1, 2, and 3 are binding to receptors by similar modes and that an analogous situation exists among the latter series. On the other hand, lack of parallelism between the phenylpiperidines and structures related to morphine suggested that the binding mode of identically substituted compounds in the former series is different from those in the latter.

If identically *N*-substituted compounds in two different series are interacting with receptors in a similar manner, then the quantitative contribution to the analgesic effect by various substituents should produce, under steady-state conditions, proportionate variations of activity in both series. Such a proportionality is reflective of a linear free energy relationship. The slope of such a regression should be near unity, since identical basic groups are expected to contribute to the pharma-

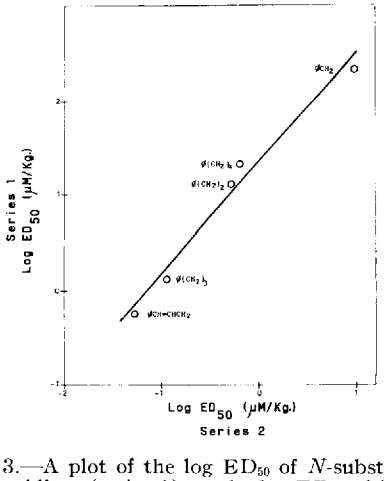
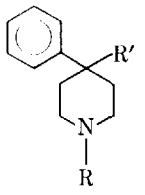


Fig. 3.—A plot of the log ED_{50} of *N*-substituted normeperidines (series 1) *vs.* the log ED_{50} of identically substituted reversed esters (series 2).

TABLE IX.—REGRESSION ANALYSIS OF THE LOGARITHM OF THE ANALGESIC ACTIVITY IN VARIOUS *N*-SUBSTITUTED PHENYLPIPERIDINE SERIES[a]

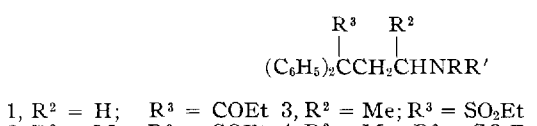


1, R′ = CO_2Et
2, R′ = OCO—Et
3, R′ = OCO—Me

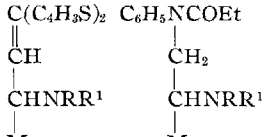
Correlation[b]	Slope[c]	S.E.	S.D.	r[d]	n[e]	Data Source[f]
1 *vs.* 2[i]	1.20	0.09	0.16	0.99	5	*g*
	1.01	0.13	0.25	0.96	6	*h*
1 *vs.* 3[j]	0.93	0.24	0.37	0.90	4	*g*
	0.87	0.21	0.42	0.87	6	*h*
3 *vs.* 2[k]	1.09	0.20	0.27	0.96	4	*g*
	1.06	0.19	0.25	0.94	5	*h*

[a] Adapted from *Reference 28*. [b] Series 1, 2, and 3 were plotted as the logarithm of the activity (μm./Kg.). [c] Values were calculated by the method of least squares. [d] Represents the linear correlation coefficient; when $r = 1$ there is a perfect correlation; if $r = 0$ there is no correlation. [e] Denotes the number of points in the regression. [f] All data were obtained from *Reference 90*; mice were the test animals. [g] Eddy's data. [h] Janssen's data. [i] R=φCH=CHCH₂; φ(CH₂)₃; φ(CH₂)₂; φ(CH₂)₄; φCH₂ · R=φCH(OCOEt)CH₂CH₂; φCH=CHCH₂; φ(CH₂)₃; φCH=CH(CH₂)₂; CH₃. [j] R=φ(CH₂)₃; φ(CH₂)₂; φ(CH₂)₄; φCH₂ · R=φCH(OAc)CH₂CH₂; φCH=CHCH₂; φ(CH₂)₃; φ(CH₂)₂; φCH=CH(CH₂)₂; CH₃. [k] R=φ(CH₂)₃; φ(CH₂)₂; φ(CH₂)₄; φCH₂ · R=φCH=CHCH₂; φ(CH₂)₃; φ(CH₂)₂; φCH=CH(CH₂)₂; CH₃.

TABLE X.—RELATIVE ANALGESIC ACTIVITY[a,b] OF STRUCTURES RELATED TO METHADONE[c]



1, R^2 = H; R^3 = COEt 3, R^2 = Me; R^3 = SO_2Et
2, R^2 = Me; R^3 = COEt 4, R^2 = Me; R^3 = CO_2Et



R	R^1	1[d]	2[d]	3[d]	4[d]	5[e]	6[f]
Me	Me	1.2[g], 2.5[g,h]	7.8,[g] 5.6[g,h]	6	1.3,[g] 0.55[h]	5	0
Me	Benzyl	0, 0[h]	...	..	...	<0.1	1.4[i]
Et	Et	0.3,[g] 0.82[h]	8.1	..	...	5	0
n-Pr	*n*-Pr	<0.33, <0.25[h]	...	..	...	<0.1	...
Allyl	Allyl	0.5[j]	...	..	...	0.7	...
C_4H_8		4.0, 1.9[g,h]	4,[k] 5.7[g,h]	..	...	3.5	0
C_4H_8O		7.0, 8.5[g,h]	19,[g] 4.5[g,h]	..	<0.1[h]	1	0
C_5H_{10}		2.6,[g] 2.5[g,h]	20, 5.4[g,h]	6	0.2[h]	5.5	0

[a] Analgesic activity relative to meperidine; a value of 10 signifies the compound is 10 times more potent than the reference compound. [b] Unless otherwise specified compounds were administered subcutaneously to rats. [c] Adapted from *Reference 28*. [d] Data were obtained from Janssen, P. A. J., "Synthetic Analgesics," Part I, Pergamon Press, Inc., New York, N. Y., 1960, Table V, p. 63. [e] Values were calculated from *Reference 4*. [f] With the exception of the methyl benzyl analog, the above compounds were inactive at 25 mg./Kg. The authors thank Dr. W. Wright, Jr., Lederle Laboratories, Pearl River, N. Y., for providing this information. [g] Average value. [h] Mice were employed as test animals. [i] Calculated from *Reference 15*. [j] Animal species not revealed. [k] Administered intraperitoneally.

cological effect by the same mechanism. The above quantitative relationship is, of course, dependent on the assumption that identical changes in substituents on two different analgesiophores will affect the distribution of the compounds in a similar fashion. This assumption is quite reasonable in view of the successful application of substituent constants for predicting drug availability at the site of action (89). When the mode of binding is not similar, a nonparallel relationship should be obtained which may be characterized by point scattering and the absence of a regression.

It is important to point out that a regression cannot be properly constructed unless the pharmacological data have well-defined confidence limits and are derived from a single source. The analgesic data of Janssen and Eddy (90) appeared to fulfill these requirements. The regressions obtained from these data are shown graphically (Fig. 3) and in Table IX. The high correlation coefficients (r) corroborate the postulate that parallelism in activity is indicative of similar modes of binding and that this concept (28, 87) is of utility in distinguishing between similar [Cases 1(*a*) and 2(*a*)] and different modes [Cases 1(*b*),

2(*b*), or 3] of drug–receptor interactions. Although series 3, 4, and 5 (Table VIII) also display parallelism, these data cannot be quantitated because the activities were obtained from different literature sources.

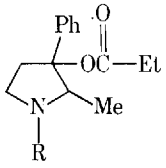
It was discussed earlier that inversion of configurational selectivity is indicative of differences in the mode of interaction between analgesic molecules and receptors. Further support for this proposal was obtained by qualitatively assessing whether parallelism exists between different series of open-chain compounds (Table X) whose *N*-substituents were varied in the same way. Because these data were obtained from a variety of sources, only substantial changes in potency were interpreted as being meaningful.

Qualitative inspection suggests that series 1, 2, 3, and 5 exhibit a roughly parallel variation of analgesic activity. This has been interpreted as being reflective of similar modes of interaction with receptors [Cases 1(*a*) or 2(*a*)] and is consistent with the fact that, among the series containing an asymmetric center (series 2, 3, and 5) the more active enantiomers are configurationally related to R-alanine (Table III). Since series 4 and 6 (Table X) show little correlation with series 2, 3, and 5, the mode of interaction of compounds in the former series is probably different from those in the latter. Significantly, the more active enantiomers (series 4 and 6) possess the S-configuration. In the above case, dissimilar binding modes are characterized by both inversion in the stereoselectivity of the receptors and by nonparallel variations in activity. It is important to realize that an identical stereochemical relationship between more active enantiomers does not necessarily imply that analgesic molecules are interacting with receptors in a similar fashion, since this may be coincidental. A more rigorous procedure would, in addition, involve the correlative procedures discussed above. For example, the more active enantiomers of the carbethoxy analog of methadone (series 4; R = R^1 = Me) and the basic anilide compounds (series 6; R = Me, R^1 = benzyl) possess identical configuration but variation of the *N*-substituent does not appear to affect analgesic activity in the same way. This is suggestive of different binding modes despite the fact that the more active optical isomers have the same configuration.

Similar analysis on phenolic and nonphenolic morphinans (I: *Scheme VIII*) and benzomorphans (II: *Scheme VIII*) has also been carried out by the substituent variation method (28). The phenolic compounds show an enhancement of activity on replacing an *N*-methyl with a phenethyl group, while the nonphenolic structures exhibit a decrease in activity when an identical change is made (81). This suggests that the phenolic and nonphenolic compounds are binding to analgesic receptors by different modes. Lack of correlation with other series is also seen among the tetrahydroisioquinoline analgesics (*Scheme IX*), where it has been reported (91) that replacement of the *N*-methyl group by a variety of substituents causes a loss of activity. The pyrrolidine analgesics (Table XI) exhibit a pattern of activities on substituent variation, which is unlike its close relative, the prodines (92). Nonparallelism is also found in the highly potent benzimidazole analgesics (XVI) (93) where variation of the basic group causes changes in activity unlike those seen in other types of analgesic compounds. All of the above phenomena can be rationalized in terms of differing modes of drug–receptor binding. These possibilities include Cases 1(*b*), 2(*b*), and 3, which were described earlier.

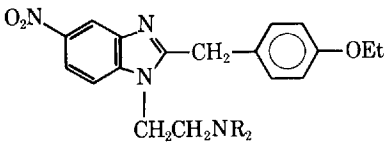
It has also been mentioned (28) that differing modes of interaction were likewise possible among compounds in a single series. In such a case, the binding mode of an analgesiophore would be modified when the basic group is changed. One criterion for detecting transitions in the mode of binding is a large change in enantiomeric potency ratio. It has been shown (18) recently that as the number of methylenes in the *N*-aralkyl group of the basic anilide analgesics (Table XII) is increased from one to three, the enantiomeric potency ratio approaches unity. These marked

TABLE XI.—PYRROLIDINE ANALGESICS[a]



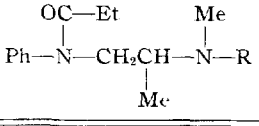
R	Activity[b]
Me	0.8
Ph—CH_2CH_2	0.4
Ph—CH=CH—CH_2	0.4
H_2N—C$_6$H$_4$—CH_2CH_2	1.3
Ph—$CH(OH)CH_2CH_2$	0.8

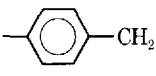
[a] *Reference 92.* [b] Relative to codeine (base/base), 30 min. after i.p. injection.



XVI

TABLE XII.—RELATIONSHIP OF LENGTH OF THE *N*-ARALKYL GROUP ON CONFIGURATIONAL SELECTIVITY OF ANALGESIC RECEPTORS[a]



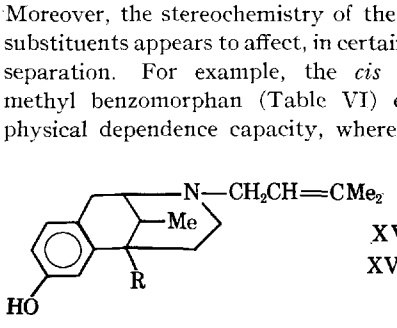
R	Enantiomer[b]	Configuration	AD_{50}[c]
Ph—CH_2	(+)	(S)	4.3[d]
	(−)	(R)	Inactive at 50
Me—  —CH_2	(+)	(S)	1.6
	(−)	(R)	Inactive at 50
Ph—CH_2CH_2	(+)	(S)	3.6[d]
	(−)	(R)	11.7
Ph—$CH_2CH_2CH_2$	(+)	(S)	8.9
	(−)	(R)	11.9

[a] Adapted from *Reference 18*. [b] Optical rotation of the free base. [c] The subcutaneous dose which elevates the rat tail radiant heat response time by 100% in 50% of the animals. [d] *Reference 16*.

changes in potency ratio, which were observed on increasing the chain length, represent a decrease in the stereoselectivity of the receptors which is attributed to differing modes of analgesic–receptor binding [possibilities are Cases 1(*b*), 2(*b*), or 3].

Archer (94, 95) has reported a significant observation regarding the effect of stereochemistry on analgesic antagonist activity. The *cis* and *trans* 5,9-dimethyl (XVII) and the *cis*-5-ethyl-9-methyl (XVIII) compounds all show antagonism against narcotic analgesics, while *trans* XVIII is a meperidine antagonist but does not antagonize morphine. This suggests that the mode of interaction of *trans* XVIII differs from that of the other dimethallyl benzomorphans and provides support that more than one receptor species (Cases 2 or 3) may be involved in analgesic action.

It is conceivable that the great variability in the physical dependence associated with various narcotic analgesics could be related to differing modes of interaction. May (66, 70) has shown that analgesic activity can be separated from physical dependence by merely altering the 5,9-dialkyl substituents in *N*-methylbenzomorphans. Moreover, the stereochemistry of the 5,9-dialkyl substituents appears to affect, in certain cases, this separation. For example, the *cis* 5-propyl-9-methyl benzomorphan (Table VI) exhibits no physical dependence capacity, whereas the corresponding *trans* isomer shows high physical dependence capacity (70).



XVII, R = Me
XVIII, R = Et

CONCLUDING REMARKS

Modification of the pain threshold by strong analgesics is a complex phenomenon which is not well understood. The large variety of structurally unrelated compounds which possess morphine-like activity attests to the complexity of this phenomenon. The structural diversity of compounds having high analgesic potency may be due to a combination of factors. Induced fit (2, 28, 94, 96) and differing modes of analgesic-receptor association (2, 28) have been discussed as possible contributing causes for the apparent overall lack of consistency in the relationship between structure and activity. For this reason the topographical characteristics of analgesic receptors remain obscure. The problem of delineating the geometry of analgesic receptors and ideally, elucidating the chemical components which comprise such entities, will challenge the best efforts of the medicinal chemist for years to come.

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Pharmacology and Mechanism of Action of Cryptenamine

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Cryptenamine, an alkaloidal preparation from *Veratrum viride*, was investigated for hypotensive activity. Simultaneous bilateral denervation of the carotid sinus-body complex and bilateral vagotomy abolished the effects of cryptenamine. Adrenalectomy or pretreatment of the animals with *N,N*-di-isopropyl-*N'*-isoamyl-*N'*-diethylaminoethylurea (P-286), bretylium, reserpine, or α methyldopa abolished or markedly inhibited the hypotensive effects of cryptenamine, whereas guanethidine, which does not induce depletion of catecholamines from the adrenal medulla, failed to block the hypotensive effects of cryptenamine. Central depressor effects of cryptenamine were not abolished either in dog cross-circulation preparations or in the perfused lateral ventricle preparation of the cat. Although cryptenamine induced a depressor response in the body of the recipient following the administration of the drug into the carotid inflow to the recipient's head in the cross-circulation experiment, these responses were essentially abolished by bilateral denervation of carotid sinus-body complex. Cryptenamine potentiated epinephrine-induced depressor responses in the dog and isoproterenol-induced relaxation of both the cat nictitating membrane and the vasculature of the isolated denervated hind limb of the dog. Pronethalol decreased the duration of the hypotensive activity of cryptenamine. The data suggest that sensitization of β adrenergic receptors, a possible increased release of epinephrine from the adrenal medulla, and stimulation of carotid reflex mechanism contribute to the over-all hypotension induced by cryptenamine.

THE DETERRENT to the wider use of the veratrum alkaloids in therapy is the narrow range between their therapeutic and emetic doses. Cryptenamine, an alkaloidal preparation prepared from *Veratrum viride* by a nonaqueous benzene triethylamine extraction procedure, has been reported to have a ratio of emetic to effective hypotensive dose superior to that of other veratrum preparations (1, 2). Finnerty (2) reported that in humans the divergence between the hypotensive and emetic doses of cryptenamine was apparent on intravenous administration. McCall and his colleagues (3) studied the effects of cryptenamine on cerebral circulation and cerebral oxygen consumption in patients with toxemia of pregnancy and reported that cryptenamine induced fewer side effects than other veratrum preparations. The satisfactory ambulatory treatment of hypertension has been reported (4). Abreu *et al.* (5), however, failed to demonstrate any superiority of cryptenamine over protoveratrine A in comparing ratio of emetic to hypotensive dose in dogs.

The pharmacological advantages of cryptenamine have been attributed to the presence of amorphous hypotensive ester alkaloids reportedly lost by hydrolysis during alternative ammonia benzene extraction procedures (1). The present investigation was mainly concerned with the study of the hypotensive activity of cryptenamine and possible mechanism of action.

METHODS

Effects of Cryptenamine on Blood Pressure and Heart Rate of Anesthetized Dogs.—Mongrel dogs of either sex were anesthetized with sodium pentobarbital, 35 mg./Kg., i.v., and the blood pressure recorded from a cannulated femoral artery *via* a Statham pressure transducer onto a Grass polygraph. Heart rate was computed from blood pressure recordings. The intensity and duration of the hypotensive effects of control doses of cryptenamine, 5 mcg./Kg., were determined for each preparation. After arterial pressure returned to normal baseline, the animals were treated with one of the following: atropine sulfate, 1 mg./Kg.; *N,N* - di - isopropyl - *N'* - isoamyl - *N'* - diethylaminoethylurea (P-286), 10 mg./Kg.; bretylium tosylate, 10 mg./Kg.; or pronethalol, 10 mg./Kg.; and 5 mcg./Kg. of cryptenamine repeated within 10 min. All compounds were administered into a cannulated femoral vein. In several additional

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experiments, the animals were debuffered by denervation of the carotid sinus-body complex and bilateral vagotomy or bilateral adrenalectomy performed.

Effects of Cryptenamine on Dogs Pretreated with Reserpine, Guanethidine, or α Methyldopa.—In order to study the role of catecholamines on the mechanism of cryptenamine activity, dogs were pretreated with one of the following: reserpine phosphate, 1 mg./Kg., i.m., 24 hr. prior to the experiment; α methyldopa, 200 mg./Kg., 6 hr. prior to the experiment; or guanethidine, 10 mg./Kg., i.p., 12 hr. prior to the experiment. The animals then were anesthetized with sodium pentobarbital administered by slow intravenous infusion. Blood pressure and heart rate were recorded, and cryptenamine, 5 mcg./Kg., was administered intravenously. Several of these experimental animals were also pretreated with atropine sulfate, 1 mg./Kg., i.v., prior to the administration of cryptenamine.

Effects of Cryptenamine on Epinephrine-Induced Depressor Responses on Blood Pressure of Dogs.—Small doses of epinephrine may produce depressor responses by stimulation of β adrenergic receptors. This property of epinephrine was utilized in the following experiment to study the effects of cryptenamine on β adrenergic receptors.

Mongrel dogs were anesthetized with sodium pentobarbital, 35 mg./Kg., i.v., and prepared for the recording of blood pressure as previously described and mean blood pressure recorded on a Grass polygraph. Four doses of *l*-epinephrine bitartrate, 0.025, 0.05, 0.10, and 0.15 mcg./Kg., were administered intravenously. Cryptenamine, 5 mcg./Kg., i.v., was then administered and the doses of epinephrine repeated 30 min. later.

Effects of Cryptenamine on β Adrenergic Receptors in the Cat Nictitating Membrane.—Effects of cryptenamine on β adrenergic receptors in the cat nictitating membrane were studied using the procedure described by Gyorgy *et al.* (6). Cats were anesthetized with sodium pentobarbital, 35 mg./Kg., i.p. A femoral artery was cannulated for recording of blood pressure as previously described. Preganglionic superior cervical sympathetic nerve was isolated and stimulated at a frequency of 6 c.p.s., 0.2 msec. duration, and 1–3 v., thereby producing a state of continuous contraction of the nictitating membrane without damage to either nerve or muscle. Under these conditions, relaxation of the membrane was elicited with the administration of 5 to 20 mcg./Kg. of isoproterenol, i.v. Cryptenamine, 5 mcg./Kg., was then administered and the same dose of isoproterenol repeated. Both the blood pressure and the activity of the nictitating membrane were recorded on a Grass polygraph.

Effects of Cryptenamine on Isoproterenol-Induced Relaxation in the Denervated Perfused Hind Limb of the Dog.—Mongrel dogs of either sex were anesthetized with sodium pentobarbital, 35 mg./Kg., i.v., and a femoral artery was cannulated for the recording of blood pressure. One hind limb was denervated by severing the femoral and sciatic nerve trunks, and vascularly isolated by clamping the muscles with a 21-gauge stainless steel wire placed under the femoral artery and vein and ligated with a Schiffrin wire tightener. The distal segment of the femoral artery of the isolated limb was cannulated and perfused with the blood drawn from the central segment of the same artery. A sigmamotor pump was utilized to perfuse the leg at a constant rate of flow. Perfusion pressure was measured between the pump and isolated limb by means of a Statham pressure transducer. The changes in perfusion could be related to the changes in the vascular resistance, since blood flow to the limb remained relatively constant.

In order to study the effects of cryptenamine on β adrenergic receptors, four different doses of isoproterenol, 1, 2, 5, and 10 mcg., were administered intra-arterially into the limb followed by cryptenamine, 5 mcg./Kg., i.v. After a 30-min. stabilization period, the doses of isoproterenol were repeated.

Effects of Cryptenamine in the Dog Cross-Circulation Preparation.—Recipient dogs were anesthetized with sodium pentobarbital, 35 mg./Kg., i.v., and prepared for the recording of blood pressure as previously described. The neck musculature was removed utilizing electrocautery to expose the vertebral column from C-2 to C-5. A dorsal laminectomy was performed between C-3 and C-4; the vertebral venous sinuses and vertebral arteries were occluded utilizing 21-gauge stainless steel wire, as described by Bickerton and Buckley (7). Donor dog was similarly anesthetized and prepared for the recording of blood pressure. Circulation was established between the right common carotid artery of the donor and the head of the recipient *via* the recipient's two common carotid arteries and from the two jugular veins of the recipient's head to the right jugular vein of the donor animal. This resulted in a neurally intact, vascularly isolated recipient's head preparation. Circulatory leakage between the recipient's head and the trunk was determined utilizing ^{131}I (radio-iodinated serum albumin) administered into the recipient's carotid inflow. In several of these preparations, the carotid sinus-body complex of the recipient was denervated. Cryptenamine, 5 mcg./Kg., was administered into the arterial inflow of the recipient's head.

Effects of Intraventricular Administration of Cryptenamine on Blood Pressure of Cats.—The method of administration of compounds into the cerebrolateral ventricle, as described by Feldberg (8) and Bhattacharya and Feldberg (9), was utilized in these experiments. Cats were anesthetized with sodium pentobarbital, 35 mg./Kg., i.p., and prepared for the recording of blood pressure on an Offner dynograph as described earlier. A 22-gauge unbeveled needle, 34 mm. in length, employed as a cannula, was stereotaxically implanted in the left cerebrolateral ventricle and secured firmly to the skull with dental cement. The cisterna magna was exposed and the dura carefully cut allowing the cerebrospinal fluid to escape. Then, the lateral ventricle was perfused with artificial cerebrospinal fluid (NaCl, 8.1 Gm.; KCl, 0.25 Gm.; $CaCl_2$, 0.14 Gm.; $MgCl_2$, 0.11 Gm.; NaH_2PO_4, 0.081 Gm.; $NaHCO_3$, 0.07 Gm.; urea, 0.13 Gm., and dextrose, 0.61 Gm.; glass distilled water, 1000 ml.) maintained at 38°. The rate of perfusion was kept constant at 0.1 ml./min., utilizing a Phipps Bird constant-infusion pump.

Lateral ventricles were perfused 60 to 90 min. prior to the administration of cryptenamine. Doses of cryptenamine ranging from 1 to 10 mcg. were administered into the ventricles utilizing a Baltimore microinjection unit.

TABLE I.—EFFECTS OF CRYPTENAMINE, 5 mcg./Kg., i.v., ON BLOOD PRESSURE AND HEART RATE OF DOGS PRETREATED WITH SEVERAL PHARMACOLOGICAL AGENTS

Pretreatment	Dose, mg./Kg.	Animals, No.	Prior to Pretreatment: Mean % Decrease in Blood Pressure	Prior to Pretreatment: Mean % Decrease in Heart Rate	Prior to Pretreatment: Mean Duration, min.	Following Pretreatment: Mean % Decrease in Blood Pressure	Following Pretreatment: Mean % Decrease in Heart Rate	Following Pretreatment: Mean Duration, min.
Atropine	1	4	49.4	35.02	132.0	35.1	24.4	113.8+
Adrenalectomy	...	3	49.3	32.7	83.3	29.5	21.1	59.0
Adrenalectomy + atropine	... 1	3	49.8	29.3	115.0	37.2	3.7	85.0
Adrenalectomy + atropine	... 1	3	48.0	43.5	134.3	17.9	7.8	2.5
P-286	10	3	43.2	37.5	156.7	15.5	23.0	36.3
P-286 + atropine	10 1	3	44.3	29.2	186.7	19.0	5.0	4.3
Bretylium	10	3	52.2	32.0	87.3	28.7	10.1	82.7
Bretylium + atropine	10 1	3	52.5	28.6	60.3	0	0	...
Pronethalol	10	4	44.0	37.7	119.5	28.7	17.9	85.2
Pronethalol + atropine	10 1	4	50.5	37.9	131.2	39.2	1.3	85.2
Carotid sinus denervation + vagotomy		3	54.3	51.4	105.0	0	0	...
Reserpine	1	3				31.2	30.8	34.0
Reserpine + atropine	1 1	4				5.1	0	5.0
α Methyldopa	200	3				34.9	35.8	44.0
α Methyldopa + atropine	200 1	4				10.4	2.5	1.9
Guanethidine + atropine	10 1	4				41.9	0	41.2+

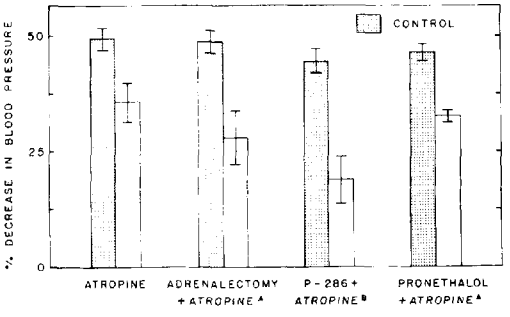

Fig. 1.—Effect of cryptenamine (5 mcg./Kg., i.v.) on blood pressure of dogs pretreated with certain pharmacological agents. Key: A, shorter duration compared with control responses; B, duration less than 10 min.

RESULTS

Effects of Cryptenamine on Blood Pressure and Heart Rate of Anesthetized Dogs.—Single doses of cryptenamine, 5 mcg./Kg., produced marked bradycardia and hypotension, persisting for 2 to 3 hr. A return of the bilateral carotid occlusion response was taken as an index for the recovery of the animals from the effects of cryptenamine. Tachyphylaxis was not observed when the second dose of cryptenamine was administered after return of this reflex.

Pretreatment of the animals with atropine sulfate partially blocked the bradycardia and the depressor effect. When cryptenamine was administered after adrenalectomy or pretreatment with P-286 or bretylium, the onset of action was delayed and only a moderate decrease in blood pressure occurred for a relatively short period of time. Pretreatment with either bretylium or P-286 produced complete blockade of the effects of cryptenamine in atropinized dogs. The cryptenamine depressor effect was blocked in three of six atropine-treated adrenalectomized animals, and the duration of activity was significantly reduced in the other animals. Pretreatment with pronethalol or pronethalol plus atropine sulfate decreased the duration of the hypotensive effects of cryptenamine. Bilateral vagotomy or bilateral denervation of the carotid sinus-body complex by itself was not sufficient to completely block the effects of cryptenamine; however, the effects were abolished by complete debuffering (Table I, Fig. 1).

Effects of Cryptenamine on Dogs Pretreated with Reserpine, Guanethidine, or α Methyldopa.—Pretreatment of the animals with either reserpine or α methyldopa significantly reduced the intensity and duration of the hypotensive activity of cryptenamine. Pretreatment with the above compounds followed by atropinization completely eliminated the depressor activity of cryptenamine. Guanethidine plus atropine abolished the effects of cryptenamine on heart rate but failed to reduce markedly the hypotensive activity of cryptenamine (Table I).

Effects of Cryptenamine on Epinephrine-Induced Depressor Responses on Blood Pressure of Dogs.—Depressor responses of epinephrine increased with the doses in the dosage range utilized. After cryptenamine, the depressor responses produced by each dose of epinephrine were potentiated suggesting a sensitization of β adrenergic receptors by cryptenamine (Table II).

Effects of Cryptenamine on β Adrenergic Receptors in the Cat Nictitating Membrane Preparation.—Isoproterenol induced a transient relaxation

of the electrically contracted nictitating membrane, and administration of cryptenamine did not alter the state of the contraction of the membrane. However, following cryptenamine, the administration of isoproterenol produced a biphasic response consisting of an initial relaxation of the membrane approximately equal to the control responses followed by a secondary relaxing effect of greater intensity and duration of action, suggesting a potentiation of the isoproterenol response on the nictitating membrane. When isoproterenol was administered following a return of the cat's blood pressure to precryptenamine levels, the effects on the nictitating membrane were similar to control responses both in intensity and duration (Table III).

Effects of Cryptenamine on Isoproterenol-Induced Relaxation in the Denervated Perfused Hind Limb of the Dog.—Administration of isoproterenol intra-arterially into the denervated perfused hind limb produced a decrease in perfusion pressure. The decrease in vascular resistance as indicated by a decrease in perfusion pressure was proportional with the dose of isoproterenol in that there was a linear relationship between the decrease in perfusion pressure and log dose of the β adrenergic stimulant. Doses of isoproterenol were repeated 20 to 30 min. after the administration of cryptenamine, and the decrease in perfusion pressure originally produced by each dose was significantly potentiated, approximately 25 to 30%. On graphical representation, the log-dose response relationship shifted to the left indicating the potentiation facilitated by cryptenamine on isoproterenol-induced relaxation of the vasculature of the limb (Table IV, Fig. 2).

Effects of Cryptenamine in Dog Cross-Circulation Preparation.—The administration of cryptenamine into the carotid inflow of the recipient's head produced a significant decrease in blood pressure in the recipient's trunk followed by a decrease in the donor blood pressure (N = 4). When the animals were debuffered by denervation of the carotid sinus-body complex, administration of cryptenamine into the head produced a pressor response in the body of the recipient followed by a depressor response in the donor (Table V).

Effects of Intraventricular Administration of Cryptenamine on Blood Pressure of Cats.—Doses ranging from 1 to 10 mcg. of cryptenamine were administered into the perfused lateral ventricle of cats. The lower doses (1–2 mcg.) produced a pressor response, and higher doses (3–10 mcg.) had no effect on blood pressure of the cat. Depressor responses were not observed following the administration of cryptenamine (Table VI).

TABLE II.—EFFECTS OF CRYPTENAMINE, 5 MCG./KG., I.V., ON EPINEPHRINE-INDUCED DEPRESSOR RESPONSES ON BLOOD PRESSURE OF ANESTHETIZED DOGS

	Before Cryptenamine—Decrease in Mean Blood Pressure to Epinephrine, mm. Hg				After Cryptenamine—Decrease in Mean Blood Pressure to Epinephrine, mm. Hg				Change, %			
Wt., Kg., Sex	0.025	0.05	0.10	0.15	0.025	0.05	0.10	0.15	0.025	0.05	0.10	0.15
	mcg./Kg.				mcg./Kg.				mcg./Kg.			
13.7 M	5	9	12.5	...	10	15	18	...	100	66.6	44.0	...
15.4 F	5	5	10.0	15.0	15	10	20	25	200	100.0	100.0	66.6
13.4 F	5	15	30.0	25.0	20	20	35	35	300	33.3	16.6	40.0
14.2 M	0	5	7.5	12.5	8	10	13	24	α	100.0	73.3	92.0
Mean	3.75	8.5	15.0	17.5	13.25[a]	13.75[a]	21.5[a]	28.0[a]				
± S. E.	±1.25	±2.36	±5.11	±3.83	±2.69	±2.40	±4.73	±3.52				

[a] Significantly different from pretreatment response ($P < 0.05$) when t values calculated by direct difference method (15).

TABLE III.—EFFECTS OF CRYPTENAMINE, 5 MCG./KG., ON ISOPROTERENOL-INDUCED RELAXATION OF THE CAT NICTITATING MEMBRANE

			Before Cryptenamine				After Cryptenamine			
Sex	Wt., Kg.	Isoproterenol Dose, mg./Kg.	Decrease in Blood Pressure, %	Duration, min.	Relaxation of N. M., mm.	Duration, min.	Decrease in Blood Pressure, %	Duration min.	Relaxation of N. M., mm.	Duration, min.
F	3.6	20	58	6.0	18	3.0	61	30.0	22	11.5
M	3.2	10	65	11.0	13	2.0	71	10.0	18	10.0+
F	2.8	10	51	4.0	7	4.6	52	4.0	8	10.0+
M	2.6	10	30	3.6	11	3.3	46	4.3	18	12.0+

TABLE IV.—EFFECTS OF CRYPTENAMINE, 5 MCG./KG., I.V., ON ISOPROTERENOL-INDUCED RELAXATION IN DENERVATED PERFUSED HIND LIMB OF THE DOG

Dose of Isoproterenol, mcg.	Animals, No.	Mean Decrease in Perfusion Pressure, mm. Hg ± S. E.—Before Cryptenamine	After Cryptenamine	Probability (P)[a]
1	6	34.8 ± 3.69	45.2 ± 5.62	0.01
2	6	41.0 ± 3.91	55.25 ± 5.53	0.01
5	6	48.0 ± 4.92	62.8 ± 4.21	0.01
10	6	52.5 ± 5.12	67.5 ± 5.32	0.01

[a] P was calculated by direct difference method (15).

DISCUSSION

Veratrum alkaloids have been reported to sensitize certain afferent receptors in left ventricles sending impulses *via* the vagus to the central nervous system resulting in hypotension and bradycardia (10). Cryptenamine appears to resemble other veratrum derivatives in this respect. It was necessary to perform bilateral vagotomy as well as bilateral denervation of the carotid sinus-body complex to abolish the effects of cryptenamine on blood pressure and heart rate, suggesting that cryptenamine induced reflex hypotension and bradycardia by affecting afferent receptors at the carotid sinus-body area and in the myocardium. Central hypotensive effects of cryptenamine were not demonstrated either in cross-circulation preparations or in perfused lateral ventricle preparations of the cat. The depressor effects observed in the recipient's trunk following the administration of cryptenamine into the arterial inflow of the head in the dog cross-circulation preparations were most likely due to the effects of cryptenamine on baro- or chemo-receptors in the carotid sinus-body complex. These effects were abolished in the debuffered preparations, and pressor effects resulted in the recipient's trunk.

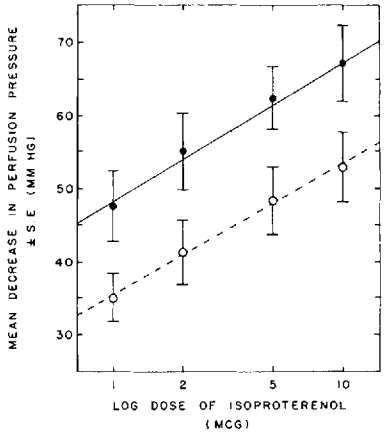

Fig. 2.—Effect of cryptenamine on isoproterenol responses in denervated perfused hind limb of dog. Crytenamine, 5 mcg./Kg. Key: ○, before; ●, after.

The efferent pathways in this reflex are still open to question. None of the pharmacological agents (atropine, pronethalol, bretylium, and P-286) when administered alone, could block the cryptenamine effects in dogs even though they all produced marked alterations in the responses, suggesting the complexity of various mechanisms involved in this reflex. The efferent parasympathetic pathways were blocked by atropinization in order to study the nature of other efferent pathways.

P-286 has been reported to block catecholamine release from the adrenal medulla induced by splanchnic stimulation (11). Pretreatment of the animals with P-286 and atropine sulfate completely abolished the effects of cryptenamine on blood pressure and heart rate. If P-286 is as specific in blocking the adrenal glands as has been reported, a combination of adrenalectomy and atropinization would be expected to produce similar effects. The data obtained in the current study do not totally disagree with this possibility, in that adrenalectomy produced marked inhibition of cryptenamine activity in atropinized animals. Since the adrenal medulla contains large concentrations of catecholamines especially epinephrine, it is quite probable that cryptenamine may reflexly release epinephrine from the adrenal medulla. The possible role of catecholamines in the hypotensive mechanisms of cryptenamine was further confirmed using animals

TABLE V.—EFFECTS OF CRYPTENAMINE, 5 mcg./Kg., i.v., ON BLOOD PRESSURE OF RECIPIENT AND DONOR DOGS IN CROSS-CIRCULATION PREPARATIONS

Expt.	Control Blood Pressure, mm. Hg		Change in Blood Pressure, %				Change in Perfusion Pressure, %	Duration, min.
	Donor	Recipient	Donor	Duration, min.	Recipient	Duration, min.		
1	125/100	120/75	−46.2	75+	−31.1	75+	−12.5	75+
2	125/95	120/95	−50.4	45+	−24.2	45+	−30.3	30
3	175/115	105/75	−45.9	85+	−47.0	47	−30.0	40
4	140/110	150/120	−45.8	55	−63.0	55+	−34.7	20
5[a]	200/135	100/60	−69.4	45	+36.9	55+	−12.5	55
6[a]	160/120	100/55	−52.6	70+	+61.4	70+	−33.3	35
7[a]	175/110	110/60	−53.0	75+	+49.3	75+	−25.0	42
8[b]	120/95	90/35	−30.0	35+	0.0	...	−10.7	35+

[a] Recipient dogs were debuffered. [b] Recipient pretreated with bretylium tosylate (10 mg./Kg. *via* femoral vein).

TABLE VI.—EFFECTS OF INTRAVENTRICULAR ADMINISTRATION OF CRYPTENAMINE ON BLOOD PRESSURE OF CATS

Sex	Wt., Kg.	Dose, mcg.	Control Blood Pressure, mm. Hg	Change, %	Duration, min.
F	2.6	1	110/60	+33.7	160
M	2.3	2	190/110	+ 7.8	150
M	2.3	3	190/110	0.0	...
M	3.4	5	190/125	0.0	...
M	3.4	10	190/125	0.0	...

pretreated with reserpine or α methyldopa. Depletion of catecholamines with reserpine or α methyldopa (12) facilitated blockade of the effects of cryptenamine in atropinized animals, whereas guanethidine which does not induce depletion of catecholamines from the adrenal medulla (13) could not block the cryptenamine effects in animals pretreated with atropine sulfate. Therefore, it appears that nonparasympathetic efferent pathways involved in the reflex hypotension produced by cryptenamine may exert their action through the release of catecholamines from the adrenal medulla.

Since the effects of epinephrine are predominantly on α receptors (14), a sensitization of β receptors could mask any pressor effect that might result from epinephrine. The data obtained indicate a sensitization of β adrenergic receptors following the administration of cryptenamine.

It is apparent that there are many mechanisms in the complex phenomena of cryptenamine-induced hypotension. The results of this investigation which included the ability of P-286, reserpine, α methyldopa, and adrenalectomy to inhibit the effects of cryptenamine suggest a role of catecholamines and the adrenal medulla. The reduction of the cryptenamine effects by pronethalol, potentiation of the isoproterenol effects in the cat nictitating membrane and isolated perfused hind limb of the dog, and potentiation of the epinephrine-induced depressor responses in the dog indicate a role of the β adrenergic receptors and suggest that cryptenamine sensitizes the β adrenergic receptors to circulating epinephrine.

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Formation of Acetylcodeine from Aspirin and Codeine

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A reaction is discussed which leads to the formation of acetylcodeine from aspirin and codeine. It is noted that the generally published methods of analysis will not differentiate between the two alkaloids. A method of separating and assaying the individual compounds is described. Furthermore, the dependence of the interaction on water is discussed.

Although an abundance of products are marketed which contain combinations of aspirin, phenacetin, caffeine, and codeine, little has been published as to the stability and reactivity of these systems. Studies (1, 2) have been conducted on the stability of aspirin *per se*, but little attention has been given to its effect on other compounds.

During the development of a capsule product containing aspirin, phenacetin, caffeine, itobarbital, and codeine phosphate,[1] thin-layer chromatography indicated the presence of an unknown product in some samples after aging. The present communication deals with an investigation of this reaction and indicates that under certain conditions acetylcodeine forms. The acetylcodeine which results from this interaction of aspirin and codeine cannot be detected by the normal analytical methods employed for the determination of codeine. A partition column separation technique of codeine from acetylcodeine is described.

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RESULTS AND DISCUSSION

Isolation and Identification of the Reaction Product.—Based upon the possible reactants, it was speculated that an interaction might occur between aspirin and codeine (Scheme I).

Although the mechanism of the reaction has not been investigated, it might proceed by a classical transesterification or might be facilitated by an

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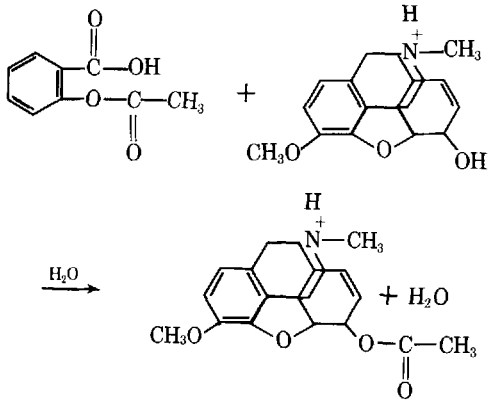

Scheme I

anhydride as reported by Higuchi *et al.* (3) for a number of systems.

Mixtures of aspirin and codeine phosphate were placed into ampuls and water was added. The samples were sealed and heated at 60° for periods up to 4 weeks. At the end of regular time intervals the samples were fractionated using a thin-layer preparative chromatographic method. Aside from the aspirin, codeine, and salicylic acid, a new product was isolated. The isolation was carried out by scraping the silica gel from the plate in the area where the spot was evident and subsequent extraction with chloroform. The chloroform was evaporated to dryness leaving a white crystalline residue. The material was analyzed and compared with known acetylcodeine with the following findings.

Acetylcodeine, m.p. 131.5–132.5°; unknown m.p. 129.0–130°.

Anal.[2]—Calcd. for C, 70.4; H, 6.8; N, 4.1; O, 18.8. Found: C, 69.1; H, 6.7; N, 4.3; O, 18.6.

A mixed melting point (1:1) was 129.0°–131.0°. The ultraviolet and infrared spectra of the known and unknown were found to be identical. The R_f values of acetylcodeine, the unknown compound found in the aspirin–codeine mixture, and the unknown compound in the analgesic combination were identical in the systems described under *Experimental*. From these data one may conclude that this degradation product is acetylcodeine. A somewhat similar reaction was reported by Troup and Mitchner (4) involving the acetylation of phenylephrine.

Analysis of Mixtures Containing Codeine and Acetylcodeine.—As previously mentioned, it was noted during initial work on the analgesic combination that although a spot, now known to be acetylcodeine, appeared on the thin-layer chromatograms, no apparent loss of codeine was detected. The analytical technique used was a modification of the method proposed by Heuermann and Levine (5) for the separation of APC plus codeine mixtures. Ultraviolet measurements of the codeine fraction did not indicate any loss of the alkaloid, nor was any change apparent when the codeine fraction was titrated under nonaqueous conditions with perchloric acid. It is evident from Fig. 1, which shows the spectra of codeine and acetylcodeine, that the two are of the same order of magnitude. It is readily apparent that the *a* 1%, 1 cm. at 286 mμ of the compounds in chloroform are essentially identical (codeine = 55; acetylcodeine = 44), and, therefore, an ultraviolet method without prior separation would not be suitable for quantitative analysis.

The acetylation of codeine does not alter the basicity of the tertiary nitrogen and consequently does not affect the nonaqueous titration values.

Codeine and acetylcodeine can be separated by partition chromatography using a Celite support with the nonmobile phase being a 4% citrate buffer system at pH 3.5. At this pH the codeine is held on the column and the acetylcodeine is eluted with chloroform. The codeine can then be eluted with ammonia-saturated chloroform. Figure 2 is a typical chromatogram showing this separation of the products. Recovery of the acetylcodeine and codeine was shown to be quantitative.

From this it is evident that when assaying for codeine in an APC mixture according to the method of Heuermann and Levine (5), the codeine fraction should be passed through the citrate column to assay for acetylcodeine, after determining the presence of this degradation product by thin-layer chromatography.

Dependence of the Reaction on Water.—In order to ascertain the effect of moisture on the rate of the reaction, aspirin and codeine phosphate were mixed in the ratio of 100:15 w/w and placed into ampuls. Varying amounts of water were added and the ampuls sealed. A control sample was included to which no moisture was added. All ampuls were placed into a constant-temperature oven at 60° and heated for periods of time up to 22 days. At intervals, ampuls were withdrawn and assayed for aspirin, salicylic acid, codeine, and acetylcodeine.

The findings are shown graphically in Fig. 3. The data indicate that, as expected, the reaction is dependent on water in the lower moisture regions, but, however, at the higher levels the increase in the rate shows less of a dependency. This can most probably be attributed to the effect of the reverse

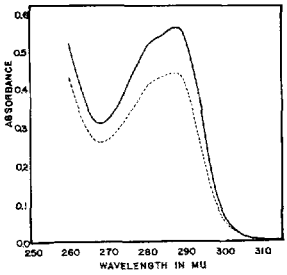

Fig. 1.—Ultraviolet spectra of codeine and acetylcodeine in chloroform. Key: ——, codeine; - - - - - -, acetylcodeine.

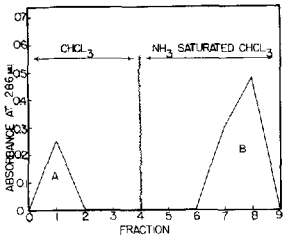

Fig. 2.—A typical chromatogram showing the separation of acetylcodeine (A) from codeine (B) by partition chromatography.

[2] The cooperation of Mr. Urs Stoeckli in performing the elemental analysis is gratefully acknowledged.

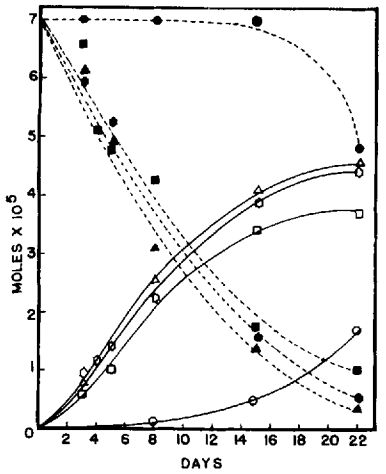

Fig. 3.—A composite representation of the degradation of codeine and formation of acetylcodeine under varying moisture conditions at 60°. Key: ●, < 0.1% moisture, codeine; ○, acetylcodeine; ■, 0.5% moisture codeine, □, acetylcodeine; ▲, 1.0% moisture, codeine; △, acetylcodeine; ⬢, 2.0% moisture, codeine; ⬡, acetylcodeine.

reaction which becomes more evident at the higher moisture concentrations.

It must be pointed out that the evaluation of such a heterogeneous system does not lend itself to a detailed kinetic analysis, but the results do indicate that moisture is essential for the reaction to proceed.

Analysis of commercial samples of the capsule product[1] stored at room temperature for 32 months indicated no evidence of acetylcodeine formation. These capsules were prepared in such a manner that the total moisture present was less than 0.2%. This bears out the findings with the simple aspirin–codeine mixture that at low moisture levels the reaction proceeds slowly even at elevated temperatures.

It should also be pointed out that based upon the work of Buckett *et al.* (6) the apparent activity and toxicity is not altered by the acetylation.

EXPERIMENTAL

Reagents

All chemicals used for the preparation of samples and analytical work were of U.S.P. or reagent grade, except for the acetylcodeine. The acetylcodeine was obtained as the hydrochloride (Merck), converted to the base, extracted with chloroform, and obtained in crystalline form by evaporation of the solvent, m.p. 131.5°–132.5°.

Analytical Procedures

Thin-Layer Chromatography.—*Plates.*—A 0.25-mm. layer of Silica Gel G (E. Merck, Darmstadt) was applied to the plates. The plates were air-dried for 10 min. and then heated in an oven for 45 min. at 120°. The plates were then stored in a desiccator until used.

Solvent System.—(*a*) Chloroform–acetone–diethylamine, 5:4:1 (7). (*b*) Chloroform–methanol, 10:1.

Spray Reagent.—One milliliter of 37% formaldehyde dissolved in 30 ml. of sulfuric acid (8).

Procedure and R_f Values.—Spot the equivalent of 100 mcg. of the alkaloid bases and develop over 10 cm. Dry the plate and spray with the reagent. Both codeine and acetylcodeine appear as purple spots. For nondestructive spraying, use distilled water to locate the compounds.

	Solvent	
R_f Values	(*a*)	(*b*)
Acetylcodeine	0.7	0.95
Codeine	0.4	0.60
Unknown[3]	0.7	0.95

Codeine Phosphate.—The nonaqueous titration as prescribed in the U.S.P. XVII was used to assay the codeine phosphate.

Aspirin.—(In mixture containing aspirin and codeine 100:15 w/w.) Place approximately 25 mg. of the mixture into a separator containing 30 ml. of chloroform and 10 ml. of 0.2 *N* sulfuric acid. Shake. Withdraw the organic layer and place into a 100-ml. volumetric flask. Repeat the extraction twice and bring the organic solution to volume with chloroform. Determine the absorbance of the solution at 280 mμ and compare with a known aspirin solution at the same wavelength.

Salicylic Acid.—Determined by the method of Levine (9).

Acetylcodeine and Codeine in Degraded Samples.—Adjust a 4% aqueous solution of citric acid monohydrate U.S.P. to pH 3.5 with 1 *N* sodium hydroxide. Mix 4.5 ml. of this solution with 7.5 Gm. of acid-washed Celite 545 (Johns-Manville Corp.) and pack into a 200 × 25 mm. chromatography tube previously plugged with glass wool. Top the Celite layer with a pledget of glass wool.

Add a 150-mg. sample of aspirin–codeine to a separator containing 10 ml. of 1 *N* sodium bicarbonate and 30 ml. of chloroform. After shaking, transfer the organic phase to the prepared column. Set a 100-ml. volumetric flask as a receiver beneath the column. Perform two additional chloroform extractions of the sodium bicarbonate layer adding each, in turn, to the column. Add sufficient water-saturated chloroform to the column to bring the eluate to 100 ml. This fraction contains the degradation product (acetylcodeine).

Elute the column with ammonia-saturated chloroform into a 100-ml. volumetric flask. This fraction contains the codeine. Read each fraction on a spectrophotometer at 286 mμ and compare with a standard.

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Solubility of the Xanthines, Antipyrine, and Several Derivatives in Syrup Vehicles

By ANTHONY N. PARUTA and BHOGILAL B. SHETH

The solubilities of the xanthine drugs, antipyrine, aminopyrine, and 4-aminoantipyrine in sucrose solutions of varying concentration have been determined. The effect of "additive" concentration, sucrose, upon the solubility of subject materials is rather dramatic, either increases or decreases in solubility being noted. The path or rate of change of solubility with increasing sucrose concentration are seen in some cases to be of a complex functionality and may indicate a combination of mechanisms. Since the dielectric constants of these sucrose vehicles were also known, the solubility as a function of the dielectric constant is also considered.

SYRUP VEHICLES still find wide use in the pharmaceutical field in liquid preparations, and in a continued effort (1) to study the characteristics of these dissolution media, this study was undertaken. The primary objective of this study was to determine if a given solute would have the same magnitude of solubility in water and simple syrup. If the solubilities were found to differ, it was felt important to determine the rate of change of solubility with sucrose concentration. Thus, syrup vehicles at concentrations intermediate to simple syrup were also tested. The solubilities of the xanthines, antipyrine, aminopyrine (4-dimethyl aminoantipyrine), and 4-aminoantipyrine in syrup vehicles at various concentration levels up to saturation have been determined.

Since it was known that the dielectric constants of sucrose solutions (1) decreased with increasing concentration, this was felt to be another parameter in which solubility could be expressed. It is implied that the usefulness of the dielectric constant resides in the expression of polarity of a given dissolution media. The dielectric constants of sucrose solutions have been determined previously (1), by other workers (2, 3), and have been repeated in this study. One main purpose of studying dielectric constants of saturated solutions of solutes was to observe if the magnitude of solubility could be correlated with the change in dielectric constant of the syrup vehicle used.

These sucrose solutions can also be considered as media in which the activity of water has been decreased by the additive, sucrose, and this may also effect the magnitude of solubility. It is possible that both decreased and increased solubility can occur and the operative mechanism being decreased dielectric constant, decreased activity, or a combination of these. It is obvious that as the sucrose concentration is increased, the water concentration is decreased; however, it is the author's view that these vehicles are *per se* dissolution media. The dependence of increased or decreased solubility probably resides in the nature of the solute and the solvent system. Whereas increased solubility had been noted in a previous study (1), both increased and decreased solubility have been found in this study.

Previous work (4–7) had indicated that the asymptotic portion of the solubility curve would have to be extended past the dielectric constant value for pure water in order to achieve decreased solubility. The results of this study show that significant solubility decreases can occur with a solvent system having dielectric constant values less than pure water.

EXPERIMENTAL

Materials.—Caffeine U.S.P. (Nepera Chemical Co.), theophylline U.S.P. (Matheson, Coleman and Bell, 7094 Tx 450), theobromine alkaloid N.F. (Penick NBT 4092), antipyrine N.F. (Penick NBT 3710), aminopyrine N.F. (Penick NBT 2376), and 4-aminoantipyrine (Eastman-Kodak white label—6902). These materials were used directly as received from the manufacturer.

Equipment.—Constant-temperature bath, Bausch & Lomb spectronic 505, WTW Multidekameter DK-06, Bantam demineralizer, BD-1, with mixed resin bed.

Solubility Determinations.—Solubility determinations were made as described previously (4–7). Equilibration time was found to be 72 hr. and all determinations made at 25° ± 0.1°. Some difficulty was encountered in pipeting samples of simple syrup due to high viscosity and these samples show a somewhat larger variation since 1-ml. samples were used in contrast to 5-ml. samples for all other syrup vehicles. After appropriate dilutions, all samples were assayed spectrophotometrically.

Dielectric Constant Determinations.—Dielectric constant determinations were done at 25° on a WTW Multidekameter, DK-06. A calibration curve was prepared from absolute ethanol and water. One advantage of this instrument is that the cali-

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TABLE I.—SUMMARY OF THE AVERAGE SOLUBILITY AND THE SOLUBILITY RANGE OF THE VARIOUS SOLUTES STUDIED IN WATER AND SYRUP VEHICLES

Solvent	Av. Solubility, mg./ml.					
	Antipyrine	4-Amino-antipyrine	Amino-pyrine	Caffeine	Theophylline	Theobromine
Water	670 ± 15	410 ± 10	48 ± 3	21 ± 1	8.0 ± 0.3	0.56 ± 0.02
7.5% w/w sucrose	605 ± 20	390 ± 10	42 ± 2	...	8.2 ± 0.3	...
18.5% w/w sucrose	500 ± 20	340 ± 10	36 ± 2	19 ± 1	8.3 ± 0.2	0.66 ± 0.05
31.5% w/w sucrose	380 ± 20	305 ± 10	30 ± 2	17 ± 1	8.3 ± 0.3	0.70 ± 0.03
41.5% w/w sucrose	...	250 ± 10	24 ± 2	...	8.2 ± 0.3	...
46% w/w sucrose	280 ± 20	205 ± 15	19 ± 2	14 ± 1	7.9 ± 0.3	0.70 ± 0.03
63.5% w/w sucrose	185 ± 20	140 ± 20	16 ± 2	10 ± 1	6.2 ± 0.5	0.83 ± 0.03

TABLE II.—SUMMARY OF THE AVERAGE SOLUBILITY RATIOS, $\frac{\text{SOLUBILITY IN SYRUP}}{\text{SOLUBILITY IN WATER}}$, FOR THE VARIOUS SOLUTES STUDIED

Solute	Av. Solubility Ratio, $\frac{\text{mg./ml. Syrup}}{\text{mg./ml. Water}}$
Caffeine	0.48
Theophylline	0.83
Theobromine	1.40
Antipyrine	0.28
Aminopyrine	0.32
4-Aminoantipyrine	0.38

bration curve is linear and although minor day to day variations are noted, the calibration curves are virtually a family of parallel lines. The error in the measurement of dielectric constants is ±0.3 dielectric constant units. Deionized water was used throughout this study as distilled water seemed to cause slight aberrations in the calibration curves, possibly due to ionic contaminants.

RESULTS AND DISCUSSION

The solubilities of the solutes under consideration in this study in water and the various syrup vehicles used are given in Table I. The average value given expresses the average of at least six determinations and in some cases, *i.e.*, theophylline and theobromine, 8–12 determinations were made. The range of the determined solubility in mg./ml. is also shown in this table for the lowest and highest solubility noted over the number of runs made.

The average solubility ratios, the solubility in mg./ml. of simple syrup/solubility in mg./ml. of water, for the solutes studied are given in Table II. In all cases, significant changes in the magnitude of solubility were noted in going from water to simple syrup.

The solubility of caffeine in mg./ml. *versus* either sucrose concentration or dielectric constants is given in Fig. 1. For caffeine it is observed that the solubility decreases dramatically, the magnitude of solubility being about 0.5 in syrup relative to pure water. The path or rate of change in solubility is a smooth nonlinear function when plotted *versus* sucrose concentration; however, approximate linearity is observed on a dielectric constant basis. The rate of change for this parameter is about 0.55 mg./dielectric constant unit. The representation of data on a dielectric constant basis simply "squeezes in" the x-axis, and the solubility curve retains the approximate shape noted on the concentration basis.

In the case of aminopyrine and antipyrine it is also observed that the solubilities decrease dramatically with either increasing sucrose concentration or decreasing dielectric constant. The data have been plotted and are shown in Figs. 2 and 3. Both solubility curves are essentially smooth nonlinear functions, and the ratio of the solubility in syrup relative to water for aminopyrine and antipyrine are 0.32 and 0.28, respectively.

The solubility of 4-aminoantipyrine in the syrup vehicles studied is given in Fig. 4. Again, the experimental solubilities are plotted in mg./ml. of solution for both increasing sucrose concentration and decreasing dielectric constants. In this case, it is observed that an essentially sigmoidal solubility curve is obtained, and a significant change in the solubility occurs. The ratio of the solubility in syrup relative to water for this solute was found to be about 0.38.

The effect of substituents, solubility ratios, and magnitudes of solubility will be discussed later in this communication with respect to the antipyrine derivatives.

In the case of theobromine, an increase in solubility is found with increasing sucrose concentration or decreasing dielectric constants. The ex-

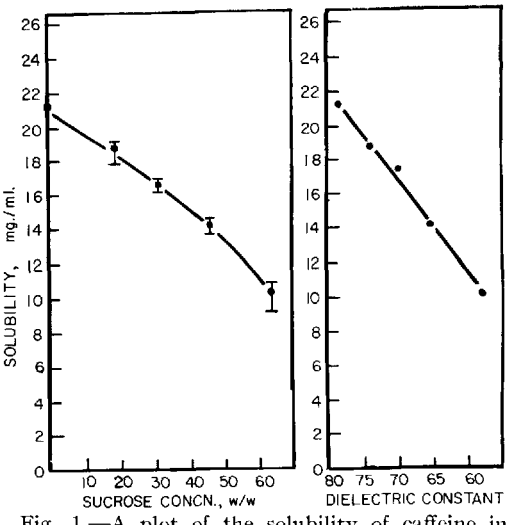

Fig. 1.—A plot of the solubility of caffeine in mg./ml. at 25° as a function of both sucrose concentration and dielectric constants.

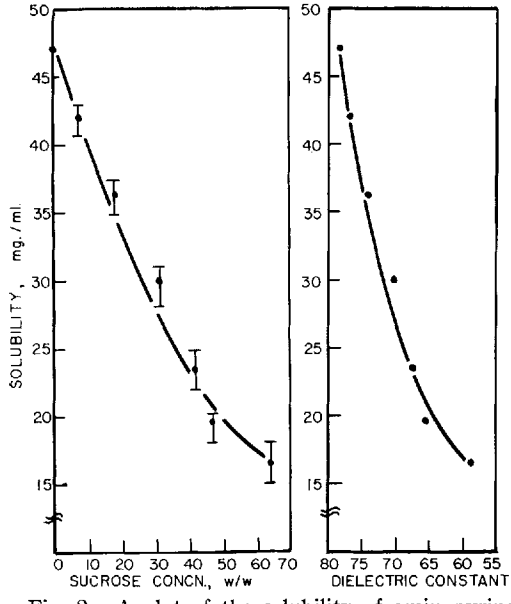

Fig. 2.—A plot of the solubility of aminopyrine in mg./ml. at 25° as a function of both sucrose concentration and dielectric constants.

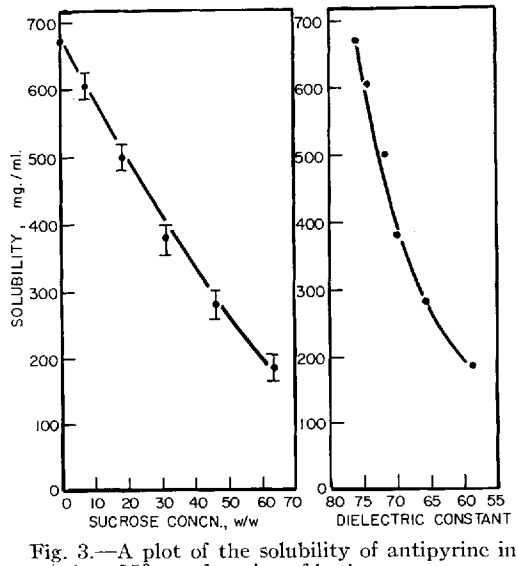

Fig. 3.—A plot of the solubility of antipyrine in mg./ml. at 25° as a function of both sucrose concentration and dielectric constants.

perimental data obtained were plotted in the usual fashion and are shown in Fig. 5. In this case, the solubility ratio has a value greater than unity, the solubility in syrup relative to water having a value of about 1.4. However, as noted in the case of 4-aminoantipyrine, the path of change is essentially sigmoidal but opposite in direction.

The solubility of theophylline presented in the usual fashion is given in Fig. 6. The solubility curve goes through a maxima and the equilibrium solubility in simple syrup finally fell to a lower value than the solubility in pure water. The solubility in simple syrup relative to water had a value of about 0.8. The maximum solubility at about 20–30% w/w sucrose represents about an 8% increase in solubility.

Mechanistically, it is felt that the additive decreases the activity of water by causing a statistically reduced number of hydrogen bonding sites which would ordinarily be available to the solute in the absence of the additive. It would seem then that any additive capable of interferring with, reducing

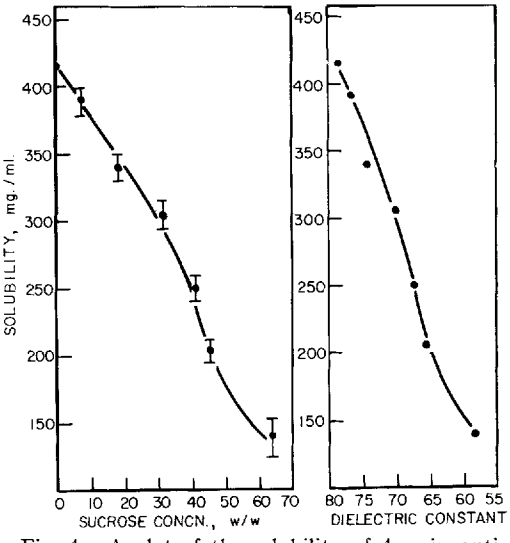

Fig. 4.—A plot of the solubility of 4-aminoantipyrine in mg./ml. at 25° as a function of both sucrose concentration and dielectric constants.

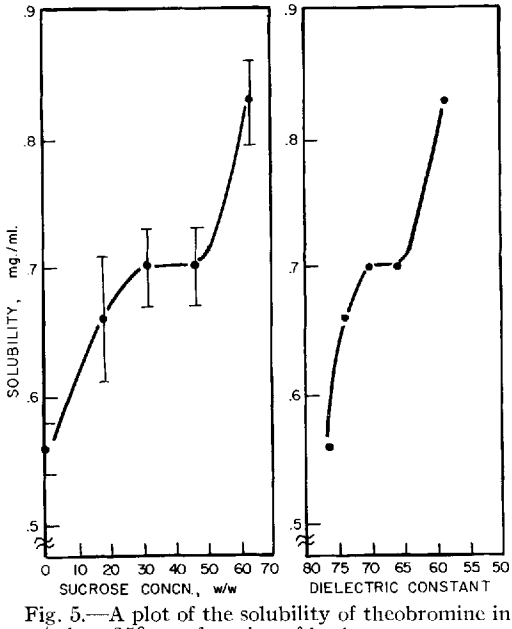

Fig. 5.—A plot of the solubility of theobromine in mg./ml. at 25° as a function of both sucrose concentration and dielectric constants.

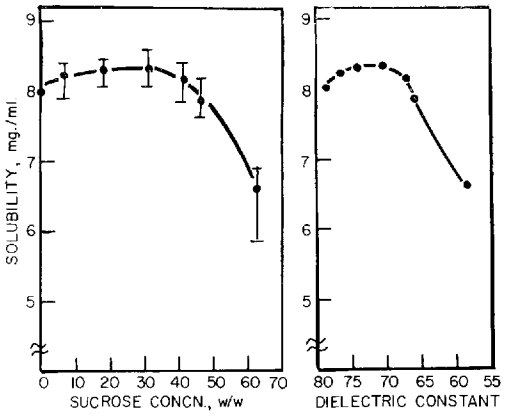

Fig. 6.—A plot of the solubility of theophylline in mg./ml. at 25° as a function of both sucrose concentration and dielectric constants.

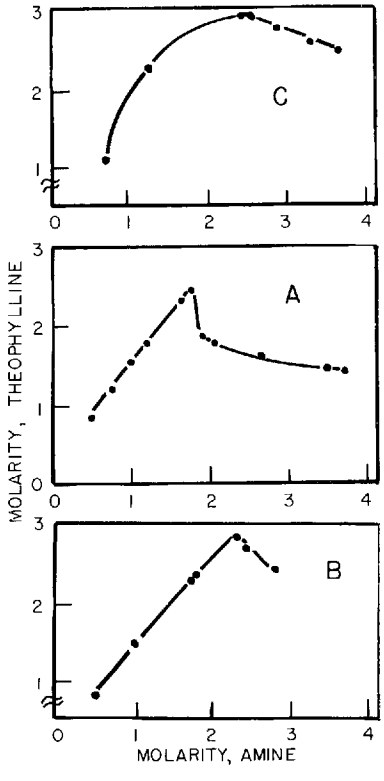

Fig. 7.—A plot of the solubility of theophylline as a function of added amine in amine–water mixtures. Key: A, monomethylamine; B, monoethylamine; C, monopropylamine.

the number, or tying up hydrogen bonding sites disturbs or shifts the equilibrium to the left, thereby decreasing the magnitude of solubility.

However, increased solubility was also found in this study, and this may be due to the decreased polarity of the syrup vehicles as measured by the dielectric constant. It is probable that a combination of mechanisms are operative. From this study, and others presently underway, it would seem that the nature of the solubility curve for a given solute in syrup vehicles depends strongly upon the original magnitude of solubility in pure water.

On the dielectric constant basis, maximum solubility is seen to occur in the dielectric constant range of 70–75. Some data (8) were available in which the solubility of theophylline had been studied in alkylamine solutions. Although the authors, Leuallan and Osol, were apparently determining the "complex" formation of theophylline and amines and solubilization therefrom, their data have been reanalyzed to apply the dielectric constant concept. The data have been replotted and shown in Fig. 7.

In the presence of monomethylamine, a maxima appears in the solubility curve at about 1.8 Gm. moles amine/L. of water. In this case and in other solubility curves presented for the amines, it has been assumed that the dielectric constant variation is linear with respect to the weight/weight composition notation. In other words, the decrease in dielectric constant from pure water to any amine concentration is proportional to the weight of the added amine. For monomethylamine, the peak solubility occurs at a dielectric constant value of about 75 which agrees with the span of 70–75 noted in the syrup vehicles. In the case of monoethyl and monopropylamines, also shown in Fig. 7, peak solubility occurs at dielectric constant values of about 71 and 68, respectively. These values concur with the range of 70–75 found in syrup vehicles. It should also be noted that the peak dielectric constants in these amine systems decrease with increasing size of the *n*-alkyl substitution.

The above data have been summarized in Table III using dielectric constant values from the literature (9). The approximate correlation of peak solubility dielectric constants for some of the amine–theophylline systems and theophylline–syrup systems is considered to be fair. The remaining theophylline–amine systems were also analyzed; however, no conclusion could be drawn from the data within the above context. Furthermore, it may be instructive to note, that both the amine and sucrose can be considered as "additives" having an approximately common effect on the solubility of theophylline. It is also rather surprising that irrespective of the possible formation of a more soluble "amine compound" or just simply adding sucrose, the peak solubility dielectric constants are to a fair degree correlatable.

Table III.—Summary of the Peak Solubility Dielectric Constants Found for Several Theophylline–Amine Systems[a]

Amine	ϵ^b Amine	Dielectric Constant Range	Peak Solubility Dielectric Constant
Monomethylamine	9.4	78.5–69.7	74.6
Monoethylamine	7.9	77.0–69.6	71.2
Monopropylamine	~5.0	75.5–62.5	68.0

[a] Data from *Reference 8*. [b] *Reference 9*.

DIELECTRIC CONSTANTS

After sample withdrawal for those solutes, the saturated solutions were tested in the dekameter at 25°, by a circulating water bath, relative to their dielectric constant values.

It had been hoped that, as the concentration of a given solute would increase or decrease, the dielectric constant of that sample would increase or decrease proportionally relative to the dielectric constant value of the syrup vehicle. In other words, the 10 mg./ml. of caffeine in simple syrup should affect the dielectric constant of simple syrup to a lesser extent than the 19 mg./ml. of caffeine in the 20% w/w syrup vehicle. This was not found to be the case in general, and no apparent conclusion could be observed from these data. The dielectric constants of the saturated solutions did, however, follow the shape of the solubility curve, but there was no general pattern in incremental increases or decreases relative to the syrup vehicles themselves. It should be noted that these determinations are over a relatively short dielectric constant span and overlapping small incremental changes with the error involved and day-to-day calibration variation are too close to delincate any patterns of change.

There was a notable exception to the above, that being the case of the highly water-soluble antipyrine, and this is shown in Fig. 8. In this figure the solubility curve and the Δ-dielectric constant curve for the saturated solutions (difference between the dielectric constant of saturated solution and the syrup vehicle) cross each other and are approximately mirror images. In this case then, as the solubility of antipyrine decreases in the syrup vehicles going to simple syrup there is proportionately less effect on the dielectric constant of the syrup vehicle itself. The very high solubility of antipyrine relative to the other solutes studied is probably the main factor in this type of analysis.

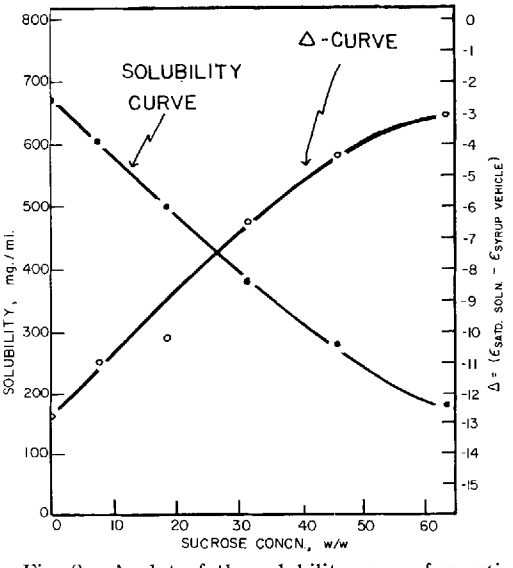

Fig. 8.—A plot of the solubility curve for antipyrine in syrup vehicles and the dielectric constant difference (Δ-curve) between saturated solutions of antipyrine and the syrup vehicles used.

PREDICTION OF SOLUBILITY CHANGE

The data on antipyrine and its derivatives may be considered from an alternate approach. Antipyrine and two derivatives, the 4-amino and 4-dimethyl amino, were studied in order to find the effect of substituent groups relative to solubility effects. In Table IV, the average determined solubility in water and simple syrup is listed. By allowing the solubility of the aminopyrine in water and simple syrup to be represented by unity, the ratios of solubility of the other solutes in the same solvent system were calculated. As can be seen from the table, these ratios are fairly constant irrespective of the solvent system. By taking the difference between the solubility in water and the solubility in syrup a delta value (Δ) is obtained. If the Δ value is divided by the original solubility, the percentage decrease in solubility can be calculated. These values are shown in the final column of this table. The percentage decrease is also seen to be fairly constant. It would seem that the derivative effects for these solutes are relatively constant and predictive solubility is a possibility, at least for the range and systems studied.

In other words, in so far as the approximate rule is valid, only the solubility of antipyrine in syrup need be determined; and the solubility of the derivatives in syrup can be approximately calculated from the ratios in pure water.

Although this type of analysis could be done for antipyrine and its derivatives, the xanthines could not be viewed in the same fashion since the solubility curves obtained were more complex and they are essentially positional isomers, with caffeine containing an extra methyl group in the 1-position.

This study is the first in a series of investigations of the solubility of various solutes in syrup vehicles and has shown that both decreases and increases in solubility are possible with this additive. Furthermore, it seems that the path in syrup vehicles in certain systems has a complex functionality. It must be pointed out that this work deals only with one highly water soluble material, *i.e.*, sucrose, and conclusions attendant to this must be viewed for the

TABLE IV.—SUMMARY OF THE SOLUBILITIES AND SOLUBILITY RATIOS OF ANTIPYRINE AND SEVERAL DERIVATIVES IN WATER AND SIMPLE SYRUP

Substance	Solubility, mg./ml.		Solubility Ratio		Δ mg./ml.	% Decrease = Δ/Soly. in Water
	Water	Simple Syrup	Water	Syrup		
Antipyrine	670	185	14.0	11.6	485	72
4-Aminoantipyrine	411	141	8.6	8.8	270	66
4-Dimethylamino-antipyrine	48	16	1	1	32	67

range and the system studied. It would be interesting to note the effect of additives, especially other sugar moieties, upon the solubility of various solutes.

Whether the dielectric concept, or activity concept mechanisms, or a combination of these are strictly involved cannot be completely delineated in this work. Although the fair correlation given for theophylline has some basis in dielectric constants, no sweeping involvement of dielectric constants for all their systems is apparent. In addition, the observations made may eventually show a very strong solute nature and solvent system dependence. Studies attendant to these points are being carried out in these laboratories and will be the subject of future communications.

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Solubilizing Properties of Bile Salt Solutions II

Effect of Inorganic Electrolyte, Lipids, and a Mixed Bile Salt System on Solubilization of Glutethimide, Griseofulvin, and Hexestrol

By THEODORE R. BATES*, MILO GIBALDI†, and JOSEPH L. KANIG

Studies of the influence of inorganic electrolyte on the solubilization of griseofulvin, glutethimide, and hexestrol in four individual bile salt solutions at 37° showed that sodium chloride had little effect on the solubility of the former two drugs but significantly increased the solubility of hexestrol. Based on these findings the possible location of the drug molecules within the micelle is considered. A mixed bile salt system was found to possess a significantly lower critical micelle concentration (CMC) than any of the individual bile salts previously studied. However, the affinities of this mixed micellar system for the drugs were comparable with those obtained with individual bile salts. The addition of lipids to the mixed bile salt system resulted in a decrease in hexestrol solubility but had little effect on griseofulvin and glutethimide solubility. The biological implications of the results obtained in the present communication are explored, and a mechanism for the role of dietary lipids and bile salts in the absorption of drugs is proposed.

BORGSTRÖM (1) has proposed a theory for the fate of ingested triglycerides prior to absorption. According to this theory, the breakdown products of pancreatic lipolysis (*i.e.*, 1- and 2-monoglycerides and fatty acids) are solubilized by bile salt micelles, present in the upper segment of the small intestine, prior to their absorption across the intestinal mucosa. In connection with this theory of fat absorption, several *in vitro* investigations have appeared in the literature demonstrating the marked micellar solubilizing properties of conjugated bile salts for fatty acids and monoglycerides (2–5). There has also been *in vivo* and *in vitro* evidence that the intestinal mucosa is capable of uptaking fatty acids and monoglycerides from a mixed micellar solution composed of these substances and conjugated bile salts (6–8).

In a previous communication (9) the effects of bile salt concentration and type, and temperature on the micellar solubilizing properties of bile salt solutions for the relatively water-insoluble drugs, griseofulvin, glutethimide, and hexestrol were reported. This report proposed that bile salts play a role in the dissolution step of the intestinal absorption mechanism for water-insoluble drugs.

In the present communication the results of findings on the influence of a mixed bile salt system, inorganic electrolyte concentration, and pancreatic lipolytic products and bile components on the degree of micellar solubilization of griseofulvin, glutethimide, and hexestrol are presented.

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range and the system studied. It would be interesting to note the effect of additives, especially other sugar moieties, upon the solubility of various solutes.

Whether the dielectric concept, or activity concept mechanisms, or a combination of these are strictly involved cannot be completely delineated in this work. Although the fair correlation given for theophylline has some basis in dielectric constants, no sweeping involvement of dielectric constants for all their systems is apparent. In addition, the observations made may eventually show a very strong solute nature and solvent system dependence. Studies attendant to these points are being carried out in these laboratories and will be the subject of future communications.

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Solubilizing Properties of Bile Salt Solutions II

Effect of Inorganic Electrolyte, Lipids, and a Mixed Bile Salt System on Solubilization of Glutethimide, Griseofulvin, and Hexestrol

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Studies of the influence of inorganic electrolyte on the solubilization of griseofulvin, glutethimide, and hexestrol in four individual bile salt solutions at 37° showed that sodium chloride had little effect on the solubility of the former two drugs but significantly increased the solubility of hexestrol. Based on these findings the possible location of the drug molecules within the micelle is considered. A mixed bile salt system was found to possess a significantly lower critical micelle concentration (CMC) than any of the individual bile salts previously studied. However, the affinities of this mixed micellar system for the drugs were comparable with those obtained with individual bile salts. The addition of lipids to the mixed bile salt system resulted in a decrease in hexestrol solubility but had little effect on griseofulvin and glutethimide solubility. The biological implications of the results obtained in the present communication are explored, and a mechanism for the role of dietary lipids and bile salts in the absorption of drugs is proposed.

BORGSTRÖM (1) has proposed a theory for the fate of ingested triglycerides prior to absorption. According to this theory, the breakdown products of pancreatic lipolysis (*i.e.*, 1- and 2-monoglycerides and fatty acids) are solubilized by bile salt micelles, present in the upper segment of the small intestine, prior to their absorption across the intestinal mucosa. In connection with this theory of fat absorption, several *in vitro* investigations have appeared in the literature demonstrating the marked micellar solubilizing properties of conjugated bile salts for fatty acids and monoglycerides (2–5). There has also been *in vivo* and *in vitro* evidence that the intestinal mucosa is capable of uptaking fatty acids and monoglycerides from a mixed micellar solution composed of these substances and conjugated bile salts (6–8).

In a previous communication (9) the effects of bile salt concentration and type, and temperature on the micellar solubilizing properties of bile salt solutions for the relatively water-insoluble drugs, griseofulvin, glutethimide, and hexestrol were reported. This report proposed that bile salts play a role in the dissolution step of the intestinal absorption mechanism for water-insoluble drugs.

In the present communication the results of findings on the influence of a mixed bile salt system, inorganic electrolyte concentration, and pancreatic lipolytic products and bile components on the degree of micellar solubilization of griseofulvin, glutethimide, and hexestrol are presented.

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EXPERIMENTAL

Materials.—Hexestrol,[1] griseofulvin,[2] and glutethimide[3] were used as received. The pure bile salts, sodium cholate,[4] sodium desoxycholate,[4] sodium taurocholate,[5] sodium glycochenodesoxycholate,[6] sodium glycodesoxycholate,[6] sodium taurochenodesoxycholate,[6] and sodium taurodesoxycholate[6] were dried *in vacuo* for 36 hr. prior to use. The first four bile salts were used individually to study the effect of inorganic electrolyte concentration on the degree of micellar solubilization of the three drugs, whereas, the latter six conjugated bile salts, representing those found in the human intestinal bile content, were used as a mixture to examine their influence on solubilization, alone and in the presence of lipid additives.

Reagent grade (Fisher Scientific Co.) sodium chloride, anhydrous sodium phosphate, and sodium biphosphate were used as received.

The lipid additives chosen for this study were those which are normal components of human bile or the end products resulting from the enzymatic action of pancreatic lipase during the process of digestion of ingested dietary triglycerides. The former compounds are represented by cholesterol,[7] and lecithin,[7] and the latter by lauric acid,[8] myristic acid,[8] palmitic acid,[8] 1-monolaurin,[9] 1-monomyristin,[9] and 1-monostearin.[9]

Effect of Inorganic Electrolyte on Solubilization.—Solutions of 0.04 *M* sodium cholate, sodium desoxycholate, sodium glycocholate, and sodium taurocholate were prepared and adjusted to 0.06, 0.08, or 0.16 *M* total sodium ion concentration by the addition of sodium chloride.

Effect of a Simulated Intestinal Bile Salt Mixture on Solubilization.—A stock bile salt solution, reported to approximate the human intestinal bile content (10) was prepared. It contained, per liter of final solution: sodium glycocholate, 0.030 mole; sodium glycochenodesoxycholate, 0.030 mole; sodium glycodesoxycholate, 0.015 mole; sodium taurocholate, 0.010 mole; sodium taurochenodesoxycholate, 0.010 mole; sodium taurodesoxycholate, 0.005 mole; and sodium chloride, 0.05 mole. The final stock solution was thus 0.1 *M* with respect to total bile salt concentration and 0.15 *M* with respect to sodium ion concentration. This solution was kept refrigerated when not in use.

A pH 6.4 phosphate buffer solution was prepared by mixing the following solutions in appropriate volumes to give the desired pH. A 0.3 *M* NaH_2PO_4 solution and a 0.15 *M* Na_2HPO_4 solution were mixed in a ratio of 55 parts of the former solution to 45 parts of the latter solution. The resulting solution was thus 0.3 *M* with respect to sodium ion concentration. This stock buffer solution was diluted 1:1 (v/v) with distilled water immediately before use.

The concentration of the simulated intestinal bile salt mixture was varied from 0–0.06 *M* by volumetrically mixing the 0.1 *M* stock solution with the appropriate volume of diluted pH 6.4 buffer. The pH of a 0.06 *M* solution prepared in this manner was approximately 6.48.

Preliminary experiments with this mixture showed that it supported the growth of bacteria under the conditions posed by the solubility experiments. Therefore, all solutions containing this mixture were sterilized by means of Millipore filtration (0.45 μ pore size) in the presence of ultraviolet light. Solutions sterilized in this manner showed no evidence of bacterial growth during the time required for the samples to reach equilibrium.

Effect of Lipid Additives on Solubilization.—In these experiments the simulated bile salt mixture (0.04 *M*), adjusted to the pH (about 6.4), and total sodium ion concentration (0.15 *M*) of the intestinal contents was employed. Predetermined amounts of the lipid additives were rotated for 1 to 2 hr., at 37°, in measured quantities of the bile salt mixture until solution was effected. The resulting clear solutions were then subjected to sterile filtration, and an excess quantity of drug added. These solutions were rotated at 37° until equilibrium was established.

The lipid additives, and the concentration in which they were employed are as follows: (*a*) 1-monolaurin, 0.40%; (*b*) 1-monomyristin, 0.20%; (*c*) 1-monostearin,[10] about 0.025%; (*d*) lauric acid, 0.40%; (*e*) myristic acid, 0.20%; (*f*) palmitic acid,[10] about 0.05%; (*g*) lecithin, 0.20%; (*h*) cholesterol,[10] about 0.025%.

The percentages of lipid additives selected were such that the mixed bile salt micelles were nearly saturated with these compounds. This condition is quite similar to *in vivo* conditions during fat digestion where the bile salt micelles are saturated with the digestive products (*i.e.*, fatty acids and monoglycerides).

A concentration of 0.04 *M* bile salt approximates the molarity of total bile salts present in the small intestine within 30 min. after the administration of a test meal containing corn oil (10).

Equilibration.—In each of the above experiments an excess amount of drug was added to bile salt solution contained in suitably sealed tubes. For those experiments in which the simulated intestinal bile salt mixture was employed, the drug was added under sterile conditions. The tubes were then placed in a shaker-incubator[11] and equilibrated for periods usually not less than 1 week's duration. Equilibrium was determined by repetitive sampling.

Assay Procedure.—The procedure employed for sampling and determining the amount of solubilized drug has been previously reported (9). Sodium chloride and all of the lipid additives, with the exception of lecithin, had no effect on the Beer's law curves for

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[1] Obtained from Gallard-Schlesinger Chemical Mfg. Co., New York, N. Y.

[2] Marketed as Fulvicin by the Schering Corp., Bloomfield, N. J.

[3] Marketed as Doriden by the Ciba Pharmaceutical Co., Summit, N. J.

[4] Obtained from Mann Research Laboratories, Inc., New York, N. Y.; special enzyme grade; reported to be > 99% pure.

[5] Obtained from Southeastern Biochemicals, Morristown, Tenn. Reported to be 98–99% pure by thin-layer chromatography.

[6] Obtained from Gallard-Schlesinger Chemical Mfg. Co., New York, N. Y. These bile salts were reported to be not less than 98% pure. Synthesized by T. J. Sas & Son, Ltd., London, England.

[7] Cholesterol S.C.W. (standard for clinical work) and the vegetable lecithin (95% pure) were obtained from Nutritional Biochemical Co., Cleveland, Ohio.

[8] Obtained from Mann Research Laboratories, Inc., New York, N. Y. Reported to be >99% pure.

[9] Supplied by the Distillation Products Industries, Rochester, N. Y., in 99.5% purity.

[10] Due to the very low solubility of these compounds in the bile salt mixture these percentages are approximate and essentially represent their saturation solubility.

[11] Gyratory incubator shaker, model G-25, New Brunswick Scientific Co., New Brunswick, N. J.

TABLE I.—EFFECT OF INORGANIC ELECTROLYTE (NaCl) ON THE MICELLAR SOLUBILIZING PROPERTIES OF 0.04 *M* BILE SALT SOLUTIONS ON GRISEOFULVIN, HEXESTROL, AND GLUTETHIMIDE AT 37°

Drug	Solubilizer	Total Sodium Ion Concn. 0.04 *M*	0.06 *M*	0.08 *M*	0.16 *M*
Griseofulvin[a]	Sod. cholate	6.9	6.3	6.8	6.5
	Sod. desoxycholate	9.1	8.3	9.2	8.7
	Sod. taurocholate	6.8	7.2	6.7	6.5
	Sod. glycocholate	7.0	6.5	7.2	6.8
	Water	...	...	...	0.7
Hexestrol[a]	Sod. cholate	122.0	127.6	136.2	175.6
	Sod. desoxycholate	139.4	142.5	152.0	...
	Sod. taurocholate	148.0	166.9	178.0	196.0
	Sod. glycocholate	143.0	164.6	170.9	192.1
	Water	...	...	...	0.8
Glutethimide	Sod. cholate	1.98	2.04	2.04	2.00
	Sod. desoxycholate	2.51	2.45	2.41	2.42
	Sod. taurocholate	1.90	1.93	1.93	1.86
	Sod. glycocholate	1.82	1.77	1.75	1.69
	Water	...	...	...	1.08

[a] Solubilities expressed as mg./100 ml. [b] Solubility expressed as mg./ml.

the three drugs, in the concentrations present in the solutions subjected to spectrophotometric analysis. In the case of lecithin, which does absorb, a blank solution containing the same concentration of lecithin and simulated intestinal bile salt mixture as the sample being analyzed was employed.

RESULTS AND DISCUSSION

Effect of Inorganic Electrolyte on the Degree of Solubilization.—The effects of total sodium ion concentration on the solubility of griseofulvin, hexestrol, and glutethimide in 0.04 *M* concentrations of the four individual bile salts at 37° are presented in Table I. As can be seen from this table, the solubility of griseofulvin and glutethimide, in all of the individual bile salts, is essentially constant over the entire sodium ion concentration range (0.04–0.16 *M*) studied. However, the solubility of hexestrol significantly increased with increasing total sodium ion concentration, in each of the bile salt solutions examined. Sodium chloride, in itself, has no solubilizing potential. For example, the solubility of each of the drugs in water and 0.16 *M* sodium chloride solution, respectively, at 37°, are: griseofulvin (1.4 mg./100 ml., 0.7 mg./100 ml.), glutethimide (1.20 mg./ml., 1.08 mg./ml.), and hexestrol (1.0 mg./100 ml., 0.8 mg./100 ml.).

According to the theory proposed for micellar solubilization by typical ionic surfactants, inorganic electrolytes function to shrink effectively the double layer surrounding the like-charged polar head groups of the surfactant molecules comprising the micelle. As a result, the electrical repulsions existing between adjacent charged surfactant molecules are reduced and the surfactant molecules are able to approach one another more closely in the micelle. This condition would, in effect, allow more surfactant molecules to enter the micelle with the result that the size of the micelle and therefore the volume of the hydrocarbon center of the micelle would increase. As a consequence of the increased volume, the solubility of a material which is solubilized by a mechanism involving incorporation into this region of the micelle (*i.e.*, nonspecific solubilization) should be enhanced. The enhancement noted in the solubilization of hexestrol, in the four individual bile salts, upon the addition of sodium chloride, is consistent with this theory (Table I). This suggests that hexestrol is solubilized by a "nonspecific" mechanism. A concomitant effect of inorganic electrolytes is to increase the degree of packing of the surfactant molecules in the micelle and thereby effectively reduce the volume in the palisade layers of the micelle. Consequently, the solubility of a compound which is solubilized by a mechanism involving incorporation into the palisade layers of the micelle (*i.e.*, specific solubilization) would either remain the same as in the absence of added electrolyte or decrease, depending on the concentration of electrolyte added to the surfactant system. The data given in Table I showing the effect of total sodium ion concentration on the solubility of griseofulvin and glutethimide in the four individual bile salts are consistent with the premise that these solubilizate molecules are solubilized by a mechanism in which they are more closely associated with the palisade layers of the bile salt micelle. This hypothesis, however, is not in agreement with tentative conclusions based solely on relative saturation ratio data (9, 11, 12).

Effect of a Simulated Intestinal Bile Salt Mixture on Solubilization.—The solubilization curves for griseofulvin, hexestrol, and glutethimide, in varying concentrations of the simulated intestinal bile salt mixture, at 37° are shown in Figs. 1–3, respectively. The CMC values for this mixture as determined from the solubilization of griseofulvin and hexestrol at 37° are 0.004 and 0.003 *M*, respectively. These values are in excellent agreement with that determined by Hofmann from the solubilization of azobenzene (5). This investigator obtained, under the same experimental conditions of pH, temperature, and total sodium ion concentration, a CMC value of 0.0035 *M* for this simulated intestinal mixture.

The CMC values obtained for the conjugated bile salt mixture are considerably lower than those previously obtained for the individual, conjugated bile salts, sodium glycocholate and sodium taurocholate at 37° (9). The lower CMC can be attributed to the effect of sodium ion concentration, the presence of more than one surfactant in the system, and the pH on the process of micelle formation. Inorganic electrolytes have been shown to

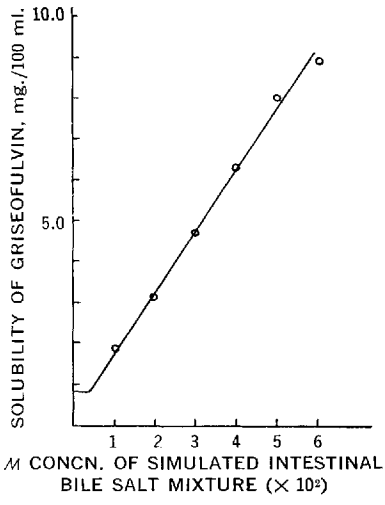

Fig. 1.—Solubility of griseofulvin as a function of simulated intestinal bile salt mixture concentration at 37°.

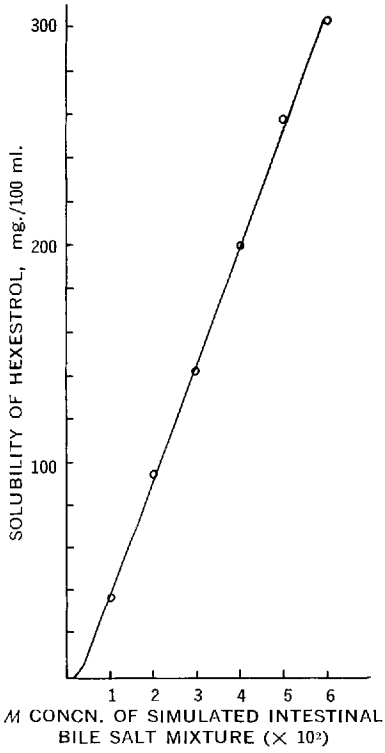

Fig. 2.—Solubility of hexestrol as a function of simulated intestinal bile salt mixture concentration at 37°.

decrease the CMC of ionic surfactants by decreasing the repulsive forces existing between adjacent charged surfactant molecules in the micelle (11, 12). The effect of mixtures of ionic surfactants on the CMC has a twofold effect (11, 12). First, at low concentrations the surfactants that have the lesser tendency to form micelles act as though they were salts to lower the CMC of the other surfactants. Second, at higher concentrations, the surfactants that have the lesser tendency to form micelles become important constituents of the micelle because they themselves are solubilized (12). The presence of these surfactants within the palisade layers of the micelle acts to stabilize the resulting mixed micelle through the formation of intermolecular van der Waal and hydrogen bonding. This stabilization also results in a decrease in the CMC. Normally, the effect of reducing the CMC is to increase micellar solubilization since at a given concentration of surfactant the number of micelles available are increased.

In the case of ionic surfactants the extent of ionization will be influenced by pH. The pH of an aqueous solution of the six conjugated bile salts, in water, is approximately 7.2. Since they are anionic in nature a decrease in the pH to 6.4 will significantly reduce the extent of ionization of some of the bile salts. In the micelle, those bile salts which are least ionized act to screen the repulsive forces between the bile salts which are ionized to a greater extent at this pH. The charge density on the micelle is also reduced. Thus, the over-all effect of pH is to increase the stability of the resultant mixed micelle and thereby lower the CMC.

The saturation ratios for griseofulvin, hexestrol, and glutethimide are included in Table II. For

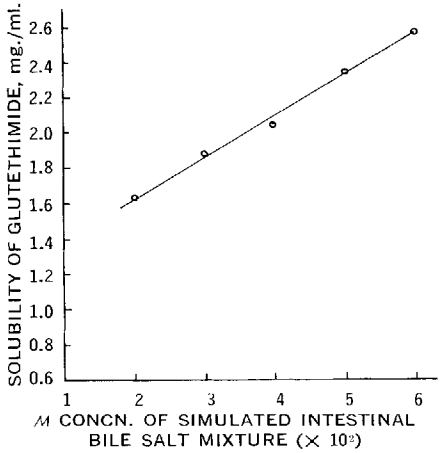

Fig. 3.—Solubility of glutethimide as a function of simulated intestinal bile salt mixture concentration at 37°.

TABLE II.—MAXIMUM SOLUBILIZING POWER OF BILE SALTS FOR GRISEOFULVIN, HEXESTROL, AND GLUTETHIMIDE AT 37°

Solubilizer	Saturation Ratio[a] × 10³ (moles of Drug/mole of Solubilizer) Griseofulvin	Hexestrol	Glutethimide
Sodium cholate[b]	6.18	195	104
Sodium desoxycholate[b]	6.18	167	163
Sodium taurocholate[b]	4.90	225	108
Sodium glycocholate[b]	5.13	231	71.8
Simulated intestinal mixture	4.22	197	108

[a] Slope of linear portion of solubilization curve, above the CMC, determined by the method of least squares. [b] Values obtained from *Reference 9*.

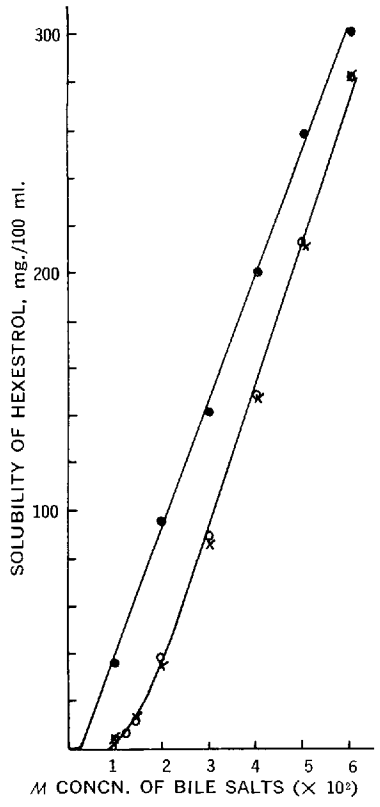

Fig. 4.—Solubility of hexestrol as a function of sodium taurocholate, sodium glycocholate, and simulated intestinal bile salt mixture concentration at 37°. Key: ●, simulated intestinal bile salt mixture; ○, sodium taurocholate; ×, sodium glycocholate.

griseofulvin, a comparison of the saturation ratios of all of the bile salts listed in Table II shows that at 37° the simulated intestinal mixture has the lowest saturation ratio, even though its CMC is the lowest. For hexestrol the simulated intestinal mixture has a saturation ratio lower than that for the conjugated bile salts, sodium taurocholate and sodium glycocholate, even though its CMC is considerably lower. In the case of glutethimide, the presence of the mixed bile salt system had little effect on the maximum solubilizing power (*i.e.*, saturation ratio).

As noted, the presence of more than one surfactant in the system should result in a decrease in the CMC of the system. As a result of the lowering of the CMC, solubilization should begin at a concentration lower than that for a system containing one surfactant. The results of this investigation are consistent with this theory. In addition, as predicted by theory, the amount of hexestrol solubilized at any one simulated intestinal bile salt mixture concentration is higher than that at the same concentration of sodium taurocholate or sodium glycocholate (Fig. 4). However, the increased degree of solubilization based on individual solubilities at a particular surfactant concentration should not be construed as indicating that the simulated intestinal bile salt mixture is a more efficient solubilizer than is sodium taurocholate or sodium glycocholate. As may be noted in Table II the saturation ratio, for hexestrol, obtained with the simulated intestinal bile salt mixture is significantly lower than that obtained with either conjugated bile salt, both of which are components of the mixture. This is indicative of a reduced affinity of the micelle for the solubilizate molecules. The same situation exists in the case of griseofulvin (Table II).

The formation of a mixed surfactant micelle is usually accompanied by a closer degree of packing of the surfactant molecules in the micelle. It is quite possible that this increased packing reduces the maximum solubilizing ability (*i.e.*, saturation ratio) of the simulated intestinal mixture as compared to a system containing one surfactant. The inclusion of surfactant molecules within the palisade layer of the micelle may preclude the effective penetration of solubilizate molecules.

Effect of Lipid Additives on Solubilization at 37°.—The effects of saturated fatty acids, 1-monoglycerides, lecithin, and cholesterol on the solubilization of griseofulvin, glutethimide, and hexestrol in 0.04 *M* simulated intestinal bile salt mixture at 37° are manifest in the data presented in Table III.

Nonpolar additives which are solubilized in the hydrocarbon center of the micelle would, according to theory, "swell" the micelle and effectively increase the volume available in the palisade layers of the micelle for the solubilizate molecules. Consequently, the solubility of a material normally solubilized by a "specific" process, should be enhanced. Whereas solubilizate molecules, normally solubilized by a "nonspecific" mechanism, should show a decrease in solubility upon the addition of nonpolar additives to the system. This results from a competition existing between the solubilizate and nonpolar additive for the space available in the hydrocarbon center of the micelle.

Amphiphilic additives (*i.e.*, compounds which are believed to be solubilized by a "specific" mechanism) act on the surfactant molecules comprising the micelle in a manner similar to inorganic electrolytes and would therefore essentially have the same effect on the solubility of solubilizate molecules. The nature of the effect would depend on the region of the micelle in which the solubilizate molecule normally resides (*i.e.*, specific or nonspecific solubilization).

All of the lipids listed in Table III, with the exception of lecithin and 1-monolaurin, have been shown to function as nonpolar additives in a system containing one bile salt at 37° (2–4). The decrease in the solubility of hexestrol in their presence can therefore be attributed to a competition between hexestrol and the nonpolar additives for the space available in the hydrocarbon center of the micelle. Although lecithin and 1-monolaurin have been reported to function as amphiphiles in a system containing a single bile salt (4, 5), it is conceivable that in a mixed surfactant system they are functioning as nonpolar additives. In the case of glutethimide and griseofulvin the addition of these lipids to the simulated intestinal bile salt system had little or no effect on the solubility of the drugs. However, a sufficient quantity of nonelectrolytes were present in the system such that they nearly saturated the mixed bile salt micelles. Under these saturated

TABLE III.—EFFECT OF LIPID ADDITIVES ON THE SOLUBILIZATION OF GRISEOFULVIN, HEXESTROL, AND GLUTETHIMIDE IN 0.04 *M* SIMULATED INTESTINAL BILE SALT MIXTURE (SIM) AT 37° (pH 6.4 [Na^+] TOTAL 0.15 *M*)

Solvent	Drug— Griseofulvin, mg./100 ml.	Hexestrol, mg./100 ml.	Glutethimide, mg./ml.
pH 6.4 buffer, Na^+ total 0.15 *M*	0.8	1.1	1.08
SIM, alone	6.3	200.0	2.04
SIM plus the fatty acids:			
Lauric acid, 0.40%	6.7	144.9	2.29
Myristic acid, 0.20%	6.3	160.6	1.94
Palmitic acid, 0.05%	5.5	176.4	1.84
SIM plus the 1-monoglycerides:			
Monolaurin, 0.40%	6.3	184.3	2.29
Monomyristin, 0.20%	5.9	178.7	2.21
Monostearin, 0.025%	5.5	192.1	1.88
SIM plus cholesterol (0.025%)	6.3	204.7	2.04
SIM plus lecithin (0.20%)	5.5	182.6	2.04

conditions it is highly probable that these nonpolar, lipid compounds, which are normally solubilized by a nonspecific mechanism, also penetrate into the palisade layers of the micelle. If such penetration occurs then competition with the griseofulvin and glutethimide solubilizate molecules would be expected. As a result, the increase in the solubility of these solubilizates which would normally occur in the presence of nonpolar additives, is counterbalanced by the decrease in solubility resulting from the competition between the solubilizate molecules and the nonpolar additives. It is conceivable that the solubilities of griseofulvin and glutethimide reported in Table III represent the net effect of these two opposing factors.

BIOLOGICAL IMPLICATIONS

The significantly high micellar phase to nonmicellar phase (*i.e.*, pH 6.4 buffer) partition coefficients found at 37° for hexestrol, griseofulvin, and glutethimide, (*viz.*, 2.69×10^5, 1.03×10^4, and 1.2×10^3, respectively) indicates that these relatively water-insoluble drugs are preferentially partitioned to the simulated intestinal bile salt micelle, in agreement with the theory proposed for the physical state of the pancreatic lipolytic products during fat digestion and absorption. Based on this similarity, it is quite reasonable to predict that bile salts play a role in the intestinal absorption of water-insoluble drugs.

The absorption and serum levels of griseofulvin have been shown to be enhanced, in humans, when the drug was administered in conjunction with high fat meals (13). A similar effect was observed in rats when griseofulvin was administered in corn oil (14). The present studies conducted to examine the influence of fatty acids, 1-monoglycerides, lecithin, and cholesterol have shown that these lipid substances have no critical effect on the solubilization of griseofulvin and glutethimide, but a decreasing effect on hexestrol solubility. Even though the solubility of hexestrol decreased in the presence of added lipid, its solubility is still significantly higher than that in the absence of bile salts and lipids (*i.e.*, 132–182 times its solubility in pH 6.4 buffer).

The present findings suggest a mechanism to explain the reported enhancement of drug absorption following the administration of a high fat meal. It is well known that triglycerides and other fatty material stimulate the flow of bile into the small intestine. The elevated bile salt concentration then serves to solubilize the drug to a degree far greater than its water solubility. From the limited observations with three different drug molecules it would appear that the concurrent solubilization of fatty material does not preclude significant solubilization of the drug molecules. The increased solubility results in an increased rate of dissolution (15) and, in turn, an increased absorption rate for any compound which manifests a dissolution rate-limited absorption process.

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Comparison of Different Photobeam Arrangements for Measuring Spontaneous Activity of Mice

By NATHAN WATZMAN, HERBERT BARRY, III, WILLIAM J. KINNARD, JR., and JOSEPH P. BUCKLEY

Effects of chlorpromazine (1, 2, 4, 8 mg./Kg., p.o.) and aggregation (one or five animals) were tested on activity of mice in a circular photocell activity cage. The use of two right-angle (crisscross) beams yielded a better delineation of low drug doses than the use of three parallel beams. Fewer counts were recorded from the middle of three parallel beams than from the same beam in the crisscross arrangement, suggesting that the tendency of rodents to stay near the periphery was enhanced when the audible clicks of the counter were generated by interruptions of the peripheral beams. Both beam arrangements showed higher counts for aggregated than single animals and also showed a significant dose-aggregation interaction, with chlorpromazine decreasing activity of aggregated more than single animals. The findings indicate certain optimal conditions for testing the effects of chlorpromazine on activity.

THE PHOTOCELL activity cage summates, on digital counters, light beam interruptions due to animals in motion. This instrument has been used extensively in the preliminary evaluation of potential psychotropic agents, for measuring their ability to alter the normal spontaneous locomotor activity of small animals. Variations of this apparatus, used for testing drug effects, have included a rectangular single-beam cage (1), and a circular arena with two beams (2) or six beams (3, 4) activating a single counter. Investigation of the conditions which maximize the sensitivity and reliability of this measuring instrument may contribute scientific knowledge about the interactions of experimental variables with drug effects and also may be of practical use in evaluating compounds.

The present paper reports on the use of a photocell activity cage with a separate counter recording from each of several beams. Thus, it is possible to compare different arrangements of beams recording the activity of the same animals simultaneously. The principal objective of this study was to determine whether photocell beams in different locations show significant differences in the magnitude of drug effects that are recorded. Studies with the six-beam photocell activity cage (3, 4) gave evidence that chlorpromazine (CPZ) may have a greater effect on activity of animals tested in aggregations of five rather than singly; the present study provided a test of this drug–aggregation interaction in the independent-beam photocell activity cage.

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Presented to the Pharmacology and Biochemistry Section, A.PH.A. Academy of PharmaceuticalSc iences, Dallas meeting, April 1966.

EXPERIMENTAL

Subjects.—The subjects were 360 male, Swiss-Webster albino mice (Taconic Farms, New York, N. Y.).

Apparatus.—Spontaneous locomotor activity was measured by the photocell cage available from Aidiation Electronics, Alexander, Va. This unit is a circular arena 13 in. in diameter and physically similar to the six-beam Actophotometer (Metro Industries, New York, N. Y.). The counters, which produce an audible clicking sound, are mounted on the front of the apparatus, near the base of beam D, shown in Fig. 1. Spontaneous activity can be measured by a single beam, or a combination of any two or three beams operating simultaneously. Also, the positions of any of the light sources may be changed so that a parallel or right-angle (crisscross) arrangement of the beams may be utilized. The experimental room was sound attenuated.

Experimental Design.—The animals were divided into two groups, one tested with two beams in a crisscross arrangement (Fig. 1, left), the other with three beams in a parallel arrangement (Fig. 1, right). Each group was subdivided into five subgroups which were given one of four dosages of CPZ (1, 2, 4, 8 mg./Kg. orally) or saline placebo (0.9%, 0.1 ml./10 Gm. body weight). These groups were further subdivided into two test aggregation conditions: singly or in groups of five. Thus, the experiment comprised a complete factorial design with every combination of two beam arrangements, five dosage levels (including placebo), and two aggregation conditions; the design was repeated 6 times. The animals were given a 60-min. test session in the photocell cage in a dark environment, beginning 60 min. after drug administration.

Statistical Treatment of Data.—The number of activity counts for the first 30-min. period were punched on IBM cards, then converted into square roots, and evaluated by the BMDO2V analysis of variance program on the IBM 7090 computer. In accordance with prior findings (3, 4), the use of the square root transformation, for the first 0.5 hr.

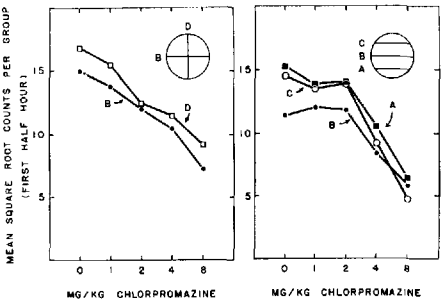

Fig. 1.—Effects of CPZ and beam arrangement on spontaneous activity recorded from each separate beam in the crisscross and parallel arrangements.

of the session, yielded a minimally skewed distribution of scores, with stable performance and large drug effects. For testing the dosage conditions (0, 1, 2, 4, 8 mg./Kg.), and interactions between dosage conditions and any other variables, the statistical significance test was based on the linear orthogonal polynomials, with 1 degree of freedom (5, 6), as used in the prior papers by Watzman *et al.* (3, 4). This test for linear trend assumes a progressive dose-response relationship.

RESULTS

Figure 1 shows the effects of the different dose conditions on activity recorded by each beam, separately for the crisscross and parallel beam arrangements. Different activity counts were recorded on beam B, depending on whether it was one of two crisscross beams or the middle one of three parallel beams. The analysis of variance, comparing this one beam in the different arrangements, shows a statistically significant over-all difference, with higher counts in the crisscross arrangement ($F = 10.1$, d.f. $= 1, 95$, $p < .01$).

A comparison between different beams simultaneously recording the activity of the mice also reveals some highly significant differences. The left graph of Fig. 1 shows a consistent difference between the two crisscross beams, with higher activity recorded on beam D ($F = 25.5$, d.f. $= 1, 50$, $p < .001$). This over-all difference was due to a highly significant interaction between beams and aggregation ($F = 17.5$, d.f. $= 1, 50$, $p < .001$), with beam D recording higher activity levels than beam B for the aggregated but not for the singly tested mice. No significant interaction was found between these two beams and the dosage conditions.

With the parallel arrangement, as shown in the right graph of Fig. 1, the two peripheral beams did not differ significantly from each other, but the middle beam produced lower activity counts, in spite of the fact that it covers the longest span. The quadratic term in the analysis of variance, which provides a comparison between the middle and peripheral beams, shows a significant over-all difference ($F = 20.8$, d.f. $= 1, 100$, $p < .001$). The greater drug decrement for the peripheral beams, which registered substantially higher activity counts than the middle beam for the groups given placebo and the lower doses but not for the groups given the higher doses, resulted in a significant interaction of beams with dosage conditions ($F = 8.35$, d.f. $= 1, 100$, $p < .01$). No significant interaction was found between these three beams and aggregations.

For testing the effects of drug doses and aggregation on activity, the two crisscross or three parallel beams were averaged together. For both arrangements (Fig. 2), the difference among dosage conditions was highly significant, with a similar order of magnitude ($F = 58.1$, d.f. $= 1, 45$, $p < .001$ for the crisscross arrangement; $F = 62.1$, d.f. $= 1, 45$, $p < .001$ for the parallel arrangement). However, an important difference may be seen in the pattern of drug effect. The crisscross arrangement shows a progressive decrease in activity with increasing doses, including a clear delineation between placebo and the lowest doses, but the parallel arrangement shows no consistent decrease in activity with the two lower doses. An analysis of variance limited to the placebo and lower two doses showed a significant dose–response relationship for the crisscross arrangement ($F = 10.9$, d.f. $= 1, 25$, $p < .01$) but not for the parallel arrangement ($F < 1$, d.f. $= 1, 25$).

Figure 2 also shows that higher activity counts were recorded with aggregated than single animals; this aggregation effect was greater with the crisscross arrangement ($F = 86.2$, d.f. $= 1, 45$, $p < .001$) than with the parallel arrangement ($F = 20.6$, d.f. $= 1, 45$, $p < .001$). With both beam arrangements, the drug produced a greater activity decrement in aggregated than single animals, as shown by a significant linear trend for the interaction between dosage and aggregation ($F = 8.76$, d.f. $= 1, 45$, $p < .01$ for the crisscross arrangement; $F = 6.64$, d.f. $= 1, 45$, $p < .05$ for the parallel arrangement).

DISCUSSION AND CONCLUSIONS

The natural tendency of rodents to stay near the periphery of the arena is indicated by the lower number of counts on the middle beam than on the peripheral beams in the parallel arrangement. Figure 1 shows that a significantly larger number of counts, with a superior delineation of small CPZ doses, was recorded from the same location (beam B) when it was one of two crisscross rather than one of three parallel beams. The different locations in which the animals activated the clicking noise of the counters thus produced a significant difference in locomotor behavior, with the crisscross and parallel arrangements. Mice have been shown to behave

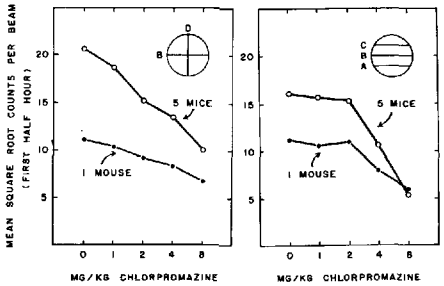

Fig. 2.—Effects of CPZ and aggregation on spontaneous activity; the separate beam recordings in the crisscross and parallel arrangements are pooled together.

so as to elicit noises of low intensity and pitch, comparable to the clicking sound of the counters (7). The present results indicate that the greater coverage of the peripheral portion of the arena, with the parallel beams, enhanced the natural tendency to stay near the periphery, and thus reduced the number of counts on the middle beam. With the crisscross arrangement, the more uniform coverage of all quadrants of the arena by the noise-producing beams apparently elicited a more consistent pattern of activity and dose–response relationship.

The higher counts produced by beam D than beam B, for the aggregated animals tested in the crisscross arrangement, indicates that the aggregated animals tended to clump together near the periphery, either near the counters (at the base of beam D) or away from them, thus activating beam D more often than beam B. The lack of any difference between the two peripheral beams (A and C), in the parallel arrangement, indicates that there was no consistent tendency either to approach or avoid the counters. A further comparison of the parallel beams also shows that the peripheral beams recorded higher counts than the middle beam for the animals under placebo and the low CPZ doses, but not for those under the highest dose. Apparently, the high doses of CPZ reduced the tendency to stay at the periphery of the arena.

With both beam arrangements there was a large dose–aggregation interaction, with CPZ producing a greater depression in activity of grouped than single mice. This indicates a greater tranquilizing effect of this compound in the stimulating social situation, in agreement with prior findings (3, 4). In the photocell activity cage with six crosscross beams recording on a single counter, the less consistent dose–aggregation interaction (4) may be due to failure of the single counter to record fully the high rate of beam interruptions during the intense activity of grouped animals in the placebo condition.

The six-beam Actophotometer is closely similar in dimensions and appearance to the independent beam instrument used in the present study. The counter which recorded activity in the six-beam unit was placed in a separate room and was inaudible to the animals. Apparently this condition of silence does not necessarily improve the delineation of drug effects; the present crisscross arrangement compares favorably with the six-beam unit in detecting the effects of low doses of CPZ. However, a separate experiment, with all other conditions equalized, would be necessary to test the effect of the audible counter clicks on spontaneous activity.

The superiority of the crisscross arrangement in detecting effects of small doses of CPZ, plus the greater drug effect with the peripheral than middle beam in the parallel arrangement, suggest that the most sensitive measure of effect of CPZ would be with an arrangement of two pairs of peripheral beams at right angles to each other, forming a tic-tac-toe pattern. The optimal conditions should probably include the use of animals in aggregations of five rather than singly as well as a separate counter for each beam.

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Distribution of Quaternary Ammonium Salts Between Chloroform and Water

By JOHN A. BILES, FOTIOS M. PLAKOGIANNIS, BEVERLY J. WONG, and PAULA M. BILES

The apparent partition coefficients, $K_{app.}$, of some alkylsulfate salts of six quaternary ammonium compounds and one tertiary amine are reported. The $K_{app.}$ of the corresponding bisulfate salts were determined by extrapolation. Some comparisons of molecular structures to the $K_{app.}$ are discussed. A method of analysis of the quaternary cations in the presence of long chain anions is reported. The relationship of longer crystal spacings of the sodium salts of alkylsulfates to the molecular weight is shown.

IN PREVIOUS communications (1, 2) it was reported that the partition of organic salts or complexes was determined by the molecular weight of the organic ions, the branching effect of the aliphatic amine cations, and the nature of the organic solvent system used. The authors showed that partitioning into the organic layer from the aqueous layer could be increased by the addition of proton donor molecules.

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workers (3–7) have discussed the results of their studies concerning the intestinal absorption of quaternary ammonium compounds. It was shown that the poor absorption of quaternary ammonium compounds was attributable to the formation of nonabsorbable complexes with mucin (3). Later Levine reported that, although intestinal mucus can form nonabsorbable complexes with quaternary ammonium compounds, removal of the mucus by washing the intestine resulted in a decrease rather than an increase in the absorption of these ions (4). This led to Levine's study of the absorption of a mixture of quaternary ammonium compound and a phosphatido-peptide fraction (PPF). In two separate reports Levine reported that a mixture of PPF and either benzomethamine or *d*-tubocurarine caused more efficient absorption of either quaternary ammonium compound (5, 6). Cavallito and O'Dell reported that the administration of certain sterol acids improved the oral responses to a quaternary hypotensive agent (8). Schanker has suggested that organic ions might penetrate the gastrointestinal-blood barrier by the diffusion of the ions through the barrier in the form of a less polar complex formed with some material normally present in the lumen. He also suggested that the absorption might occur by a specialized transport process analogous to those which transport certain inorganic cations (9).

These data indicate that it is possible that a cation–anion complex is formed which may facilitate the absorption of the quaternary ammonium compound. Therefore, a program was initiated to determine to what extent the presence of anions in aqueous solution containing quaternary ammonium compounds would become more soluble in lipoid solvents and determine to what extent, if any, these salts or complexes would affect the rate of intestinal absorption of the quaternary ammonium compound. The initial communication deals with the *in vitro* study of the partitioning of the alkylsulfate salts of quaternary ammonium compounds between chloroform and water. The *in vivo* studies will be the subject of a future communication.

EXPERIMENTAL

Reagents.—The sodium alkylsulfates were furnished by E. I. DuPont Co. Additional batches were synthesized. Benzomethamine was furnished by Squibb, oxyphenonium bromide by Ciba, methantheline bromide and propantheline bromide by Searle, isopropamide by Smith Kline & French, tridihexethyl iodide by Lederle, and 2-PAM chloride by A. Kondritzer. Homatropine HBr was purchased from Mallinckrodt Chemical Works. Chloroform U.S.P. and distilled water were used as partitioning solvents.

A cationic resin,[1] was cleaned and charged by first treating the resin with 5 *M* HCl and then washing repeatedly with distilled water to remove all traces of excess HCl. The resin was stored in distilled water.

Synthesis.—The sodium alkylsulfates were synthesized by refluxing the aliphatic alcohol (Matheson, Coleman and Bell) with a moderate excess of concentrated sulfuric acid. After refluxing and cooling, the oil was neutralized with sodium bicarbonate. Excess sodium chloride was added to precipitate the sodium alkylsulfate salt (10). The salt was recrystallized several times from alcohol–ether solutions. The degree of purity of the alkylsulfates was followed using X-ray powder diffraction. Carbon and hydrogen analyses were performed.

Preparation of Solutions.—Stock solutions of each of the sodium alkylsulfates were prepared by dissolving enough of the salt in distilled water to make a 0.0005 *M* solution. Also 0.0005 *M* concentrations of each of the quaternary ammonium compounds and homatropine hydrobromide were prepared by dissolving the required amount of solute in sufficient distilled water. The tropaeolin 00 stock solution was prepared as previously described (1).

Determination of the Apparent Partition Coefficients ($K_{app.}$).—The procedures previously described were modified (1, 2). It was concluded from initial studies that association of the cations and anions in aqueous solutions was very low when using very dilute concentrations. Initial studies also indicated the salt existed as ion–ion paired monomers in the organic phase (1). Therefore, a log–log plot of the concentrations of the salt in the chloroform *versus* the concentrations of the ions in the aqueous phase would yield a slope of 2 with the y intercept being equal to $pK_{app.}$

In applying these previous observations, equivalent concentrations of a specific sodium alkylsulfate and quaternary ammonium compound or homatropine were added to 4-oz. amber bottles and enough distilled water added to bring to 40 ml. (considered to be 40 Gm.). The concentrations of each solute were either 1.5, 2.5, 3.5, or 4.5 μm./40 Gm. To the 40 Gm. of aqueous solution was added 40 Gm. of chloroform. All stoppered bottles were shaken for at least 30 min. in an Eberbach horizontal shaker. Following the shaking, the liquids were separated by decantation or by using separators. Blank solutions were also prepared.

Analyses.—Dilute aqueous solutions of the quaternary ammonium compounds and homatropine were used to prepare standard curves. An appropriate amount, usually 0.5 to 1.0 μm., of each solute was mixed with 25 ml. of a saturated solution of tropaeolin 00. The mixed solution was shaken with aliquots of chloroform for extraction. A total of 50 ml. of the chloroform extracts was collected in a 50-ml. volumetric flask. No change in the standard curve was obtained if a slurry of the standard solution of the organic cationic agent and resin was filtered and then treated with tropaeolin 00 and extracted with chloroform.

The procedure for assay of the quaternary ammonium compounds and homatropine in the presence of equivalent concentrations of alkylsulfate was

[1] Marketed as Dowex by the Dow Chemical Co., Midland, Mich.

TABLE I.—TWO LONGER CRYSTAL SPACINGS OF THE SODIUM SALTS OF ALKYLSULFATES

Alkylsulfate	d_2	d_3
Octyl	14.97 Å.	9.86 Å.
Nonyl	16.30	10.75
Decyl	17.59	11.61
Undecyl	19.07	12.46
Dodecyl	20.21	13.32
Tetradecyl	22.50	14.79

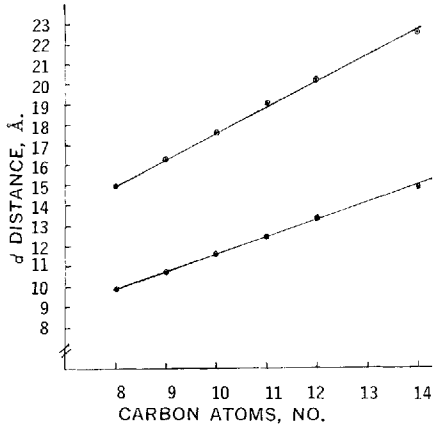


Fig. 1.—Two longer crystal spacings of the sodium salts of alkylsulfates.

modified because the anion interfered with the extraction of the "dyesalt" of the cation and tropaeolin 00. For successful analysis of the cation, enough solution containing approximately 1 μm. of cation was added to a 150-ml. beaker. The resin was then added to remove the anion. After standing for a short period of time, the solution was filtered into a 125-ml. separator. The resin collected in the funnel above the flask was washed with several small portions of distilled water. When washing was complete, 25 ml. of the saturated solution of tropaeolin 00 was added to the filtrate and the quaternary ammonium "dyesalt" was extracted with aliquots of chloroform. The chloroform extract was collected in a 50-ml. volumetric flask. All chloroform solutions were read at 425 mμ using a Beckman DU spectrophotometer.

Temperature Variation Studies.—The apparent partition coefficients of the quaternary ammonium alkylsulfates were determined at various temperatures ranging from 4° to 45°.

RESULTS AND DISCUSSION

Powder Diffraction Data.—The purification of the sodium salts of the alkylsulfates was followed by the change in the X-ray powder diffraction patterns.[2] Copper K-α radiation source was used. In general, it was found that the differences in peaks of diffraction became consistent as the alkylsulfates became pure. The d distances in angstroms for the second and third peaks are recorded in Table I and plotted in Fig. 1. The spacings obtained in these laboratories were not in complete agreement with those reported by Boyd *et al.* (11). It was also noted that distinctly different lines for the even-numbered and odd-numbered carbon atom chains were not observed as found with the fatty acids (12). This is illustrated in Fig. 1.

Apparent Partition Coefficients ($K_{app.}$).—Benzomethamine chloride was subjected to a detailed analysis since the studies of the intestinal absorption of this compound have been rather extensive (3). Both the homologous series of fatty acid salts and the alkylsulfates were studied. The fatty acids of molecular weight greater than decanoic acid were studied. The 0.0005 M solutions were prepared by adjusting the pH of the aqueous solution to 7.4 with NaOH to insure complete dissociation of the acid. It was found that partitioning did not occur with any of the fatty acid ions studied even when three or more equivalents of anion were mixed with one equivalent of cation. These observations can perhaps be explained by assuming that the partition constant of the fatty acid (even considering the almost total dissociation of acid) between chloroform and water is very large so that the fatty acid partitions in favor of the ion-ion pair of the fatty acid–ion and quaternary ammonium compound (13).

The 0.0005 M solutions of the sodium alkylsulfate salts were also used to study their effect on the partition of benzomethamine between chloroform and

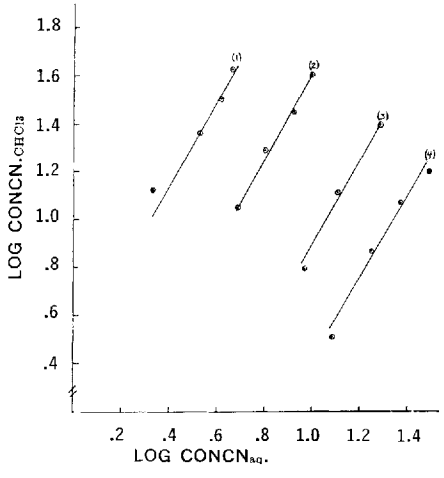


Fig. 2.—Log-log plot of the distribution of benzomethamine with 4 different concentrations of dodecyl sulfate (*1*), undecylsulfate (*2*), decylsulfate (*3*), and nonylsulfate (*4*).

TABLE II.—EXTENT OF ASSOCIATION OF ORGANIC CATIONS WITH ALKYLSULFATES IN CHLOROFORM–WATER MIXTURES

Organic Cation	Alkylsulfate Anion[a] C_9	C_{10}	C_{11}	C_{12}
Benzomethamine	1.89	1.75	1.83	1.70
Isopropamide	1.60	1.70	...	1.92
Oxyphenonium	1.84	1.40	1.50	1.60
Homatropine	...	1.78	1.85	1.80

[2] An XRD-5 spectrometer was used. The instrument was purchased with the aid of an Augustus P. Pfeiffer Foundation grant.

[a] Recorded values indicate the slope obtained by plotting log chloroform concentration against log aqueous concentration.

TABLE III.—APPARENT PARTITION COEFFICIENTS OF THE ALKYLSULFATES OF SOME ORGANIC CATIONS, EXPRESSED AS $pK_{app.}$

Organic Cation	Alkylsulfate Anion[a] C_8	C_9	C_{10}	C_{11}	C_{12}
Benzomethamine	...	3.95	4.48	5.22	5.87
Isopropamide	3.93	4.49	4.96	5.60	6.20
Oxyphenonium	4.30	4.66	5.52	5.88	6.33
Methantheline	...	4.07	...	5.86	6.68
Propantheline	...	5.60	6.63	7.54	8.56
Tridihexethyl	...	4.90	5.61	6.38	7.29
Homatropine	...	3.42	3.88	4.69	5.25

[a] $K_{app.}$ expressed as liters/mole.

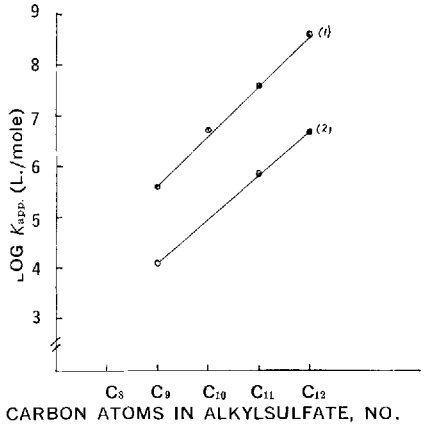


Fig. 3.—The apparent partition coefficients of alkylsulfates of propantheline (*1*) and methantheline (*2*) between chloroform and water.

water. The highest concentration of alkylsulfate used in the study was less than the concentration at which dimerization of anion has been observed (14). The sodium salts of nonyl-, decyl-, undecyl-, and dodecylsulfate were each used to determine their effect on the partition of benzomethamine. The log chloroformic concentration of benzomethamine was plotted against the log aqueous concentration of benzomethamine. The data obtained are plotted in Fig. 2.

The results for benzomethamine shown in Fig. 2 indicate that there was no apparent change in association over the 1.5–4.5 μM/40 Gm. concentration range studied. The slope for each anion varied from 1.70 to 1.89 as shown by the first entry in Table II. The slope would theoretically be 2.00 if dissociation of ions was complete in the aqueous phase and the complex existed as an ion-ion paired monomer in the chloroform. Similar studies were done using other organic cationic agents. The slopes of each cation–anion studied were calculated. The values are recorded in Table II.

The $pK_{app.}$ for the various alkylsulfates and the quaternary ammonium compounds and homatropine were determined. Since the extent of dissociation in the aqueous phase and the association in the chloroformic phase was similar for the various quaternary ammonium compounds listed in Table II, it was decided that the $pK_{app.}$ could be determined for the alkylsulfates of methantheline bromide, propantheline bromide, and tridihexethyl iodide using but one concentration, namely 2.5 μm. of quaternary ammonium ion and 2.5 μm. of alkylsulfate. The $pK_{app.}$ for all values listed in Table III were calculated from the 2.5 μM concentrations. In calculating the $pK_{app.}$ it was assumed that the slope would ideally be two, that is, that the ions were completely dissociated in the aqueous and that the ions existed as paired monomers in the chloroform. Thus, calculations were made using Eq. 1

$$pK_{app.} + 2 \log \text{concn.}_{aq.} = \log \text{concn.}_{CHCl_3} \quad \text{(Eq. 1)}$$

The calculated values are listed in Table III.

Partition studies were also performed using 2-pyridinealdoxime methiodide (2-PAM). However, the partitioning using sodium laurylsulfate was too low to determine any apparent partition coefficient. The data from Table III are plotted in Figs. 3 and 4. The data for methantheline and propantheline are plotted separately to compare more readily the two structurally similar compounds. When the linear plots in Figs. 3 and 4 are extended through the ordinate, one may posssibly conclude the intercepts to be the apparent partition constants for the acid sulfate (bisulfate) salts. These possible apparent partition constants may also be calculated knowing the change in the $pK_{app.}$ per carbon atom of the alkylsulfate. This change represents the slope of the line in Figs. 3 and 4. The information is recorded in Table IV.

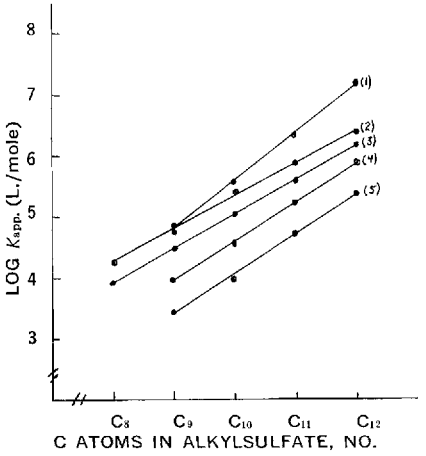
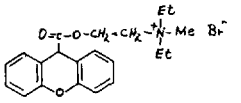


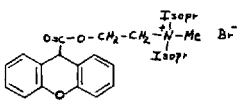
Fig. 4.—The apparent partition coefficients of alkylsulfates of tridihexethyl (*1*), oxyphenonium (*2*), isopropamide (*3*), benzomethamine (*4*), and homatropine (*5*) between chloroform and water.

TABLE IV.—APPARENT PARTITION COEFFICIENTS OF SOME ORGANIC AMINE SULFATES

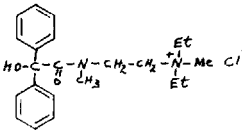
Organic Cation	$\Delta pK_{app.}$ per Carbon Atom in Alkylsulfate	$pK_{app.}$ for the Bisulfate Salt
Benzomethamine	0.64	−1.81
Isopropamide	0.55	0.50
Oxyphenonium	0.53	0.09
Methantheline	0.87	−3.76
Propantheline	0.99	−3.13
Tridihexethyl	0.81	−2.43
Homatropine	0.64	−2.37



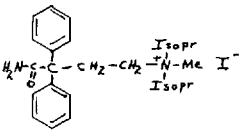
Methantheline Bromide



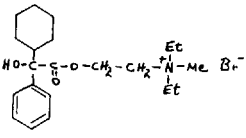
Propantheline Bromide



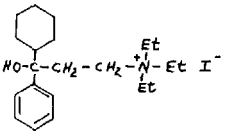
Benzomethamine



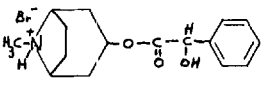
Isopropamide



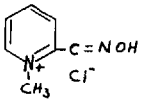
Oxyphenonium Bromide



Tridihexethyl Iodide



Homatropine HBr



2-PAM Chloride

Fig. 5.—Structures of quaternary ammonium compounds and one amine.

The $pK_{app.}$ values for the bisulfate salts were not determined because the ordinate intercept values were too small. Also the concentrations were too low in aqueous solutions for accurate determination.

The structures of the organic cationic agents listed in Table IV are illustrated in Fig. 5. When the data in Table IV are related to the structures given in Fig. 5, limited conclusions may be drawn. Some of the conclusions are made with the assumption that halide ions in the concentration present do not alter the apparent partition coefficient of the alkylsulfates. There is evidence that bromides and iodides in particular affect the distribution of organic cations between aqueous and organic solvents (7, 15, 16). A comparison of the partition studies of methantheline and propantheline bromides indicates that the $K_{app.}$ increases (*i.e.*, lipoid solubility increases) as the molecular weight increases. However, the change in $pK_{app.}$ per carbon atom change in the alkylsulfates is not the same for the two compounds. No conclusions can be drawn with respect to the $pK_{app.}$ of the alkylsulfates of tridihexethyl, benzomethamine, isopropamide, and oxyphenonium and their molecular structures. A comparison of tridihexethyl and oxyphenonium does indicate that an ester linkage and methyl group alter the $pK_{app.}$ considerably. A comparison of the structures of benzomethamine and oxphenonium does indicate that the degree of aromaticity and ester or amide alters the $pK_{app.}$ to a large extent. A comparison of the changes in structures of isopropamide and benzomethamine indicates that changes in functional groups alter the $pK_{app.}$ to a significant extent.

The apparent partition coefficients for the alkylsulfate salts of benzomethamine, tridihexethyl, and isopropamide were determined at various temperatures to determine to what extent, if any, iceberg structuring (17) around the nonpolar portion of the organic cation and anion occurred in aqueous solution. Studies were run at 4°, 23°, 32°, and 45°. No significant differences in $pK_{app.}$ values could be detected over this range of temperature. This was surprising; however, the solubility of water in chloroform may have contributed to a canceling effect. Nevertheless, it was of interest to find that significant differences occurred with one experimental study of benzomethamine and sodium laurylsulfate, but could not be duplicated with other batches of laurylsulfate. Carbon, hydrogen analysis indicated a quite small amount of adulteration of the one batch of sodium laurylsulfate. The adulterant present was not identified.

A useful analytical assay for quaternary ammonium compounds using the "dyesalt" method of analysis was developed when the aqueous solutions contained alkylsulfates. It was noted that very small amounts of the alkylsulfate would interfere with the extraction of the "dyesalt" even when huge amounts (relatively speaking) of the anionic dye was used. This indicated that the stability constant of the quaternary ammonium alkylsulfate was significantly high when compared to the tropaeolin 00 salt of the quaternary ammonium salt.

The intestinal absorption of the alkylsulfate salts of the various quaternary ammonium compounds illustrated in Fig. 5 is now being studied and will be the subject of a future communication.

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Requirements for the Growth of *Aspergillus versicolor* on Atropine Sulfate

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Three strains of *Aspergillus versicolor* were isolated from soil, obtained in pure culture, and grown with atropine sulfate as the sole source of carbon and nitrogen. Optimum growth was obtained at 25°, when cultures were aerated by continual shaking, and were buffered to pH 5.5 with 0.2 to 0.4 *M* phosphate. Optimal media contained 1.25 per cent atropine sulfate and were supplemented with vitamins and minerals. Growth rate was inhibited by higher substrate levels. Peak growth was attained in 5 days, following a 2-day lag period.

ALTHOUGH atropine is toxic for many microorganisms, microbial growth often is observed in aqueous solutions of the alkaloid. Little is known of the contaminating organisms, of other organisms capable of utilizing this alkaloid, or of the degradative pathways that are involved.

An organism that utilized atropine, hyoscyamine, scopolamine, tropine, or tropionone as sole carbon and nitrogen sources was first reported by Bucherer (1), who classified it as *Corynebacterium belladonnae* (Nov. spec.). Eighteen years later, phenylacetic acid and tropic acid were isolated from the growth medium, and the presence of atropinesterase was reported (2). In the interim, Kaczkowski (3) had reported that *Arthrobacter terregens* produced tropine, nortropine, tropic acid, and atropic acid, when grown with atropine as the sole source of carbon and nitrogen. Niemer (2) also isolated atropic acid during his studies with *C. belladonnae*, but only traces were obtained. He reasoned that atropic acid arose by dehydration during isolation and was an artifact rather than a metabolite of atropine.

Using complete disappearance from the growth medium as the criterion for degradation, Kedzia and co-workers (4) surveyed 744 strains for ability to degrade atropine. Fifty-four strains, one of which was an *Aspergillus*, degraded the alkaloid. In all instances, ability to degrade atropine was an unstable property that was reacquired when the organism was subcultured 7 to 15 times in a meat broth medium that contained atropine.

From a soil sample, the authors have isolated pure cultures of three strains of *Aspergillus* that utilize atropine as a sole source of carbon and nitrogen. The authors intend to use these strains in studies of atropine metabolism and as a source of atropine-degrading enzymes. This report describes the identification of the three strains and the establishment of optimal growth conditions for that strain which grew most rapidly in aqueous solutions of atropine sulfate.

MATERIALS AND METHODS

Isolation of Aspergillus Strains.—Harary's method (5) for obtaining nicotinic acid-dependent organisms was modified by using atropine sulfate, rather than nicotinic acid, as the sole source of carbon and nitrogen. Three strains of *Aspergillus*, a *Penicillium* strain, and one *Sclerotium* species initially grew together in atropine solutions. Standard mycological methods (6) were used to obtain the five organisms in pure culture. The ability of each to grow in atropine solutions was then tested, and only the three *Aspergilli* utilized atropine sulfate as a sole carbon and nitrogen source. These three strains were stored on sterile soil and were repeatedly subcultured on atropine-agar slants before use.

Identification of the Organisms.—For identification of organisms, cultural and morphological characteristics, determined by standard mycological methods (6), were compared with published data (7). Mycophil agar, Czapek's agar, and Czapek's agar containing 20% sucrose[1] were used routinely. Descriptions are based on observations of colonies arising from hyphal transfers, following examination of the colonies at weekly intervals for 2 months.

Measurement of Growth.—For the measurement of growth, distilled water suspensions of spores, diluted to contain 30 mcg. of nitrogen/ml., were used for inoculation. Spore suspensions were prepared from 15-day-old second transfers on

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[1] Czapek's agar is available from Difco, Detroit, Mich. Mycophil agar is available from Baltimore Biological Laboratories, Baltimore, Md.

agar slants that contained 1.5% agar, 1% atropine sulfate, and 0.025% $MgSO_4 \cdot 7H_2O$. Triplicate sets of conical flasks, each containing 20 ml. of the appropriate medium, were inoculated with 0.2 ml. aliquots of the standard spore suspension. With stated exceptions, the inoculated flasks were incubated at room temperature as stationary cultures.

At appropriate times, mats were filtered individually through previously dried and tared 8-ml. Pyrex crucibles, having fine porosity disks. Mats were washed with water until free of atropine, dried at 105° to constant weight, and progress curves were obtained by plotting mat weight as a function of duration of incubation. Each point on the progress curves was the average of at least three mat weights.

Maximum growth was read directly from the progress curves. Progress curves were extrapolated to the abscissa to estimate the time that growth was initiated. The lag phase was assumed to be that interval between inoculation and the initiation of growth. Rate of growth was determined from the slope of that tangent to the curve which passed through the time of growth initiation.

Stock Solutions and Buffers.—Reagent grade chemicals were used to prepare all solutions. Vitamin and alkaloid solutions were sterilized by filtration through a Morton bacterial filter, having an ultrafine fritted disk. All other solutions were sterilized by autoclaving for 15 min. at 15 lb. pressure.

Minerals, vitamins, and atropine as concentrated, sterile, stock solutions were added to previously sterilized buffer solutions. Phosphate buffers were used in all experiments and were 0.2 *M* final concentration, with stated exceptions.

For supplementation with $MgSO_4$, a final concentration of 0.025% of the hydrated salt was used routinely. When complete mineral supplements were used, 1 ml. of stock solution was added to each 100 ml. of culture medium. Each milliliter of the mineral stock solution contained 2 mg. of $FeCl_3 \cdot 6H_2O$, 2 mg. of $ZnSO_4 \cdot 7H_2O$, 2 mg. of $MnSO_4 \cdot H_2O$, and 40 mg. of $MgSO_4 \cdot 7H_2O$. These salts were selected to avoid the introduction of extraneous carbon and nitrogen sources and did not support growth in the absence of atropine or similar carbon and nitrogen source.

One milliliter of stock vitamin solution, which was added to each 100 ml. of medium when vitamins were used, containing 0.4 mcg. of biotin and cyanocobalamin, 40 mcg. of niacin, and 80 mcg. each of riboflavin, thiamine HCl, pyridoxine HCl, and calcium pantothenate. This concentration of vitamins did not support growth in the absence of atropine or other carbon and nitrogen source.

With stated exceptions, a final concentration of 1% atropine sulfate was used routinely. The alkaloid was sterilized as a 25% aqueous solution and an appropriate amount was added to previously sterilized buffer immediately before use.

RESULTS AND DISCUSSION

Identification of Organisms.—Cultural and morphological characteristics indicated that the three strains capable of utilizing atropine as a sole source of carbon and nitrogen were all strains of *A. versicolor*, differing in details of color, texture, and pigment formation.[2] Two of the three strains were green spored, one was rough textured, and the other was smooth textured. The third strain was an unstable, asporogenous variant that represented about 2% of the total population and quickly "reverted" to one or the other of the more stable forms. Except for data concerned with identification and descriptions, results reported here were obtained with the strain that grew most rapidly on media containing atropine as the sole source of carbon and nitrogen.[2]

Growth on Czapek's agar was very slow, colonies were 25–30 mm. in diameter after 4 weeks, sporulation was delayed, and no hulle cells were produced. The colony was flat, with few radial folds, having some floccose growth in the folds or in the center of the colony. The color changed during growth from an initial white, to light gray, through shades of yellow, orange, and green to a final, dark gray green or olive green. In some variants, the different colors appeared in definite zones, while in others there were irregular patches. Droplets of pink exudate appeared in younger colonies, particularly those with a maroon reverse. The reverse was usually maroon, but it was yellow in some strains. Relatively few well-developed conidial heads occurred, but there were many conidial chains borne on mycelial branches without vesicles.

Growth on Czapek's agar with 20% sucrose was somewhat more rapid than on Czapek's agar. Colonies were 35–45 mm. in diameter after 4 weeks, there was more abundant sporulation, and hulle cells were produced. The colony was flat, with a slightly raised center of floccose mycelium. Conidial heads were almost hemispherical, becoming radiate upon longer incubation. Color of the colony was extremely variable, with different shades of gray, green, yellow, orange, and occasionally brick red. Color occurred in definite, concentric zones or with some variant types in irregularly shaped patches. When the brick-red color occurred, the vesicles and sterigmata usually had the same color as the mycelium. Hulle cells of the *A. nidulans* type were produced abundantly throughout the mycelium, and in pale yellow clusters resembling perithecia.

Growth on Mycophil agar at pH 7.0 was very rapid, and sporulation occurred after only a few days' incubation at room temperature. After 4 weeks, colonies were 45–55 mm. in diameter. Small differences in colony color, colony texture, and degree of sporulation were much more apparent on Mycophil agar than on the other media. The colony types varied from relatively smooth and flat colonies, to raised colonies with considerable radial folding, to types having predominantly flocculose growth. Color ranged through pale green, to yellow green, to the darker shades of green, depending on age of the colony. Hulle cells were abundant throughout the mycelium, but were most abundant in clusters that appeared near the outer edges of the colony. These yellow clusters resembled irregularly shaped perithecia,

[2] Three apparently identical cultures of each type organism were examined and cataloged by Dr. C. W. Hesseltine and Dr. J. J. Ellis of A. R. S. Culture Collection, Peoria, Ill. The nine cultures were assigned A. R. S. numbers A-12232, A-12233, A-12234 (asporogenous variant), A-12235, A-12236, A-12237 (rough-textured variant), A-12238, A-12239, and A-12240 (smooth-textured variant). The organism used in the establishment of optimal growth conditions was A 12238.

TABLE I.—COMPARISON OF MORPHOLOGICAL CHARACTERISTICS[a] OF ONE ATROPINE-DEPENDENT STRAIN[b] WITH PUBLISHED DATA[c] FOR *A. versicolor*

Characteristic	*A. versicolor*	Atropine-Dependent Strain
Heads	Hemispherical to radiate. Up to 100–125 μ diam.	Hemispherical to radiate. 88–111 μ diam. Some chains of conidia borne on mycelial branches without vesicles.
Conidiophores	5–10 μ diam. Smooth	2.7–4.6 μ diam. Smooth
Vesicles	15–20 μ diam.	8.9–13.1 μ diam.
Primary sterigmata	8–9 × 3 μ	4.2–10.7 × 1.8–3.2 μ
Secondary sterigmata	5–10 × 2–2.5 μ	3–7.7 × 2.1–3 μ
Conidia	Globose, echinulate, 2.5–3 μ. Occasionally 3.5–4 μ	Globose, echinulate, 3.0–4.5 μ
Hulle cells	Up to 25 μ diam. *A. nidulans* type	Globose, thick-walled, 12.5–19.6 μ diam.
Perithecia or sclerotia	Absent	Absent. Clusters. Hulle cells resembling perithecia are present.

[a] Determined on Czapek's agar. [b] Smooth-textured variant, A. R. S. No. A-12,238. [c] Thom and Raper (7).

but contained only hulle cells with no evidence of asci or ascospores.

A comparison of the morphological characteristics of the green-spored, smooth-textured, atropine-dependent variant with published descriptions of *A. versicolor* (7) is shown in Table I. From the data, it was concluded that the strain, used to establish optimal growth conditions, is a variant of *A. versicolor*[3] capable of utilizing atropine as a sole source of carbon and nitrogen.

Effects of Hydrogen Ion Concentration on Growth.—Maximal and prolific growth was observed when the test organism was grown on dextrose and ammonium sulfate buffered to pH 6.5 for 5 to 7 days. In contrast, after 20 days' incubation in atropine sulfate solutions, growth of the organism was scanty. On the assumption that a change in substrate might be accompanied by a shift in the pH optimum, the effects of pH on growth rate and maximum growth were studied in an attempt to obtain better growth on atropine.

Stationary cultures were grown in liquid media buffered to different hydrogen ion levels in the pH range 5.0–8.0, flasks were processed for mat weight at 3-day intervals from 1 through 3 weeks, and average mat weights were determined for each pH at each incubation time. Progress curves were plotted, and maximum growth and growth rate were determined from the curves for each pH level. The effects of pH on growth rate and on growth maximum are shown in Table II.

Peak growth and rate of growth were maximal at pH 6.0, and rapidly declined within 0.5 pH unit on either side of the optimum. Maximum growth approximated that earlier seen in stationary cultures on glucose–ammonium sulfate at pH 6.5, but rate of growth on the atropine medium was much less than on the simple carbon and nitrogen source. This difference in growth rates caused the authors to consider vitamin and mineral supplementation.

Effects of Vitamin and Mineral Supplements.—Preliminary experiments disclosed a shift in the pH optimum to pH 5.5, increased growth, and increased growth rate in supplemented media. These observations were confirmed by studying the effects of supplements at both pH 5.5 and pH 6.0.

TABLE II.—EFFECT OF pH ON MAXIMUM GROWTH AND GROWTH RATE[a]

Initial pH	Max. Growth, mg. Mat Wt.	Growth Rate, mg. Mat/Day
5.0	24.6	4.3
5.5	41.0	6.0
6.0	55.0	8.4
6.5	40.2	4.5
7.0	31.9	3.5
7.5	30.9	3.0
8.0	21.8	2.0

[a] Growth of atropine-utilizing *A. versicolor* at room temperature, in stationary cultures, 0.2 *M* phosphate buffers, 1% atropine sulfate supplemented with 0.025% magnesium sulfate. Data taken from individual growth curves, as described in text.

Growth at both hydrogen ion levels, in solutions of atropine supplemented with magnesium, minerals, vitamins, and vitamins plus minerals was measured over 3 weeks, in the manner previously described. Throughout this period, growth on mineral, vitamin, and vitamin-mineral controls was insignificant (2–3 mg.). From the individual growth curves, maximum mat weights and growth rates were determined for each of the eight test media, and are shown in Table III.

Significant increases in maximum mat weight and the growth rate were observed in media supplemented with minerals or vitamins plus minerals. These increases were accompanied by a shift in the pH optimum from 6.0 to 5.5. Lysis was much more rapid at the higher hydrogen ion level, as is apparent from the two progress curves shown in Fig. 1. In formulating the "optimal" medium, vitamins were arbitrarily included, although their addition did not produce growth increases significantly greater than those produced by mineral supplements alone.

Effects of Temperature on Growth.—In early experiments, cultures were incubated at 25° because this temperature is recommended (8) for the growth of *A. versicolor* on simple carbon and

[3] The authors are indebted to Dr. C. W. Hesseltine and Dr. J. J. Ellis, A. R. S. Type Culture Investigations, Peoria, Ill., for their assistance and for confirming the identification of organisms used in this study.

TABLE III.—EFFECT OF SUPPLEMENTS ON MAXIMUM GROWTH AND GROWTH RATE AT pH 5.5 AND pH 6.0[a]

Supplement Added	Max. Growth, mg. Mat Wt. pH 5.5	pH 6.0	Growth Rate, mg. Mat/Day pH 5.5	pH 6.0
Magnesium sulfate	40.1	48.8	6.4	8.0
Minerals	56.3	45.9	9.5	7.8
Vitamins	21.6	11.1	1.9	1.6
Vitamins plus minerals	59.4	56.0	10.0	8.0

[a] Growth in stationary cultures, room temperature, 0.2 *M* phosphate buffers, 1% atropine sulfate solution, supplemented as indicated and as described in the text. Growth rates and maximum growth were determined from individual growth curves.

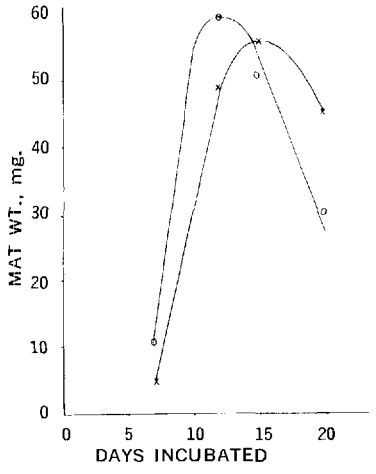

Fig. 1.—Growth of *A. versicolor* on optimal media adjusted to pH 5.5 and pH 6.0. Growth in stationary culture at 25°, 0.2 *M* phosphate buffers, 1% atropine sulfate supplemented with both minerals and vitamins. Key: O, pH 5.5; and ×, pH 6.0.

nitrogen sources. Since atropine is a complex carbon and nitrogen source, temperature effects on growth of the atropine-dependent strain were studied.

Using stationary cultures and optimal media adjusted to pH 5.5, growth curves were plotted for incubations at 0°, 8°, 20°, 25°, 30°, and 36°. Maximum mat weights, determined from the individual growth curves, were then plotted as a function of incubation temperature, as shown in Fig. 2.

It is apparent from the curve that there was a sharp temperature optimum at 25°, as reported for *A. versicolor* grown on simple carbon and nitrogen sources. Thus, the change from simple carbon and nitrogen sources to atropine did not affect the optimum incubation temperature.

Effects of Aeration.—Because fungi usually require good aeration for maximum growth, the effect of aeration by shaking was investigated. Two sets of flasks, one set incubated without shaking and the other shaken continuously on a rotary shaker, were incubated under otherwise identical conditions. At 2-day intervals or less, flasks were removed from each set in quadruplicate and were processed for mat weights. The results obtained at different time intervals, for shaken and stationary cultures, are compared in Table IV.

Aeration caused a striking increase in both growth rate and maximum mat weight, the lag phase was reduced from 6 days to 2 days, and maximum mat weights were 50% higher in shaken cultures. These conditions appeared to be nearly optimal because total mat weights, and the incubation period required, were almost identical with those observed in early experiments in which simple carbon and nitrogen sources were supplied for the organism.

Effect of Phosphate Concentration.—Because the *Aspergilli* usually grow best at phosphate levels between 0.01 and 0.05 *M*, the possible toxic effects of the phosphate concentrations used in development of the optimal medium were considered.

Cultures were grown 4 days, with continuous shaking at pH 5.5 and 25°, on media that were optimal except for varying phosphate concentrations. Four flasks for each of the following phosphate concentrations were employed: 0.00, 0.01, 0.02, 0.05, 0.10, 0.20, 0.30, and 0.40 *M*. Average mat weight was plotted as a function of phosphate concentration, as shown in Fig. 3.

Growth on atropine was very slight at phosphate levels below 0.15 *M* and increased with increasing phosphate, through the highest phosphate concentration tested. Since incubation time was constant and had not reached the optimum, maximum mat weights would not necessarily follow the same pat-

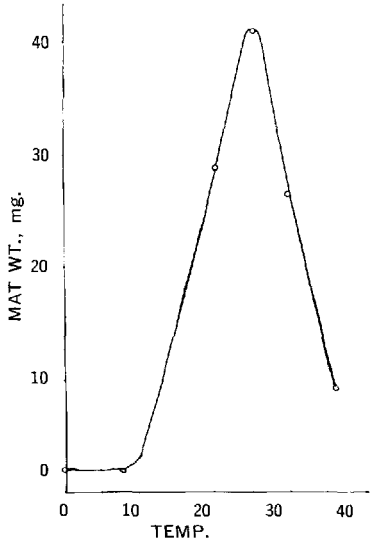

Fig. 2.—Effect of temperature on growth of *A. versicolor*. Growth on optimal medium, stationary cultures, pH 5.5. Maximum mat weight obtained from individual growth curves.

TABLE IV.—COMPARATIVE GROWTH OF *A. versicolor* IN SHAKEN AND STATIONARY CULTURES[a]

Incubation Time, Days	Max. Mat Wt., mg. Shaken Cultures	Max. Mat Wt., mg. Stationary Cultures
0	1.2	1.3
2	12.0	2.5
4	39.5	4.8
6	65.0	20.2
7	82.6	30.0
8	79.0	48.7
10	51.7	55.0

[a] Growth on optimal medium at pH 5.5, 0.2 *M* phosphate buffers. Each point is the average of 4 individually determined mat weights.

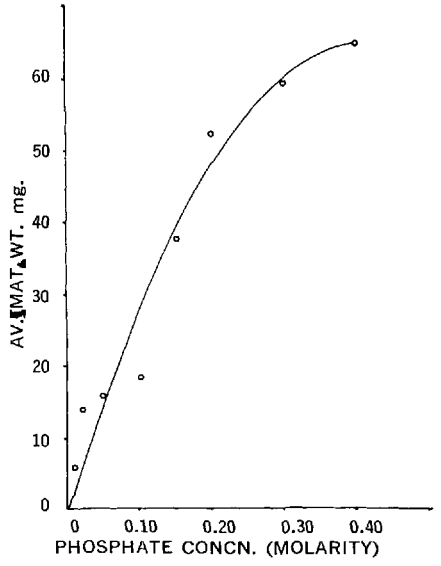

Fig. 3.—Effect of phosphate concentration on growth of *A. versicolor*. Growth for 4 days, continuous shaking, pH 5.5, on media that were optimal except for variations in phosphate levels. Each point is the average of four mat weights.

tern. In more prolonged incubations, the relative rates of growth and lysis would have an important influence on observed mat weights. Lysis was especially apparent in cultures grown in 0.3 and 0.4 *M* phosphate.

In contrast to the growth of *A. versicolor* on simple carbon and nitrogen sources, phosphate requirements are unusually high when atropine is the sole carbon and nitrogen source. This observation is unexplained, but may be associated with the complexity of the substrate, or with increased requirements for nucleic acid synthesis. More extensive studies would be required to clarify this point.

Growth as a Function of Alkaloid Concentration.—Unequivocal proof of atropine utilization by the strain of *A. versicolor* used in these experiments was obtained by relating growth to concentration of the alkaloid, in the absence of other sources of carbon and nitrogen.

Atropine concentrations were varied from 0.06 to 2.0%, using stationary cultures and otherwise optimal media and conditions. To obtain growth rate directly from mat weight, cultures were incubated for 12 days, at which time growth was submaximal and was increasing linearly with time. Growth rates, obtained by averaging triplicate mat weights at each atropine level, were plotted as a function of alkaloid concentration, as shown in Fig. 4.

There was a linear increase in growth rate throughout the concentration range 0–0.5% atropine, a maximum at 1.25%, and a sharp decrease beyond this point. In the absence of other carbon and nitrogen sources, the linear relationship between growth rate and atropine level confirms the ability of this strain of *A. versicolor* to utilize atropine as a sole carbon and nitrogen source. That growth rate is inhibited by high substrate levels is indicated by the existence of a maximum.

SUMMARY AND CONCLUSIONS

Three soil fungi, capable of utilizing atropine as a sole source of carbon and nitrogen, were obtained in pure culture, identified, and the identifications then independently confirmed. Although cultural and morphological characteristics differed somewhat from published data, all three were identified as strains of *A. versicolor*, differing in details of color, texture and pigment formation. These differences were far more apparent on the Mycophil agar used than on other media.

Optimal growth conditions, when atropine sulfate was the sole source of carbon and nitrogen, were determined for that strain which initially grew most rapidly in solutions of atropine supplemented with magnesium. For maximal growth, supplementation with vitamins and minerals was required, although the omission of vitamins had little effect. Using the supplemented medium, the organism grew best at 25° and pH 5.5. Aeration during growth greatly increased growth and decreased the induction period. Phosphate requirements were unusually high, good growth occurring at 0.2–0.4 *M* phosphate, but essentially none occurring below these levels.

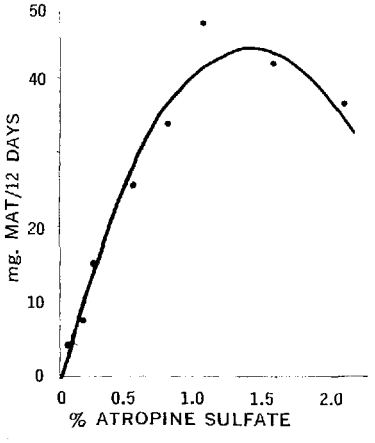

Fig. 4.—Growth of *A. versicolor* as a function of atropine concentration. Optimal media, pH 5.5, 0.2 *M* phosphate, stationary cultures. Each point is the average of three individually determined mat weights.

The need for high phosphate concentrations was not explained. Growth was optimal at an atropine concentration of 1.25% and was inhibited by higher concentrations of alkaloid.

The details of atropine utilization are being studied and will be reported at a later date, but there is evidence that an atropinesterase is involved. There is also evidence that growth requirements are quite specific for atropine, hyoscyamine, or their hydrolysis products.

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(6) Thom, C., and Raper, K. B., "A Manual of the Aspergilli," Williams and Wilkins Co., Baltimore, Md., 1946.
(7) *Ibid.*, pp. 185–186.
(8) *Ibid.*, p. 45.

Analysis of Steroids in Mixtures Using the Kinetics of Blue Tetrazolium Reduction

By DAVID E. GUTTMAN

The rate of formation of formazan resulting from the base-catalyzed reduction of blue tetrazolium by certain steroids was studied spectrophotometrically. With cortisone and hydrocortisone, the reaction rate exhibited a first-order dependency on steroid concentration. The rate constant for cortisone-containing systems was significantly larger than that found for hydrocortisone systems. Studies with cortisone acetate showed that hydrolysis of the ester was prerequisite to reaction with the tetrazolium salt. Differences in rates of color development were used to analyze mixtures of cortisone and hydrocortisone and of cortisone and cortisone acetate.

A WIDELY USED colorimetric method for the determination of the purity of corticosteroids and the potency of dosage forms containing such steroids is based on the formation of a colored formazan resulting from the base-catalyzed reduction of blue tetrazolium by the α-ketol side chain of the steroid molecule. The method consists of determining, after a specified time period, the intensity of color in a test preparation and comparing it with that produced under similar conditions in a standard preparation of the steroid under consideration. There have been a number of published studies of the rates of color formation in systems containing reducing steroids and blue tetrazolium and it has been observed that closely related steroids can exhibit significant differences in their rates of reaction with the tetrazolium salt. For example, Chen, Wheeler, and Tewell (1) presented data which suggested that color generation in cortisone-containing systems was much more rapid than in systems containing hydrocortisone. Meyer and Lindberg (6), in their extensive study, showed that the position and configuration of certain keto- and hydroxy-groups in the steroid molecule influenced reducing characteristics. Recknagel and Litteria (7) also demonstrated differences in reaction rate by their determination of the optimum incubation times for maximum color development for various steroids; *i.e.*, 30 min. for cortisone and 11-deoxycorticosterone as contrasted to 50 min. for corticosterone and hydrocortisone. Similarly, Izzo, Keutmann, and Burton (3) reported that reducing steroids with an 11-keto group developed maximum color faster than those with an 11-hydroxyl group. Martin and Salvador (5) found that acetylation of the 21-hydroxyl group decreased reaction rate relative to the parent alcohol, while Johnson, King, and Vickers (4) reported that with hydrocortisone hemisuccinate, and triamcinolone, color development was unusually slow compared to other steroids.

It has not been generally recognized that, under the conditions usually employed in the assay procedure where the steroid is present at a much lower concentration than that of the catalyst and the tetrazolium reagent, the rate of appearance of formazan exhibits a first-order dependency on the steroid concentration. This behavior is of potential analytical utility because of the lack of direct methods for analyzing mixtures of closely related steroids and because certain steroids which might be found or combined in mixtures do exhibit differences in the rate of this reaction. The present study was conducted to test the feasibility of utilizing such differences as the basis for analytical methods for the determination of steroids in mixtures. Toward this end, this laboratory has studied, under closely

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controlled conditions, the kinetics of the reaction of cortisone, hydrocortisone, and cortisone acetate with blue tetrazolium and has taken advantage of rate differences to analyze mixtures of cortisone and hydrocortisone, and of cortisone and cortisone acetate. The method of proportional equations, suggested by Garmon and Reilley (2), was used for this purpose and was found to yield reasonably accurate estimations of steroid concentration and to offer a rather convenient approach to what would normally be a difficult analytical problem.

PROCEDURE

Stock solutions of the steroids[1] were prepared in absolute alcohol at a concentration of 10 mcg./ml. Binary mixtures of two steroids were prepared by mixing aliquots of two solutions.

Cortisone–Hydrocortisone Systems.—Absorbance-time plots were obtained with a Beckman DB spectrophotometer and a Beckman linear-log recorder equipped with an event marker. The spectrophotometer was set at a wavelength of 525 mμ. A recorder speed of 1 in./min. was used. The cell compartment was maintained at constant temperature by the circulation of water at 24° ± 0.1° with a P.M. Tamson, N.V. constant-temperature circulating bath.

Twenty milliliters of steroid solution was placed in a 50-ml. glass-stoppered conical flask. Twenty milliliters of absolute alcohol was placed in a similar flask to serve as a blank. One milliliter of a 0.5% solution of blue tetrazolium in absolute alcohol was added to each flask. The recorder was started and 1 ml. of a 1% solution of tetramethylammonium hydroxide in absolute alcohol (prepared from a 10% solution in water) was added to each flask simultaneously and with mixing. The time of addition of base was marked on the recorder chart with the event marker. The solutions were placed in cells which were stoppered and placed in the cell compartment.

Cortisone–Cortisone Acetate Systems.—Absorbance-time plots for cortisone acetate-containing systems were obtained in the manner previously described. For the analysis of mixtures of these two steroids, a somewhat different procedure was used. Here, 20-ml. aliquots of steroid solution were pipeted into each of two glass-stoppered test tubes. Corresponding blank tubes were prepared to contain 20 ml. of absolute alcohol. One milliliter of the 0.5% solution of blue tetrazolium was added to each tube. The tubes were placed in a constant-temperature bath at 25° ± 0.1° and allowed to attain temperature equilibrium. One milliliter of the 1% solution of tetramethylammonium hydroxide was added to each tube, and the time of addition was taken as zero time. Exactly 8 min. after the addition of base, one steroid tube and a corresponding blank were quenched by the addition of 1 ml. of glacial acetic acid. Exactly 20 min. after the addition of base, the remaining steroid tube and its corresponding blank were similarly treated. Absorbance values at 525 mμ were determined for each steroid solution using the corresponding blank solution as the reference blank. Standard solutions of pure cortisone and pure cortisone acetate were treated in an identical manner.

Cortisone Acetate Ophthalmic Suspensions.—Two samples of commercially available cortisone acetate ophthalmic suspension were assayed by the kinetic method. The samples were obtained from a local pharmacy. One sample, according to the pharmacist "has been on the shelf for years." The other "was just received from the supplier." These will be designated as "aged suspension" and "fresh suspension," respectively. One milliliter of suspension was placed in a 50-ml. separator and diluted with 5 ml. of distilled water. The steroid was extracted with three 20-ml. portions of chloroform. The chloroform extracts were drained through a cotton plug into a 100-ml. volumetric flask. Chloroform was added to volume. Five-milliliter aliquots of the chloroform solution were placed in each of two 50-ml. glass-stoppered, conical flasks and the chloroform was evaporated using gentle heat. The residue in each flask was dissolved in 20 ml. of absolute alcohol. The analysis was conducted as previously described.

RESULTS AND DISCUSSION

Cortisone–Hydrocortisone Systems.—The time course for formazan appearance, as reflected by increase in absorbance at 525 mμ, is illustrated for each of the steroids in Fig. 1. It is apparent from this figure that formazan production occurred much more rapidly from cortisone-containing systems than from corresponding systems containing hydrocortisone. The semilog plot of Fig. 2 emphasizes this rate difference and illustrates that, under the conditions employed, the rate of appearance of formazan followed first-order kinetics. Here, the logarithm of the function $(A_\infty - A_t)$ is plotted as a function of time, where A_∞ is the asymptotic absorbance and A_t is the absorbance at a particular time. In each case, excellent linearity was observed over at least three half-lives. The pseudo first-order rate constant for cortisone was calculated to be 18×10^{-2} min.$^{-1}$ while that for hydrocortisone was 5.1×10^{-2} min.$^{-1}$. Treatment of the data by the Guggenheim method, which does not rely on an infinity value for absorbance, yielded the same rate constants.

For the analysis of the two steroids in combination, the method of Garmon and Reilley (2), that of proportional equations, was employed. In a reacting mixture containing both steroids, a common product, the formazan, is produced by two simultaneously occurring first-order processes. The absorbance at 525 mμ, which is specific for the product, is thus established at any time by:

$$A = aC(1 - e^{-k_c t}) + aH(1 - e^{-k_h t}) \qquad \text{(Eq. 1)}$$

where

A = absorbance at 525 mμ,
C = initial concentration of cortisone,
a = absorptivity of formazan at 525 mμ,
k_c = first-order rate constant for cortisone,
H = initial concentration of hydrocortisone,
k_h = first-order rate constant for hydrocortisone.

Measurement of two absorbance values at two

[1] The steroids were obtained from commercial sources and were of U.S.P. or N.F. quality.

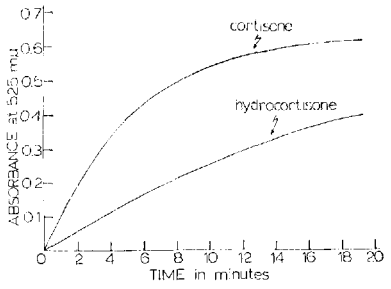

Fig. 1.—A plot illustrating the rate of reaction of cortisone and hydrocortisone with blue tetrazolium.

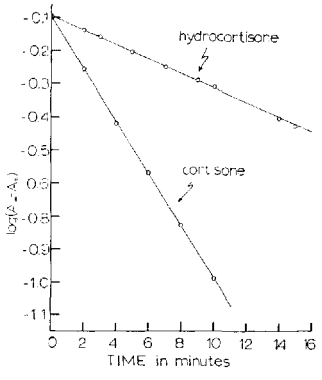

Fig. 2.—A plot illustrating the pseudo first-order appearance of formazan in the reaction of blue tetrazolium with cortisone and hydrocortisone.

times, a shorter time, t, and a longer time, t', allows the formulation of two equations which can be solved simultaneously to yield values for C and H, *i.e.*,

$$A = K_cC + K_hH \qquad \text{(Eq. 2)}$$

$$A' = K'_cC + K'_hH \qquad \text{(Eq. 3)}$$

where

A = absorbance value after the shorter reaction time, t,
A' = absorbance value after the longer reaction time, t',
$K_c = a\,(1 - e^{-k_c t})$; $K'_c = a\,(1 - e^{-k_c t'})$
$K_h = a\,(1 - e^{-k_h t})$; $K'_h = a\,(1 - e^{-k_h t'})$

Although the constants K_c, K'_c, K_h, K'_h can be calculated from a knowledge of rate constant, time, and absorptivity, it is more convenient to determine them directly from absorbance values, at the appropriate reaction times, exhibited by standard preparations of the pure steroids. Thus,

$$K_c = A_c/C_s;\quad K'_c = A'_c/C_s;$$
$$K_h = A_h/H_s;\quad K'_h = A'_h/H_s$$

where

C_s = concentration of the standard solution of cortisone,
H_s = concentration of the standard solution of hydrocortisone,

A_c and A'_c are the absorbances exhibited by the standard preparation of cortisone at times t and t', respectively.

A_h and A'_h are the absorbances exhibited by the standard preparation of hydrocortisone at times t and t', respectively.

Appropriate substitution into Eqs. 1 and 2 and solution of the simultaneous equations for C and H yields:

$$\frac{C}{C_s} = \frac{A - \frac{A_h}{A'_h}A'}{A_c - \frac{A_h}{A'_h}A'_c} \qquad \text{(Eq. 4)}$$

$$\frac{H}{H_s} = \frac{A - \frac{A_c}{A'_c}A'}{A_h - \frac{A_c}{A'_c}A'_h} \qquad \text{(Eq. 5)}$$

For the analysis of mixtures of these two steroids, the longer reaction time was chosen to be 20 min. A shorter reaction time of 5 min. was found to be optimum on the basis of the graphical approach to time selection which was recommended by Garmon and Reilley (2). Absorbance values at these reaction times were read directly from absorbance *versus* time plots which were obtained with mixtures and with standard solutions of the steroids. Results which illustrate the precision and accuracy obtained in the determinations are presented in Table I.

Cortisone–Cortisone Acetate Systems.—Absorbance-time plots obtained with cortisone acetate at a number of different concentrations of base are presented in Fig. 3. A cortisone curve is presented here for comparative purposes. It is apparent from these curves that the rate of reduction of the tetrazolium salt did not exhibit a first-order dependency on the concentration of the steroid ester.

Table I.—Analysis of Cortisone and Hydrocortisone Mixtures

Steroid Concn. Taken, mcg./ml.		Steroid Concn. Found, mcg./ml.	
Cortisone	Hydrocortisone	Cortisone	Hydrocortisone
9.00	1.00	9.30	1.04
		8.72	1.20
		8.55	1.77
		8.91	1.26
7.50	2.50	7.44	2.52
		7.78	2.20
		7.71	2.36
		7.67	2.50
7.00	3.00	7.10	2.93
5.00	5.00	5.12	4.78
		4.93	5.33
		5.41	4.60
		4.86	5.23
4.00	6.00	3.90	6.01
2.50	7.50	2.60	7.48
		2.84	7.40
		2.93	7.25
1.00	9.00	0.68	9.25
		1.33	9.00
		1.18	9.23
		1.07	9.12

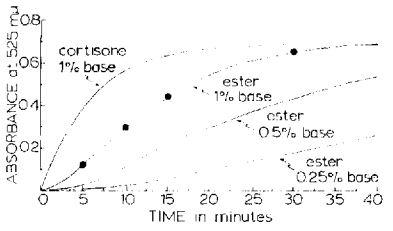

Fig. 3.—A plot illustrating the rate of reaction of cortisone and cortisone acetate with blue tetrazolium.

TABLE II.—ANALYSIS OF CORTISONE AND CORTISONE ACETATE MIXTURES

Steroid Concn. Taken, mcg./ml.		Steroid Concn. Found, mcg./ml.	
Cortisone	Cortisone Acetate	Cortisone	Cortisone Acetate
0.50	9.50	0.41	9.71
1.00	9.00	0.82	9.35
		0.92	9.10
3.00	7.00	3.05	6.85
5.00	5.00	4.61	5.02
		4.75	5.02
7.00	3.00	6.94	2.94
9.00	1.00	8.58	1.07
		8.46	1.07

TABLE III.—ANALYSIS OF CORTISONE ACETATE OPHTHALMIC SUSPENSION, 0.5%

Sample	Label Claim, mg./ml.	Cortisone Acetate Found,[a] mg./ml.	Cortisone Found[a]
"Aged" suspension	5	5.25	0
"Fresh" suspension	5	4.90	0

[a] Average of 3 determinations.

The curves are sigmoidal in shape and a lag-time in the appearance of formazan is observed. It is also seen that as the concentration of catalyst was decreased, the lag-time increased, and the sigmoidal shape of the curve was emphasized. The observed behavior indicates that here production of formazan was the result of a series of consecutive reactions. A logical assumption is that hydrolysis of the ester was prerequisite to a reaction resulting in the generation of formazan, *i.e.*,

$$\text{cortisone acetate} \xrightarrow[OH^-]{k_1} \text{cortisone} + \text{acetate}$$

$$\text{cortisone} + \text{blue tetrazolium} \xrightarrow[OH^-]{k_2} \text{formazan}$$

If the steroid is present in limiting concentration, k_1 and k_2 are pseudo first-order rate constants and the time course for increase in absorbance would be given by:

$$A = aE\left[1 + \frac{1}{k_1 - k_2}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})\right] \quad \text{(Eq. 6)}$$

where E = initial concentration of cortisone acetate.

The validity of this model was checked by calculating the value of k_1 by iteration based on a knowledge of k_2, a, E, and absorbance values at particular times. The value, in systems prepared from the 1% solution of tetramethylammonium hydroxide, was determined to be 12×10^{-2} min.$^{-1}$. The solid line of Fig. 3, corresponding to the condition of 1% base, represents observed behavior while the circles represent points predicted from the calculated rate constants.

Although the kinetics of formazan generation in reaction systems containing the cortisone ester is complex, absorbance at constant reaction time is directly proportional to the initial concentration of ester, and the method of proportional equations can, therefore, be used to analyze mixtures of cortisone and cortisone acetate. The derivation and nature of the equations used in this case are essentially identical to those discussed in the case of cortisone and hydrocortisone. Analysis of binary mixtures of cortisone and cortisone acetate were carried out and the results are shown in Table II. Here reaction times of 8 and 20 min. were found to be appropriate.

The method was also applied to aged and fresh samples of commercially available cortisone acetate ophthalmic suspension with the anticipation that the aged preparation might exhibit a detectable concentration of free alcohol. Results are shown in Table III. It was found that the cortisone acetate content in both preparations was consistent with the label claim and that negligible free alcohol was present. The low solubility of the steroid in the aqueous vehicle is probably responsible for the long-term stability of the product.

In applying this differential kinetic method to the analysis of dosage forms or to extracts from biological systems, the same types of interferences that are well recognized in the application of the conventionally used tetrazolium assay of steroids should be anticipated. Thus, for example, the presence in a sample of a nonsteroidal reducing agent, acids, or alkali would be expected to interfere and would necessitate a preliminary treatment by which steroidal components are separated from the interfering substances. In this method, as with other analytical procedures based on reaction kinetics, careful control of temperature and of the concentrations of catalyst and reagent was necessary for reproducible results.

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Absorption, Disposition, and Excretion of ^{3}H-Mineral Oil in Rats

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The fate in rats of ^{3}H-mineral oil was studied after both oral and i.p. administrations. Five hours following a single oral dose of 0.66 ml. of ^{3}H-mineral oil/Kg. about 1.5 per cent of the dose had been absorbed unchanged, and an additional 1.5 per cent of the dose was found in the carcasses as ^{3}H-nonmineral oil substances. The ^{3}H-mineral oil concentration in the carcasses decreased at first rapidly to 0.3 per cent within 2 days post-treatment, and much more slowly, thereafter, to 0.1 per cent of the amount administered by day 21. The absorption and excretion of mineral oil after chronic administration orally was similar to the absorption and excretion after a single dose. The physiologic disposition of the drug, 5 hr. after an oral dose, indicated that the liver, fat, kidney, brain, spleen, and carcass contained ^{3}H-mineral oil. After the i.p. administration of labeled mineral oil, it was excreted very slowly; 11 per cent was found in the feces during the first 8 days post-treatment; only trace quantities were found in the urine. The identity of the mineral oil following oral administration that had been isolated from tissues and excreta was made on the basis of thin-layer chromatography of tissue extracts. The similarity of the physical properties of the extracts of animals that had received mineral oil, i.p. or p.o., supported the view that it was mineral oil that had passed through the walls of the gut. The nonmineral oil nature of the substances containing ^{3}H after the administration of ^{3}H-water indicated that other substances were not mimicking ^{3}H-mineral oil in the assay. ^{3}H-mineral oil was shown to have exchanged ^{3}H with other substances. Metabolism of ^{3}H-mineral oil to more polar substances may also have occurred. The incorporation in the oil of dioctyl sodium sulfosuccinate, an emulsifying agent, tended to increase the amount of ^{3}H-mineral oil absorbed.

MINERAL OIL, liquid petrolatum U.S.P., is a mixture of thousands of compounds (1); it has been employed for over 50 years as a lubricant laxative. Despite its widespread and often chronic use, little is known of the physiologic disposition of this compound. Several reports have been published which have dealt with this problem, but the lack of a suitable assay and the indefinite composition of the mineral oil employed in the various studies made such studies difficult to evaluate, especially quantitatively (2–7). A comprehensive bibliography on this subject has been recently prepared (8). From the work of Stetten (5) and Bernhard and Scheitlin (6) it appeared that 5 to 25% of the administered doses to rats had been absorbed. This report contains data on the absorption, distribution, and excretion of mineral oil by rats that had received an oral or intraperitoneal dose of tritiated mineral oil, randomly labeled.

METHODS

Tritiated mineral oil (^{3}H-MO) which met the standards for liquid petrolatum U.S.P., except for the presence of radioactivity, specific activity 1.52 mc./ml., was administered to Sprague-Dawley and Holtzman rats of either sex at a dose of 0.66 ml./Kg., an amount equivalent to the recommended human dose. In studies of the fate of mineral oil after chronic administration, nonlabeled mineral oil was administered for 31 consecutive days at a dose of 0.66 ml./Kg. Tritiated mineral oil was given as the final dose on the 32nd day, and the animals were sacrificed at intervals thereafter. The effect on absorption of the oil of adding a wetting agent, dioctyl sodium sulfosuccinate, was studied. Radioactivity was measured in the alimentary tracts, carcasses, feces, and urines 24 hr. after the oral administration of tritiated mineral oil containing 1.7 or 8.3 mg./ml. of dioctyl sodium sulfosuccinate.

To study the excretion of the oil rats were placed in metabolism cages where food and water were provided *ad libitum*. Urine and feces were collected daily and stored at −15° until assayed.

In the distribution studies rats were anesthetized with ether and killed by exsanguination at suitable intervals following drug administration. After the hair had been removed with an electric clipper, the alimentary tract was exposed by a midline incision, carefully isolated, and ligated a few millimeters from the oral and anal ends. The tract and the remainder of the rat, defined as carcass, were separately weighed and homogenized with 4 vol. of toluene. In several studies visceral organs were removed for individual assays.

For the assays of radioactivity in the treated animals, tissues, as well as feces, were extracted with toluene 3 times. The residue of each sample, after toluene extraction, was extracted once with *p*-dioxane. Radioactivity that had been extracted from the tissues or excreta was measured in a liquid scintillation spectrometer after portions of the ex-

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tracts had been added to toluene or dioxane scintillation solutions.[1] Radioactivity that could not be extracted from the feces or tissues was measured by combusting portions of the extracted residue by the oxygen flask technique of Kelly *et al.* (10). Radioactivity in urine was measured by counting an aliquot of the urine in the dioxane scintillation solution directly.

This technique made it possible to extract in excess of 98% of the radioactive material from control tissues and from feces to which ^{3}H-mineral oil had been added or from tissues and feces of treated rats.

The presence of ^{3}H-mineral oil in the extracts of the tissues and feces of the treated rats was demonstrated by the use of thin-layer chromatography. The extracts were evaporated at temperatures <45° under reduced pressure to small volumes, usually less than 5 ml. Measured portions were applied to thin-layer chromatography plates of Silica Gel G 250 μ thick. The 30 × 20 cm. plates were activated before use by heating at 120° for 30 min. After activation, the plates were fully developed with Baker's reagent grade benzene to remove interfering impurities (11). The plates were dried at 100° for 5 min. and then spotted. They were developed in hexane–diethyl ether–glacial acetic acid (90:10:1 by volume) (12). ^{3}H-mineral oil was assayed after the plates had been developed by scraping small areas of the chromatogram into vials, adding a toluene or dioxane scintillation mixture, and measuring the radioactivity in a liquid scintillation spectrometer. Under these conditions ^{3}H-mineral oil could be detected on the chromatographic plate at a level of 5×10^{-3} mcg. at R_f 0.90 ± 0.02. The developed plates were visualized when necessary by spraying with 50% aqueous sulfuric acid followed by heating at 100° for 15 min. More polar substances remained at the origin or moved more slowly than mineral oil; they did not, however, separate into discrete areas, but remained as a long smear well separated from the ^{3}H-mineral oil. The quantitative nature of the recoveries was determined by applying appropriate standards to each of the plates before it was developed. Included among the standards were ^{3}H-mineral oil and extracts of control tissues to which ^{3}H-mineral oil had been added at the time the extract was applied to the chromatographic plate.

The lability of the ^{3}H label of the mineral oil was demonstrated by shaking 0.1 ml. of ^{3}H-mineral oil with 1 ml. of aqueous 0.01 *N* HCl. The water layer was carefully separated from the oil and washed 3 times with equal volumes of hexane. The water was vaporized at 95° and atmospheric pressure and condensed on a cold finger chilled with solid CO_2 and acetone. The condensate contained 0.1% of the radioactivity that had been present in the ^{3}H-mineral oil.

In another experiment 80 mg. of ^{3}H-mineral oil was dissolved in 1.0 or 100 ml. of propionic acid. The solutions were allowed to stand at room temperature for 18 hr.; they were then made alkaline by the addition of sodium hydroxide, 1.7 ml. to the first solution, and 17.0 ml. to a 10-ml. aliquot of the second. The alkalinized mixtures were extracted repetitively with heptane until the heptane washes were free of radioactivity. The tritium in the aqueous layers was measured by the use of Bray's solution (9). The aqueous layer of the 1-ml. solution contained 0.89% of the radioactivity, whereas the aqueous layer of the 100-ml. solution contained 3.76%.

Whether other substances accepted ^{3}H and mimicked the chromatographic characteristics of ^{3}H-mineral oil was investigated by administering orally to rats 0.5 ml. of ^{3}H-water with a specific activity of 340 μc./ml., the same dose of radioactivity that had been administered to the rats that had received ^{3}H-mineral oil. The tissues of these animals were subjected to the extraction and chromatographic separation described above for mineral oil.

RESULTS

After the administration of ^{3}H-mineral oil to rats, 85% of the radioactivity extracted from the tissues by toluene proved to have been ^{3}H-mineral oil. Most of the remaining 15% was present in unidentified, more polar substances that remained near the origin of the chromatogram. Of the material extracted with dioxane about 8% proved to have been ^{3}H-mineral oil on the basis of its chromatographic characteristics, the remaining 92% comprised unidentified, more polar substances.

Toluene and dioxane extracts of the carcasses of rats that had been dosed 24 hr. earlier with ^{3}H-water contained 0.3 and 40.9% of the radioactivity, respectively. After the extracts had been concentrated and chromatographed, however, no evidence of substances with the characteristics of ^{3}H-mineral oil was found in any of the tissues; in the carcass, *e.g.*, this was $<5 \times 10^{-4}$ mcg./Gm. based on the specific activity of ^{3}H-mineral oil.

The dispositions of ^{3}H-mineral oil and of tritium-containing substances other than mineral oil in rats that had received 0.66 ml. of tritiated drug/Kg. orally, either acutely or after chronic administration, are illustrated in Fig. 1. It is apparent that more than 80% of the total dose had not been absorbed before it was excreted in the feces. The highest concentration of mineral oil that had been absorbed by the rats at any time was 13.8 mcg./Gm. (1.6% of the dose); this was found 5 hr. after administration. Twenty-four hours after the dosing, the small amount of ^{3}H-mineral oil remaining in the rats was still largely in the alimentary tract, about 4.5% of the quantity administered; the carcass at that time contained about 0.6%, or 5.2 mcg. of mineral oil/Gm. of tissue. These levels continued to fall steadily, 48 hr. after the administration the levels of ^{3}H-mineral oil in the gastrointestinal tract and in the carcass had dropped to 0.7 and 0.3% of the dose, respectively.

Figure 1 shows that rats treated daily for 31 days with unlabeled mineral oil prior to the terminal dose of ^{3}H-mineral oil gave no evidence of facilitated absorption. The concentrations of ^{3}H-mineral oil found in the alimentary tracts and in the remainders of the animals were similar whether the rats had received the drug acutely or chronically.

[1] The toluene scintillation solution contained 4 Gm. of PPO, 0.05 Gm. of POPOP in 1 L. of toluene. The dioxane scintillation solution of Bray (9) contained 4 Gm. of PPO, 0.2 Gm. of POPOP, 60 Gm. of naphthalene, 20 ml. of ethylene glycol, 100 ml. of absolute methanol diluted to 1 L. with *p*-dioxane.

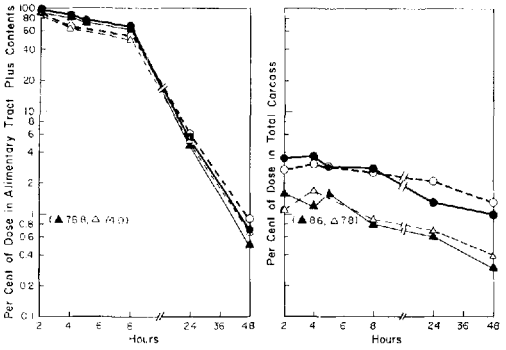

Fig. 1.—Disposition of radioactivity in alimentary tracts plus contents or in the carcasses of acutely or chronically dosed rats following an oral dose of tritiated mineral oil 0.66 ml./Kg. Each point represents the data from two animals in all the acute experiments except 24 hr. at which time five animals were assayed; three animals were assayed for each point in the chronic experiments. The difference between total ^3H and the ^3H-MO represents the radioactivity contained in substances other than ^3H-mineral oil. The figures in parentheses represent 1% of the dose expressed as mcg. of mineral oil/Gm. of tissue. Key: ●, total ^3H, acute; ○, chronic; ▲, ^3H-MO, acute; △, chronic.

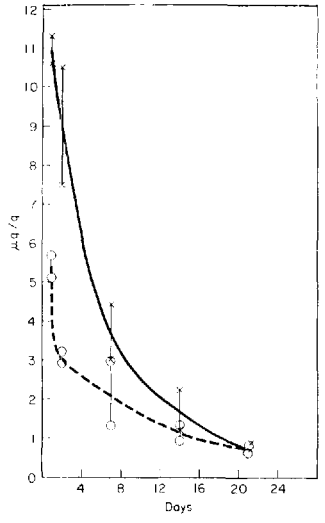

Fig. 2.—Long-term retention of ^3H-mineral oil in the carcasses of rats following a single oral dose of 0.66 ml./Kg. The curves have been drawn through the means obtained at each interval. Each mean was based on two animals as shown. Key: ———, total ^3H-substances; – – – –, ^3H-MO.

That the ^3H in the mineral oil exchanged with other substances in the rat accounted for the difference between the values of radioactivity in the ^3H-mineral oil and total radioactivity. The possiblity could not be ruled out that metabolism of ^3H-mineral oil may have contributed to the non-mineral oil substances that contained ^3H. In the alimentary tract the ratio of radioactivity of ^3H-mineral oil to the radioactivity of other substances was about 10:1 during the period when most of the drug was still in the animal; it had dropped to about 8:1 at 24 hr. and to 3:1 at 48 hr. post-treatment.

In the carcass, however, the evidence for metabolism of the oil or for exchange of ^3H from the ^3H-mineral oil to other substances was more apparent. At 24 and 48 hr. post-treatment the ratios of the radioactivity in ^3H-mineral oil to the radioactivity of other substances were 1:2 and 1:3, respectively.

The long-term retention of mineral oil in the carcasses of rats is summarized in Fig. 2. The initial

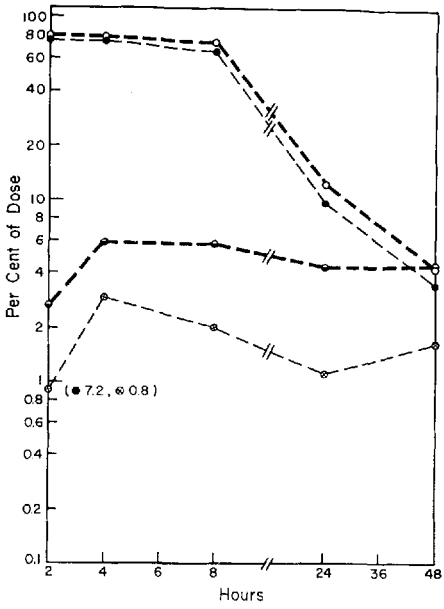

Fig. 3.—Disposition of radioactivity in the alimentary tracts plus contents or in the carcasses of rats chronically treated orally with mineral oil at various periods after the administration of a final dose of 0.066 ml. of ^3H-mineral oil. (See text for details.) Each point represents an assay of a single animal. The figures in parentheses represent 1% of the dose expressed as mcg. of mineral oil per Gm. of tissue. Key: ○, total ^3H, alimentary tracts plus contents; ◒, total ^3H, carcass; ●, ^3H-MO, alimentary tract plus content; ×, ^3H-MO, carcass.

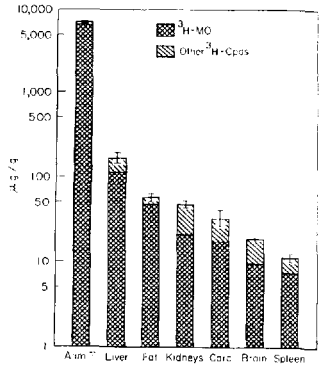

Fig. 4.—Disposition of radioactivity 5 hr. after oral administration to rats of tritiated mineral oil 0.66 ml./Kg. Height of each bar indicates the mean concentration per Gm. of tissue. Bars indicate the range of the actual values (n = 2).

sharp drop in the concentration of mineral oil may have been due to the elimination of absorbed mineral oil in the bile and also to the effects of exchange of ^3H. The slope of the curve of ^3H-mineral oil changed sharply by day 2, and, thereafter, it reflected a much slower elimination of the mineral oil. The convergence of the curve denoting the total radioactivity with the curve for ^3H-mineral oil attested to the slower rate of exchange or other change in the ^3H-mineral oil components after day 2. This was reasonably anticipated since all the ^3H atoms in the mineral oil were not equally active; those that were most active would exchange with H more rapidly than the rest. Significant concentrations of the mineral oil, 0.7 mcg./Gm. (0.1% of the amount administered), were detected in the carcasses 21 days after the single oral dose.

Evidence that the amount of mineral oil absorbed was dependent on the dose given was obtained in a chronic experiment in which rats received orally 0.066 ml. of ^3H-mineral oil/Kg., one-tenth of the usual dose. This experiment was carried out by administering orally 0.66 ml. daily of unlabeled mineral oil/Kg. for 31 consecutive days. On the 32nd day the animals received orally one-tenth the usual dose, 0.066 ml. of ^3H-mineral oil/Kg.; they were sacrificed at intervals after the final dose. The results of this experiment (Fig. 3) indicated that the carcasses of the rats that had received the smaller dose contained about 1% (0.8 mcg./Gm.) of the ^3H-mineral oil that had been administered 24 hr. earlier, whereas the carcasses of the rats that had received the normal dose contained after a comparable interval 0.7% (5.5 mcg./Gm.) of the amount administered. These data seemed to offer preliminary evidence that the passage of mineral oil through the gut wall resulted from diffusion. Clearly, additional animals would need to be tested before a firm conclusion could be reached.

The physiologic disposition in various organs and in fat 5 hr. after a single oral dose of 0.66 ml. of ^3H-mineral oil/Kg. is summarized in Fig. 4. The livers contained relatively high concentrations of the mineral oil at that time, 110 mcg./Gm., 0.5% of the dose. The concentrations in the fat, kidneys, brains, and spleens were relatively low; they ranged from 50 to 8 mcg./Gm. It was calculated that these organs contained a total of less than 0.2% of the dose. The remainder of the carcasses[2] contained 17.5 mcg./Gm., or 1.6% of the dose.

As was indicated earlier, the major portion of the dose, 75%, still remained in the alimentary tracts 5 hr. after dosing. Very little alteration in the ^3H-mineral oil occurred during that period. Of the radioactivity in the alimentary tracts, only 5% was found in substances other than mineral oil. In the carcass of the rat, however, the concentration of ^3H-nonmineral oil substances was somewhat higher. This may have been due to metabolism of the oil, to the preferential absorption of substances other than mineral oil that had been labeled with ^3H, by exchange in the gut, or to the increased opportunity for exchange after absorption of the mineral oil.

[2] The carcass in Fig. 4 represented the remainder of the animal after the other organs and tissues discussed above had been removed.

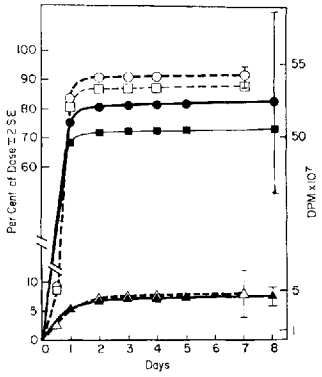

Fig. 5.—Cumulative excretion of radioactivity by rats orally dosed with tritiated mineral oil 0.66 ml./Kg. Each point in the chronic experiment represents the mean for two animals. Each point in the acute experiment represents the mean for four animals, except days 1 and 2, at which times 11 and six animals, respectively, were used. The difference between total ^3H and ^3H-mineral oil represents DPM of substances other than mineral oil, which contained ^3H, expressed as per cent of the administered dose. Only trace quantities of mineral oil were found in the urine, 0.3–2.5 × 10^{-3}% of the dose in the first 24 hr. after administration. Key: ▲, total ^3H in urine, acute; △, in urine, chronic; ●, in feces, acute; ○, in feces, chronic; ■, ^3H-MO in feces, acute; □, in feces, chronic.

The cumulative excretion of radioactivity following oral administration of the drug is illustrated in Fig. 5. The major portion of the drug was excreted during the 24-hr. period after the dose had been administered. About 80% of the dose was recovered in the feces of acutely dosed rats during the first 2 days after treatment. Levels of radioactivity recovered in the feces of chronically treated animals were slightly, but not significantly higher, than the values for acutely treated animals. Two standard errors about each point were calculated. Typical values are given about the points at days 7 and 8. As with the radioactivity in the alimentary tract over 90% of the ^3H in feces was in the form of mineral oil.

Seven to 8% of the radioactivity administered was excreted in urine during the week following drug administration. Samples collected for an additional week contained an additional 1 to 2% of the dose. Several urine samples were exhaustively extracted with toluene; the toluene extracts were carefully concentrated and portions of the concentrate were chromatographed. In five separate experiments less than 2% of the radioactivity in the urine excreted during the first day after dosing was extractable by toluene; the amount of ^3H-mineral oil in the urine varied between 0.3 and 2.5 mcg./day (0.3–2.5 × 10^{-3}% of the dose).

The fate of mineral oil administered i.p. was studied, not only to provide information about the disposition of the oil after this route of administration, but also to learn if any difference could be detected between the original ^3H-mineral oil and the ^3H-mineral oil isolated from the tissues of the body after the drug had passed through the walls of the gut.

The results of this experiment (Table I and Fig. 6)

TABLE I.—CONCENTRATION OF ^{3}H-MINERAL OIL AND NONMINERAL OIL SUBSTANCES CONTAINING ^{3}H IN THE TISSUES OF RATS AFTER THE ADMINISTRATION OF ^{3}H-MINERAL OIL[a]

	mcg./Gm.			
	Oral		I.p.	
Tissue	^{3}H-Mineral Oil	^{3}H-Nonmineral Oil Substances	^{3}H-Mineral Oil	^{3}H-Nonmineral Oil Substances
Liver	21.7	19.3	432.5	31.2
Kidney	3.3	6.2	174.9	70.8
Brain	3.4	2.7	6.5	2.9
Fat	21.4	4.0	20,235.2	<1.0

[a] Rats received 0.66 ml. of ^{3}H-mineral oil/Kg. orally (n = 2) or i.p. (n = 3). Twenty-four hours later they were sacrificed and the tissues assayed as described in the text. The specific activity of the nonmineral oil substances was assumed to have been the same as that of ^{3}H mineral oil.

revealed that the mineral oil isolated after an i.p. injection exhibited the same characteristics in the extraction and assay as that isolated after oral administration; this provided additional evidence that mineral oil had been absorbed from the gut.

It may be seen from Table I that the concentrations of labeled material in the brain were relatively unaffected by the route of administration. It is also apparent that after oral administration the nonmineral oil fractions of the total radioactivity, found in the respective tissues, were greater than after i.p. administration.

The excretion of mineral oil after an i.p. injection was found to have been relatively slow. Only 11% of the dose of radioactivity was excreted in the feces during 8 days post-treatment (Fig. 6). About 95% of the radioactivity in the feces was ^{3}H-mineral oil. The chronic i.p. administration of mineral oil did not alter the pattern of excretion exhibited after a single acute i.p. dose. Because of the relatively wide variation from animal to animal there was no significant difference in the quantities excreted in the feces following a single acute injection or following chronic i.p. administration of the drug.

The urine excreted during the first 8 days post-treatment contained 8% of the total radioactivity injected. During the first 24 hr. after i.p. dosing 0.4 mcg. of ^{3}H-mineral oil was excreted in the urine (0.3×10^{-3}% of the dose).

The influence of an emulsifying agent on the absorption and disposition of mineral oil was tested by treating rats with ^{3}H-mineral oil containing 1.7 or 8.3 mg. of dioctyl sodium sulfosuccinate/ml. The results of this experiment are summarized in Fig. 7. Total radioactivity excreted in the urine and feces, which accounted for the major portion of the dose, was not significantly altered from control values by the addition of dioctyl sodium sulfosuccinate to the mineral oil. When one considered only the relatively small fraction of the dose that had been absorbed, however, the levels of ^{3}H-mineral oil in the carcasses were found to have been higher in both groups that had received mineral oil containing the emulsifying agent.

In terms of total radioactivity in the carcasses both preparations containing the additive seemed better absorbed than the oil alone. The increased quantities of ^{3}H-mineral oil and of ^{3}H-nonmineral oil substances found in the carcasses amounted to a total of 0.7% of the dose, a barely significant increase. In terms of ^{3}H-mineral oil in the carcasses, the presence of the additive produced concentra-

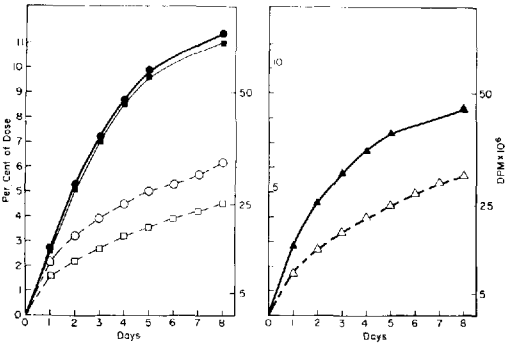

Fig. 6.—Cumulative excretion of radioactivity by rats intraperitoneally dosed with tritiated mineral oil, 0.66 ml./Kg. Each point represents the mean of three to five animals. A trace of mineral oil was found in the urine, 3.0×10^{-4}% of the amount administered in the first 24 hr. after dosing. Key: ▲, total ^{3}H in urine, acute; △, in urine, chronic; ●, in feces, acute; ○, in feces, chronic; ■, ^{3}H-MO in feces, acute; □, in feces, chronic.

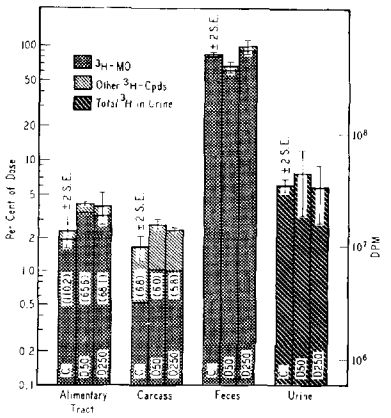

Fig. 7.—Disposition of radioactivity in rats 24 hr. after oral administration of ^{3}H-MO (C) or tritiated mineral oil containing dioctyl sodium sulfosuccinate, 1.7 mg./ml. (D50) or 8.3 mg./ml. (D250). Height of each bar indicates the mean percentage of the dose recovered. In control animals the means ±2 S.E. are listed (n = 3). In experimental animals, the mean, and the value for each animal are given (n = 2). The figures in parentheses represent 1% of the dose expressed as mcg. of mineral oil per Gm. of tissue.

tions that were almost 2 times higher than those found in the control group—6.1 and 5.8 mcg./Gm. in the groups that had received the low and high dose of the emulsifying agent, respectively, and 3.4 mcg./Gm. in the control group.

DISCUSSION

Although the literature contains reports on the disposition of mineral oil in animals (2, 5, 6), in all of the studies, doses far in excess of those normally employed by patients were given. Administration of high levels of oil was often required in such studies because of the poor specificity and low sensitivity of the methods of assay available. With the availability of a radioactive product it was possible to administer the oil at dosage levels equivalent to those recommended for humans and to assay tissues and excreta with a sensitivity previously unattainable.

Following oral administration approximately half of the radioactivity in the carcasses was shown to have been ^{3}H-mineral oil. The remaining half was ^{3}H-containing substances other than mineral oil which had been formed by the exchange of ^{3}H which had been shown to occur *in vitro*. These nonmineral oil substances might have been absorbed after undergoing an exchange reaction with the mineral oil in the gut, or they might have been formed after absorption. It is also possible that some polar metabolites of ^{3}H-mineral oil may have been included in the radioactive nonmineral oil fraction. That some ^{3}H in the ^{3}H-mineral oil exchanged rapidly was demonstrated *in vitro* by mixing the oil with a dilute aqueous acid solution or by allowing the mineral oil to mix with propionic acid overnight and measuring the ^{3}H content of the aqueous layer after separating the oil. The metabolic alteration of the mixture of saturated hydrocarbons that constituted ^{3}H-mineral oil was not demonstrated by any of the procedures described in this report, yet such a change cannot be ruled out. In any case, the specific activity of the ^{3}H-mineral oil was reduced by the amount of radioactivity found in the nonmineral oil fraction. Since efforts to determine the specific activity of the ^{3}H-mineral oil after it had been absorbed were uniformly unsuccessful because of the low concentrations present, the values of the ^{3}H-mineral oil reported in these experiments have been based on the original specific activity. These data represent, therefore, a minimum concentration of ^{3}H-mineral oil.

If the entire exchange reaction had occurred only after the absorption of ^{3}H-mineral oil, the quantity of oil that had been absorbed would be calculated on the basis of the sum of the radioactivity of the unchanged ^{3}H-mineral oil, the radioactivity of the nonmineral oil substances, the radioactivity found in the urine, and the radioactivity excreted in the bile. It has been calculated that in such a condition the concentration of ^{3}H-mineral oil reported here to have been 17.5 mcg./Gm. in the carcasses (1.6% of the dose) 5 hr. after an oral dose (Fig. 4) would have been increased to not more than 42.5 mcg./Gm. (4% of the dose) by the concentration of nonmineral oil substances containing the tritium in the carcass and by the contribution of radioactivity found in the urine (Fig. 5).

It is likely, however, that the actual value of ^{3}H-mineral oil in the carcasses lies well below the higher figure for a number of the following reasons. Exchange had been demonstrated in the lumen of the gut (Fig. 4). The ready absorption of ^{3}H-nonmineral oil substances had been demonstrated after the administration of ^{3}H-water. The i.p. administration of ^{3}H-mineral oil to rats produced relatively large quantities of ^{3}H-mineral oil in the organs; nevertheless, in comparison with results after oral administration, the concentrations of ^{3}H-nonmineral oil substances were rather low. This suggested that much of the ^{3}H-nonmineral oil substances found in the organs of the rat after the oral dose arose by a reaction that had occurred in the gut prior to absorption.

The failure of the concentration of ^{3}H-mineral oil in the brain to reflect the large amount present in the carcasses after i.p. administration may have been due, to some extent, to an immobility of the oil after it had been injected.

The kidney was able to excrete only trace quantities of mineral oil. This was demonstrated whether the drug had been given orally or i.p. Mineral oil was clearly demonstrated in homogenates of kidneys after either route of administration.

That the excretion of mineral oil after it had been absorbed by the rat was slow is apparent on examination of Fig. 6. The major portion of the dose remained in the animal 8 days following i.p. administration. At that time only 6–10% of the dose had been excreted. It is likely that one route of excretion of mineral oil is in the bile. The relatively slow rate of excretion by this route may have been due in part to the immobility of the oil after it had been deposited in the tissues, a suggestion consistent with that proposed for explaining the low concentrations found in the brains after i.p. administration; or it may have been due in part to the inability of the biliary system to collect it.

The mechanism of the absorption of mineral oil remains unknown. Frazer and Stewart (13) demonstrated that if mineral oil were emulsified with oleic acid and cholesterol to form particles not greater than 0.5 μ in diameter, absorption of large amounts of the oil would occur. These authors commented also that in the absence of emulsification negligible absorption of mineral oil had been noted. The data in this report substantiate the first observation; the presence of an emulsifying agent increased the absorption of mineral oil. With respect to the second observation, these data, contrary to those of Frazer and Stewart (13), indicate that a small but significant amount of mineral oil had been absorbed in the absence of an exogenous emulsifying agent.

The levels of radioactivity in the carcasses of rats chronically treated with ^{3}H-mineral oil were similar to those found after an acute dose. This evidence suggests that chronic administration did not alter the ability of rats to absorb or to excrete mineral oil.

At the dosage level employed in these studies only a mild laxative action was observed. In the chronic studies this effect disappeared 1 or 2 days after initiating the treatment.

The high levels of radioactivity excreted in the feces of the orally dosed rats reflected the poor absorption and the rapid elimination of ^{3}H-mineral oil.

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Enzyme Inhibitors XIV

Syntheses of Some 9-(*m*-Substituted Benzyl)-6-substituted Purines and Their Evaluation as Inhibitors of Adenosine Deaminase

By HOWARD J. SCHAEFFER and R. N. JOHNSON

Recent studies have shown that 9-(*p*-bromoacetamidobenzyl)adenine is an irreversible inhibitor of adenosine deaminase. In order to study the effect of isomers on the inhibition of adenosine deaminase, a variety of reversible inhibitors of adenosine deaminase have been synthesized which are 9-(*m*-substituted benzyl)-6-substituted purines. In addition, it was found that 9-(*m*-bromoacetamidobenzyl)adenine is an irreversible inhibitor of adenosine deaminase, but the rate of irreversible inactivation by the *meta* derivative was lower than that by the corresponding *para* isomer. This decreased rate of irreversible inhibition by 9-(*m*-bromoacetamidobenzyl)-adenine may be rationalized by assuming that in the reversible E...I complex the alkylating group is not positioned as near a nucleophilic group on the enzyme as it is in the case of the corresponding *para* isomer or that the *meta* derivative alkylates a different amino acid on the enzyme than does 9-(*p*-bromoacetamidobenzyl)adenine.

In a recent study, it was found that 9-(*p*-bromoacetamidobenzyl)adenine was an irreversible inhibitor of adenosine deaminase, whereas iodoacetamide was not an irreversible inhibitor of this enzyme (1, 2). Kinetic analysis of the data indicated that the irreversible inhibition of adenosine deaminase by 9-(*p*-bromoacetamidobenzyl)adenine occurred only after the inhibitor had reversibly complexed with the enzyme. In this complex, then, the bromoacetamido moiety of the inhibitor is held near a nucleophilic group on the enzyme, and a reaction related to a neighboring-group reaction occurs with the formation of a covalent bond. Such inhibitors, which Baker has called active-site-directed irreversible inhibitors (3), should be quite specific in their irreversible inactivation of an enzyme. For example, when a comparison is made of isomers of some potential irreversible inhibitors, the environment on the enzyme in which the alkylating or acylating group of the inhibitor is held in the reversible E...I complex (enzyme–inhibitor) could be quite different. Thus, it is possible that the reactive group of one isomer could be held near an appropriate nucleophilic group on the enzyme, whereas the reactive group of an isomeric inhibitor could be held in the reversible E...I complex in such a position that it cannot form a covalent bond with the enzyme. In an attempt to determine the specificity of 9-(*p*-bromoacetamidobenzyl)adenine for adenosine deaminase, it was decided to investigate the possible reversible and irreversible inhibition of adenosine deaminase by some 9-(*m*-substituted benzyl)-6-substituted purines.

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DISCUSSION

Chemistry.—Previous studies have shown that adenosine deaminase (calf intestinal mucosa) has a hydrophobic region to which the 9-alkyl group of some 9-alkyladenines can bind (4). Furthermore, it has been found that some 9-(*p*-substituted benzyl)-6-substituted purines were capable of inhibiting

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DISCUSSION

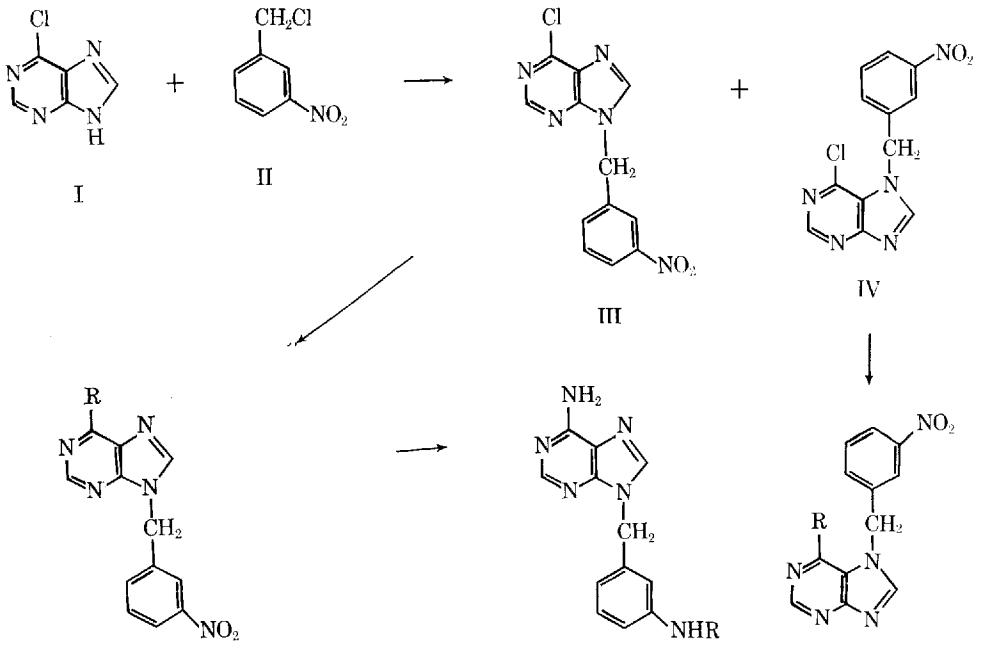
Chemistry.—Previous studies have shown that adenosine deaminase (calf intestinal mucosa) has a hydrophobic region to which the 9-alkyl group of some 9-alkyladenines can bind (4). Furthermore, it has been found that some 9-(*p*-substituted benzyl)-6-substituted purines were capable of inhibiting

adenosine deaminase (1, 2). In order to study the effect on inhibition of this enzyme by other 9-(substituted benzyl)-6-substituted purines, the preparation of some purines which contained at the 9-position a *meta*-substituted benzyl group was undertaken. The key compound for such a study is 9-(*m*-nitrobenzyl)-6-chloropurine, since this compound could be caused to undergo nucleophilic displacement at the 6-position to give a variety of products. In addition, the nitro group of appropriate inhibitors could be reduced to an amino group which could be converted into a potential alkylating or acylating group, such as a bromoacetamido or phenoxycarbonylamino group.

The general method which was employed for the synthesis of these compounds is a modification of a method that has previously been employed (5) and is outlined in Scheme I. Condensation of 6-chloropurine (I) with *m*-nitrobenzyl chloride (II) gave a mixture of 9- and 7-(*m*-nitrobenzyl)-6-chloropurines (III and IV) which was separated by chromatography on alumina. When III was allowed to react with ammonia, methylamine, dimethylamine, 1 *N* hydrochloric acid, or thiourea, the corresponding 6-substituted derivatives (V–IX) were obtained in good yields. Catalytic hydrogenation of V gave the corresponding *m*-aminobenzyl derivative (X) which on reaction with bromoacetic anhydride, phenyl chloroformate, and acetic anhydride gave XI, XII, and XIII, respectively. The assignment of structure to XI, XII, and XIII is based on the observation that N^6-acylation of adenine compounds shifts the ultraviolet maxima to longer wavelengths (6). Thus, it has been found (6) that *N*, $O^{3'}$-diacetyl deoxyadenylic 5′-acid exhibited an ultraviolet maximum at 273 mμ at pH 8, whereas XI, XII, and XIII exhibited ultraviolet maxima either at shorter wavelengths than X or at essentially the same wavelength as X.

In addition, it was found that when 7-(*m*-nitrobenzyl)-6-chloropurine (IV) was allowed to react with 20% methanolic ammonia, a mixture of the 6-amino and 6-methoxy derivatives (XIV and XV) was obtained. This unusual formation of a 6-methoxy derivative from the reaction of methanolic ammonia with a 7-substituted-6-chloropurine has been observed previously (7). However, treatment of IV with liquid ammonia at 65° gave, in moderate yield, 7-(*m*-nitrobenzyl)adenine (XIV). When IV was allowed to react with aqueous formic acid, a good yield of XVI was obtained.

Finally, the fact that III and IV, and therefore the compounds which were prepared from them, are the 9- and 7-substituted purine isomers, respectively, was established in the following manner. A 9-substituted adenine has a unique ultraviolet spectrum which allows it to be distinguished from a 1-,3-, or 7-substituted adenine (8, 9). Similarly, by ultraviolet spectroscopy a 1-substituted adenine may be distinguished from a 3-,7-, or 9-substituted



Scheme I

adenine. However, a 3- and a 7-substituted adenine have similar ultraviolet spectra but may be distinguished by their differences in pKa′ values (9).

A new alkylated adenine (XVII) was prepared by allowing adenine to react with *m*-nitrobenzyl chloride in the absence of an acid acceptor. In a similar alkylation of adenine with benzyl bromide, it was found that 3-benzyladenine was formed (10). A comparison of the ultraviolet spectra and pKa′ data of these isomerically alkylated adenines with certain known compounds is given in Table I. From this table, it can be seen that V corresponds to the 9-substituted isomer, XIV corresponds to the 7-substituted isomer, and that XVII corresponds to the 3-substituted isomer. These data constitute another example that the alkylation of 6-chloropurine in the presence of an acid acceptor produces a mixture of the 9- and 7-substituted-6-chloropurines (2, 5, 7).

TABLE I.—ULTRAVIOLET AND pKa′ DATA OF SOME SUBSTITUTED ADENINES

Compd. (Adenine Derivative)	—H⁺— $\lambda_{max.}$ mμ	—H⁺— $\epsilon \times 10^{-3}$	—OH⁻— $\lambda_{max.}$ mμ	—OH⁻— $\epsilon \times 10^{-3}$	pKa′
1-Methyl[a]	259	11.7	270	14.4	
3-Methyl[a]	274	17.0	273	13.3	5.3[a,b]
3-(*m*-Nitrobenzyl) (XVII)	274	23.0	272	19.6	5.7[c]
7-Methyl[a]	272	15.0	270	10.5	3.6[a,b]
7-(*m*-Nitrobenzyl) (XIV)	270	19.5	267	16.6	3.6[c]
9-Methyl[a]	260	14.2	260	14.7	
9-(*m*-Nitrobenzyl) (V)	261	23.3	262	23.0	

[a] These data were taken from *Reference 9*. [b] Determined titrimetrically in 50% DMF in H_2O. [c] Determined spectrophotometrically in 4% DMF in H_2O.

EXPERIMENTAL[1]

9- and 7-(m-Nitrobenzyl)-6-chloropurine (III and IV).—A mixture of 1.53 Gm. (9.95 mmoles) of I, 1.80 Gm. (10.5 mmoles) of II, and 1.46 Gm. (10.5 mmoles) of anhydrous potassium carbonate in 20 ml. of dimethylformamide was stirred for 20 hr. at room temperature. The reaction mixture was poured into 150 ml. of ice cold water, which caused the precipitation of a yellow solid. This material was collected by filtration. A chloroform solution of the crude material was chromatographed on a column of neutral alumina (48.0 Gm). 9-(*m*-Nitrobenzyl)-6-chloropurine (III) was eluted with chloroform (180 ml.); yield, 1.97 Gm. (68.6%), m.p. 135°. The 9-isomer was recrystallized from methanol and gave 1.36 Gm. (47.5%) of the pure material, m.p. 141–142°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 265 (1.66); pH 7, 265 (1.73); pH 13, 265 (1.67). ν in cm.$^{-1}$ (KBr): 1580 and 1550 (C=N and C=C); 1520 and 1340 (NO_2).

Anal.[2]—Calcd. for $C_{12}H_8ClN_5O_2$: C, 49.74; H, 2.78; N, 24.18. Found: C, 49.52; H, 2.81; N, 24.34.

7-(m-Nitrobenzyl)-6-chloropurine (IV).—This was eluted from the alumina column with chloroform containing 1% methanol (150 ml.). After removal of the solvent *in vacuo*, 529 mg. of the 7-substituted product was obtained; yield, 18.4%. The analytical sample was obtained by two recrystallizations of the crude material from ethanol. It melted at 191–192°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH, 1, 267 (1.53); pH 7, 267 (1.53); pH 13, 267 (1.53). ν in cm.$^{-1}$ (KBr): 1600 and 1570 (sh) (C=N and C=C); 1530 and 1350 (NO_2).

Anal.—Calcd. for $C_{12}H_8ClN_5O_2$: C, 49.74; H, 2.78; N, 24.18. Found: C, 49.42; H, 2.88; N, 24.00.

9-(m-Nitrobenzyl)adenine (V).—A mixture of 1.00 (3.46 mmoles) of III and 50 ml. of 20% methanolic ammonia was heated in a steel bomb at 90° ± 2° for 20 hr. The white solid was collected by filtration, washed with water, and dried. One recrystallization of the crude material from methyl cellosolve gave 716 mg. (76.5%) of the pure product (V), m.p. 276–278° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 261 (2.33); pH 7, 262 (2.30); pH 13, 262 (2.30). ν in cm.$^{-1}$ (KBr): 3320, 3150, and 1660 (NH); 1590 and 1570 (C=N and C=C); 1530 and 1350 (NO_2).

Anal.—Calcd. for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.13; H, 3.83; N, 30.94.

9-(m-Nitrobenzyl)-6-methylaminopurine (VI).—A solution of 145 mg. (0.50 mmole) of III in 5 ml. of ethanol and 8 ml. of aqueous methylamine (40%) was heated in a steel bomb at 98° ± 3° for 23 hr. The yellow product which precipitated was collected by filtration and washed with water. Addition of 20 ml. of water to the filtrate caused the precipitation of an additional amount of purine. Total yield, 101 mg. (71.1%). Two recrystallizations of the crude product from 1-propanol gave the analytical sample (VI); yield, 75 mg. (53%), m.p. 210–212°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 265 (2.68); pH 7, 268 (2.55); pH 13, 268 (2.49). ν in cm.$^{-1}$ (KBr): 3300 (NH); 1635 and 1570 (C=N and C=C); 1520 and 1340 (NO_2).

Anal.—Calcd. for $C_{13}H_{12}N_6O_2$: C, 54.92; H, 4.26; N, 29.57. Found: C, 55.03; H, 4.40; N, 29.73.

9-(m-Nitrobenzyl)-6-dimethylaminopurine (VII).—A solution of 96 mg. (0.33 mmole) of III in 5 ml. of ethanol and 5 ml. of aqueous dimethylamine (25%) was heated in a steel bomb at 96° ± 1° for 16 hr. The yellow solid which precipitated from the reaction mixture was collected by filtration and washed with water. To the filtrate was added an additional 20 ml. of water which caused the precipitation of a second crop of product. Total yield, 86 mg. (88%). One recrystallization of the crude material from ethanol–water gave the pure product (VII); yield, 64 mg. (65%), m.p. 156–157°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 269 (2.55); pH 7, 275 (2.51); pH 13, 275 (2.47). ν in cm.$^{-1}$ (KBr): 1590 and 1560 (sh) (C=N and C=C); 1520 and 1340 (NO_2).

Anal.—Calcd. for $C_{14}H_{14}N_6O_2$: C, 56.36; H, 4.73; N, 28.18. Found: C, 56.10; H, 4.90; N, 28.01.

[1] The infrared spectra were determined on a Perkin-Elmer model 137 spectrophotometer; the ultraviolet spectra were determined on a Perkin-Elmer model 4000 A spectrophotometer; the enzyme studies were done on a Gilford instrument, model 2000 spectrophotometer. The melting points, unless otherwise noted, were taken in open capillary tubes on a Mel-Temp apparatus and are corrected. All analytical samples exhibited only one spot on thin-layer chromatography.

[2] The analyses reported in this paper were performed by Galbraith Microanalytical Laboratories, Knoxville, Tenn.

9-(m-Nitrobenzyl)-6-hydroxypurine (VIII).—A solution of III (873 mg.; 3.08 mmoles) in 90 ml. of 1 *N* hydrochloric acid was refluxed for 1 hr. Evaporation of the volatile materials *in vacuo* and two recrystallizations of the crude material from ethanol gave the pure product (VIII); yield, 587 mg. (72.5%), m.p. 271–271.5°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 252 (1.72); pH 7, 251 (1.85); pH 13, 257 (1.92). ν in cm.$^{-1}$ (KBr): 3500 (OH); 2800–2300 (acidic hydrogen); 1690 (C=O); 1590 and 1550 (sh) (C=N and C=C); 1530 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_9N_5O_3$: C, 53.13; H, 3.34; N, 25.83. Found: C, 53.12; H, 3.31; N, 25.63.

9-(m-Nitrobenzyl)-6-mercaptopurine (IX).—A mixture of 146 mg. (0.504 mmole) of III, 41 mg. (0.54 mmole) of thiourea in 3.0 ml. of 1-propanol was refluxed for 0.75 hr. The product which precipitated from the reaction mixture was collected by filtration and dried; yield, 140 mg. The analytical sample was obtained by dissolving the purine in aqueous sodium hydroxide and precipitating it by addition of 5% aqueous hydrochloric acid; yield, 126 mg. (81.3%), m.p. 294–294.5° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/15% DMSO 325 (2.45); H_2O/15% DMSO 322 (2.20); 0.1 *N* NaOH/15% DMSO 314 (2.26). ν in cm.$^{-1}$ (KBr): 2800–2650 (acidic hydrogen); 1590 and 1570 (sh) (C=N and C=C): 1530 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_9N_5O_2S$: C, 50.18; H, 3.16; N, 24.39. Found: C, 50.35; H, 3.30; N, 24.32.

9-(m-Aminobenzyl)adenine (X).—A solution of 210 mg. (0.778 mmole) of V in 50 ml. of acetic acid containing 66 mg. of 5% palladium-on-charcoal catalyst was hydrogenated for 1.75 hr. under an initial pressure of 60 p.s.i. The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. An ethanolic solution of the residual solid was heated with decolorizing carbon, filtered, and allowed to crystallize. The product was collected by filtration and after drying gave 111 mg. (59.7%) of the desired product (X), m.p. 230–231°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 259 (1.53); pH 7, 262 (1.65); pH 13, 262 (1.66). ν in cm.$^{-1}$ (KBr): 3300, 3150, and 1660 (NH); 1600 and 1550 (sh) (C=N and C=C).

Anal.—Calcd. for $C_{12}H_{12}N_6$: C, 59.99; H, 5.04; N, 34.98. Found: C, 59.73; H, 4.83; N, 34.76.

9-(m-Bromoacetamidobenzyl)adenine (XI).—To a cold solution of 200 mg. (0.832 mmole) of X in 5 ml. of tetrahydrofuran and 0.8 ml. of 10% aqueous acetic acid was added a solution of 351 mg. (1.97 mmoles) of bromoacetic anhydride in 2 ml. of tetrahydrofuran. The solution was stirred for 1.5 hr. at 0°, then for 0.5 hr. at room temperature. After filtration, 15 ml. of chloroform was added to the filtrate to cause precipitation of the crude product which was collected by filtration; yield, 271 mg. (81.5%). Two precipitations from tetrahydrofuran–hexane gave 118 mg. (39.4%) of the analytical sample (XI), which did not melt below 400°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/10% EtOH, 257 (2.28); H_2O/10% EtOH, 259 (2.30); 0.1 *N* NaOH/10% EtOH, 259 (2.24). ν in cm.$^{-1}$ (KBr): 3310, 3160 (NH); 1700 (amide I); 1655 (NH) and 1530 (amide II); 1590 and 1570 (C=N and C=C).

Anal.—Calcd. for $C_{14}H_{13}BrN_6O$: C, 46.55; H, 3.62; Br, 22.12; N, 23.26. Found: C, 46.57; H, 3.81; Br, 21.91; N, 23.52.

9 - (m - Phenoxycarbonylaminobenzyl)adenine (XII).—To a solution of 95 mg. (0.40 mmole) of X and 50 mg. (0.50 mmole) of triethylamine in 25 ml. of *p*-dioxane was added 62 mg. (0.40 mmole) of phenyl chloroformate. The mixture was stirred at room temperature for 45 min., filtered, and the filtrate evaporated *in vacuo* at room temperature. One recrystallization of the crude material from methanol–water gave 60 mg. (42%) of pure material (XII), decomposition point about 310°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/10% EtOH, 239 (2.08); 259 (1.58); H_2O/10% EtOH, 240 (2.04); 259 (1.58); 0.1 *N* NaOH/10% EtOH 239 (2.54); 259 (1.70). ν in cm.$^{-1}$ (KBr): 3310 and 3140 (NH); 1700 (C=O and NH); 1530 (amide II); 1610, 1590, and 1570 (C=N and C=C); 1200 (C—O—C, ester).

Anal.—Calcd. for $C_{19}H_{16}N_6O_2$: C, 63.32; H, 4.48; N, 23.32. Found: C, 63.17; H, 4.74; N, 23.51.

9-(m-Acetamidobenzyl)adenine (XIII).—To a cold solution of 150 mg. (0.652 mmole) of X in 5 ml. of tetrahydrofuran and 0.60 ml. of 10% aqueous acetic acid was added 154 mg. (1.51 mmoles) of acetic anhydride in 2 ml. of tetrahydrofuran over a period of 7 min. The solution was stirred at 0° for 2 hr., and the resulting precipitate was collected by filtration to give 108 mg. (58.6%) of product melting at 234–238°. Recrystallization from ethanol–water gave 77 mg. (42%) of the analytical sample (XIII), m.p. 237–239°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/10% EtOH, 255 (1.84); H_2O/10% EtOH, 254 (1.90); 0.1 *N* NaOH/10% EtOH, 254 (1.99). ν in cm.$^{-1}$ (KBr): 3340, 3180 (NH); 1670 (amide I); 1650 (sh) (NH) and 1540 (amide II); 1600 and 1570 (C=N and C=C).

Anal.—Calcd. for $C_{14}H_{14}N_6O$: C, 59.56; H, 5.00; N, 29.77. Found: C, 59.67; H, 5.01; N, 29.95.

7-(m-Nitrobenzyl)adenine (XIV) and 7-(m-Nitrobenzyl)-6-methoxypurine (XV).—A mixture of 0.29 Gm. (1.0 mmole) of IV and 21 ml. of 20% methanolic ammonia was heated in a steel bomb at 106° ± 3° for 3 hr. The solution was filtered to give 36 mg. (13%) of the 6-methoxypurine derivative (XV), m.p. 192–193.5°. One recrystallization from water gave 23 mg. (8%) of the analytical material, m.p. 195–196°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 260 (1.66); pH 7, 261 (1.49); pH 13, 262 (1.45). ν in cm.$^{-1}$ (KBr): 1610 and 1550 (C=N and C=C); 1520 and 1350 (NO_2); 1250 and 1070 (=C—O—CH_3).

Anal.—Calcd. for $C_{13}H_{11}N_5O_3$: C, 54.73; H, 3.89; N, 24.55. Found: C, 55.00; H, 4.03; N, 24.75.

Evaporation of the methanolic ammonia solution from which the 6-methoxypurine was obtained, produced a yellow solid which was extracted with hot methanol[3] (2 × 1 ml.). The residue (57 mg., 21%) which did not dissolve melted at 254–259°.

One recrystallization from methanol gave 35 mg. (13%) of the pure 7-(*m*-nitrobenzyl)adenine (XIV), m.p. 265–267° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 270 (1.95); pH 7, 268 (1.67); pH 13, 267 (1.66). ν in cm.$^{-1}$ (KBr): 3380, 3150, and 1660

[3] By thin-layer chromatography, this extract was shown to be a mixture of 7-(*m*-nitrobenzyl)-6-methoxypurine and 7-(*m*-nitrobenzyl)adenine. Since the desired 6-aminopurine was prepared by an alternate method, no attempts were made to further separate this mixture.

(NH); 1600 and 1540 (C=N and C=C); 1520 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_{10}N_6O_2$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.26; H, 3.90; N, 30.90.

7-(m-Nitrobenzyl)adenine (XIV).—A mixture of 290 mg. (1.00 mmole) of IV in about 20 ml. of liquid ammonia was heated in a steel bomb at 65° ± 5° for 19 hr. After evaporation of the ammonia, the crude product was recrystallized from water; yield, 169 mg. (62.6%), m.p. 255–259° dec. An additional recrystallization from methanol gave 130 mg. (48.1%) of the pure product (XIV), m.p. 264–265° dec. ν in cm.$^{-1}$ (KBr): 3390, 3150, and 1660 (NH); 1600 and 1540 (C=N and C=C); 1520 and 1340 (NO_2).

7-(m-Nitrobenzyl)-6-hydroxypurine (XVI).—A solution of 205 mg. (0.710 mmole) of IV in 10 ml. of 88% aqueous formic acid was heated under reflux for 20 min. The yellow solution was evaporated *in vacuo*, and the residue was recrystallized from water to yield 142 mg. (73.2%) of the product, m.p. 254–256°. An additional recrystallization from water gave the analytical sample, 110 mg. (56.7%), m.p. 255–256°. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): pH 1, 257 (1.48); pH 7, 260 (1.62); pH 13, 265 (1.63). ν in cm.$^{-1}$ (KBr): 1690 (C=O); 1630 and 1580 (C=N and C=C); 1530 and 1340 (NO_2).

Anal.—Calcd. for $C_{12}H_9N_5O_3$: C, 53.13; H, 3.34; N, 25.83. Found: C, 53.11; H, 3.35; N, 26.07.

7-(m-Nitrobenzyl)-6-hydroxypurine (XVI).—Dry hydrogen chloride was passed into a solution of 3.2 mg. (0.011 mmole) of XV in 1.5 ml. of methanol. The solution was evaporated *in vacuo*, and the residue recrystallized from water to give 2.6 mg. (72%) of the hydroxypurine (XVI), m.p. 255–255.5°. $\lambda_{max.}$: pH 1, 257; pH 7, 260; pH 13, 265. ν in cm.$^{-1}$ (KBr): 1690 (C=O); 1630 (sh) and 1580 (C=N and C=C); 1530 and 1340 (NO_2). This sample was identical with a sample prepared by the previous procedure.

3-(m-Nitrobenzyl)adenine Hydrochloride (XVII).—A mixture of 6.33 Gm. (37.0 mmoles) of adenine dihydrate and 19.0 (111 mmoles) of II in 100 ml. of *N,N*-dimethylacetamide was heated at 115° for 20 hr. The solution was evaporated *in vacuo* to a yellow solid which was triturated with water and then with ethanol. The residue, 9.16 Gm. (80.5%), m.p. 264–267° dec., was recrystallized from water to give 6.79 Gm. (59.5%) of the analytical material, m.p. 264–266° dec. $\lambda_{max.}$ in mμ ($\epsilon \times 10^{-4}$): 0.1 *N* HCl/4% EtOH, 274 (2.30); H_2O/4% EtOH, 272 (1.82); 0.1 *N* NaOH/4% EtOH, 272 (1.96). ν in cm.$^{-1}$ (KBr): 3090 (NH); 1670 (C=NH^+); 1610, 1580, and 1520 (C=C and C=N); 1540 and 1350 (NO_2).

Anal.—Calcd. for $C_{12}H_{11}ClN_6O_2$: C, 46.98; H, 3.62; Cl, 11.56; N, 27.40. Found: C, 46.86; H, 3.64; Cl, 11.45; N, 27.26.

CHEMICAL REACTIVITY OF THE ALKYLATING AGENTS

These experiments were performed by a modification of a procedure described in the literature (11–13). Equal volumes (10 ml.) of the following preheated (37°) solutions were mixed: (*a*) 5% 4-(*p*-nitrobenzyl)pyridine in 2-methoxyethanol, (*b*) 0.05 *M* phthalate buffer (pH 4.2) in water, and then (*c*) 0.81 m*M* solution of the alkylating agent in 2-methoxyethanol.

The reaction mixture was incubated at 37° and at appropriate time intervals, a 3-ml. aliquot was removed and cooled briefly in an ice bath. Then 1 ml. of triethylamine was added to generate the colored quinoid-like free base, and the absorbance was immediately determined at 573 mμ against a blank which had been treated in an identical manner and contained all of the reagents except the alkylating agent. A comparison of the initial rates of reaction is given in Fig. 2.

REAGENTS AND ASSAY PROCEDURE

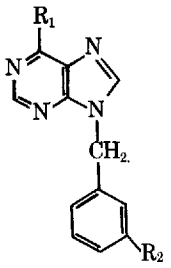
Adenosine and adenosine deaminase (type I) from calf intestinal mucosa were purchased from the Sigma Chemical Co. The assay procedure for the reversible inhibitors has been described by Kaplan (14). The measurements of the rates of the enzymic reactions were performed at 25° in 0.05 *M* phosphate buffer at pH 7.6. The stock solutions of the enzyme, substrate, and inhibitors were prepared in 0.05 *M* phosphate buffer at pH 7.6. Those inhibitors which were only slightly soluble in phosphate buffer were dissolved in phosphate buffer containing 10% dimethylsulfoxide. The addition of dimethylsulfoxide caused a slight decrease in the initial rate of the enzyme reaction. Consequently, in those experiments where it was necessary to employ dimethylsulfoxide to dissolve the inhibitor in phosphate buffer, the stock solutions of enzyme, substrate, and inhibitors were all prepared in phosphate buffer containing 10% dimethylsulfoxide. In this way, a constant 10% concentration of dimethylsulfoxide is maintained during the determination of the velocities of the enzymic reactions. In order to determine that the dimethylsulfoxide did not cause variation in the index of inhibition, the following experiment was performed. For an inhibitor which was readily soluble, the index of inhibition was determined in one set of experiments where all reagents were dissolved in 0.05 *M* phosphate buffer and in another set of experiments where the enzyme, substrate, and inhibitor were dissolved in 0.05 *M* phosphate buffer containing 10% dimethylsulfoxide. In this way, it was found that the index of inhibition did not vary in the two different determinations.

The method employed to study the irreversible inactivation at 37° of adenosine deaminase has been described previously (2) and is a modification of a published procedure (15).

RESULTS AND DISCUSSION

The results of the enzymic evaluation of these compounds are given in Table II. For those compounds which were substituted at the 9-position of the purine nucleus by a *m*-nitrobenzyl group, it was found that the 6-amino and 6-methylamino analogs (V and VI) were significantly inhibitory; the 6-amino compound (V) being approximately three times more effective than the 6-methylamino derivative (VI). Those compounds which were substituted at the 6-position of the purine nucleus by a chloro, dimethylamino, hydroxy, or mercapto group (III, VII, VIII, or IX) when tested at 0.12 m*M* concentration were either noninhibitory or at

TABLE II.—INHIBITION INDEX OF SOME 9-(*m*-SUBSTITUTED BENZYL)-6-SUBSTITUTED PURINES WITH ADENOSINE DEAMINASE



Compd.[a]	R_1	R_2	m*M* Concn. for 50% Inhibition[b]	$[I/S]_{0.5}$[b]	$K_i \times 10^5$ *M*
V	NH_2	NO_2	0.016 ± 0.004[c]	0.30 ± 0.03[c]	
VI	NHMe	NO_2	0.063 ± 0.001	0.96 ± 0.02	
X	NH_2	NH_2	0.20 ± 0.02	3.0 ± 0.3	
XI	NH_2	$NHCOCH_2Br$	0.056 ± 0.003	0.85 ± 0.05	3.6 ± 0.9
XII	NH_2	$NHCOOC_6H_5$	0.038 ± 0.004	0.58 ± 0.05	
XIII	NH_2	$NHCOCH_3$	0.095 ± 0.004	1.4 ± 0.1	
XVII	NH_2	H	0.10 ± 0.01	1.5 ± 0.2[d]	

[a] None of these compounds served as substrates of adenosine deaminase. [b] The concentration of adenosine in all experiments was 0.066 m*M*. In no experiment of reversible inhibition did the concentration of inhibitor exceed 0.12 m*M*. In that case where a higher concentration is shown for 50% inhibition, the value was obtained by extrapolation of a plot of V_0/V *vs.* I, where V_0 = initial velocity of the uninhibited reaction, V = initial velocity of the inhibited reaction at various inhibitor concentrations, and I = the various concentrations of inhibitors. $[I/S]_{0.5}$ = the ratio of the m*M* concentration of the inhibitor for 50% inhibition to the m*M* concentration of the substrate. [c] Average deviation. [d] Data taken from *Reference 2*.

best, very weakly inhibitory relative to the corresponding adenine derivative (V). Thus, it would appear that the 6-amino group of V makes a contribution, either directly or indirectly through the purine nucleus, to the binding of the inhibitor to adenosine deaminase. In addition, when the 7-substituted isomers (IV, XIV, XV, and XVI) were evaluated as inhibitors of adenosine deaminase at 0.12 m*M* concentrations, they were essentially noninhibitory or at best, very weakly inhibitory relative to V. In the case of XIV, one could rationalize this result by assuming that the enzyme has little bulk tolerance for a group at the 7-position of the purine nucleus of an inhibitor or that an important binding group at the 9-position of the inhibitor is absent. Similar results have been obtained earlier (7).

With regard to the *meta* substituent on the 9-benzyl group of the 6-aminopurines, it was found that the reversible inhibitory power of the compounds decreases in the following order: $-NO_2$ (V)> $-NHCOOC_6H_5$(XII)> $-NHCOCH_2Br$ (XI)> $-NHCOCH_3$(XIII)≥ H (XVII)> NH_2(X). It was observed by the double reciprocal plot method (16) that XI is a competitive inhibitor of adenosine deaminase with a K_i of 3.6×10^{-5} *M*. This order of decreasing inhibition is different from that obtained with the corresponding *para* derivatives (2). As one example, the $[I/S]_{0.5}$ for the *m*-nitro derivative (V) is 0.3, whereas the $[I/S]_{0.5}$ of 9-(*p*-nitrobenzyl)adenine is 3.6 (2). Thus, of the adenine derivatives prepared in the *meta* series, the *m*-nitro derivative (V) is the most potent reversible inhibitor of adenosine deaminase whereas in the corresponding 9-(*p*-substituted benzyl)adenines, the *p*-nitro derivative is the weakest reversible inhibitor of the series. The reasons for the differences in the order of reversible inhibition in the two series is not yet clear. Further studies on this problem are planned and will be the subject of a future paper.

Finally, when the two potential irreversible inhibitors (XI and XII) were incubated with adenosine deaminase, it was found that the *m*-phenoxycarbonylamino derivative (XII) did not cause irreversible inactivation of the enzyme whereas the *m*-bromoacetamido derivative (XI) irreversibly inhibited the enzyme but at a rate which was very low relative to 9-(*p*-bromoacetamidobenzyl)adenine. It was found, however, that both XI and 9-(*p*-bromoacetamidobenzyl)adenine were capable of causing more than 90% irreversible inhibition of adenosine deaminase. The inactivation of the enzyme by these compounds was not reversed by dialysis. A comparison of the apparent first-order loss of enzyme activity caused by XI and 9-(*p*-bromoacetamidobenzyl)adenine is given in Fig. 1. Because the reaction of XI with adenosine deaminase is slow, these enzyme inactivation experiments have larger experimental errors than in the case of 9-(*p*-bromoacetamidobenzyl)adenine. Since the irreversible inhibitors have limited solubility in phosphate buffer, the reactions were performed in phosphate buffer containing 10% dimethylsulfoxide but even with the addition of dimethylsulfoxide, a concentration of 0.20 m*M* was near the upper range of solubility of XI. However, an examination of Fig. 1 reveals that XI irreversibly inhibited adenosine deaminase at concentrations of 0.10 and 0.20 m*M* at a much lower rate than did 9-(*p*-bromoacetamidobenzyl)adenine at a concentration of 0.03 m*M*. This significant difference in the rate of irreversible inactivation of the enzyme is not caused by a lack of chemical reactivity of the *meta* isomer since the

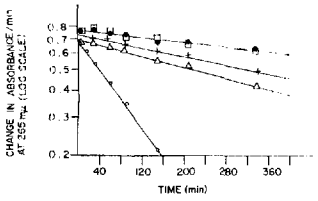


Fig. 1.—Irreversible inactivation of adenosine deaminase. Key: ●, enzyme control; □, iodoacetamide (1.0 mM); +, 9-*m*-bromoacetamidobenzyladenine (0.10 mM); △, 9-*m*-bromoacetamidobenzyladenine (0.20 mM); ○, 9-*p*-bromoacetamidobenzyladenine (0.030 mM).

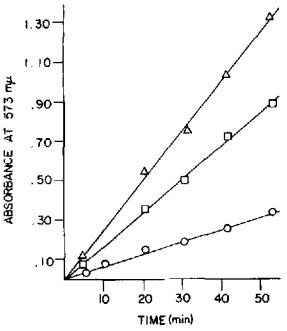


Fig. 2.—Comparative chemical reactivities of some alkylating agents with 4-(*p*-nitrobenzyl)-pyridine at pH 4.2. Key: ○, iodoacetamide (0.27 mM); □, 9-(*p*-bromoacetamidobenzyl)adenine (0.27 mM); △, 9-(*m*-bromoacetamidobenzyl)-adenine (0.27 mM).

meta isomer (XI) is actually more reactive than the *para* isomer when 4-(*p*-nitrobenzyl)pyridine (11–13) was used as the nucleophilic reagent (Fig. 2). In addition, the differences in the rates of irreversible inactivation of adenosine deaminase by XI and 9-(*p*-bromoacetamidobenzyl)adenine cannot be explained by the suggestion that XI is rapidly destroyed by the phosphate buffer solution. This suggestion was excluded when it was found that less than 10% of the alkylating ability of XI is lost during a 4-hr. incubation of XI with phosphate buffer containing 10% dimethylsulfoxide at 37°. Because iodoacetamide does not significantly irreversibly inhibit adenosine deaminase even at concentration of 1.6 mM, the authors believe that the irreversible inactivation which is caused by XI occurs mainly through a reversible E . . . I complex and not significantly by a random biomolecular process. In addition, XIII did not cause an irreversible inactivation of the enzyme. Thus, the irreversible inactivation of the enzyme by XI appears to be specifically related to its alkylating ability through an E . . . I complex.

When a comparison of the irreversible inactivation of the enzyme is made on the basis of the amount of the enzyme in the reversible E . . . I complex, it is found that 9-(*p*-bromoacetamidobenzyl)adenine is still much more reactive than the corresponding *meta* derivative (XI). Baker (17) has derived Eq. 1 for the calculation of the fraction of the total enzyme that is in the E . . . I complex.

$$[E\ldots I] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad \text{(Eq. 1)}$$

In the case where a 0.10 mM solution of XI is incubated with the enzyme, the amount of the total enzyme (E_t) in the reversible E . . . I complex is 0.73 E_t, and the half-life of the inactivation is > 600 min. When a 0.030 mM solution of 9-(*p*-bromoacetamidobenzyl)adenine is employed under identical conditions with identical total enzyme concentration, the amount of the total enzyme in the reversible E . . . I complex is 0.70 E_t, and the half-life for the inactivation is 94 min. Thus, even when the concentration of the reversible E . . . I complex for the two inhibitors is nearly equal, the rate of inactivation by the *meta* derivative is much lower than that of the *para* isomer. The authors believe that these results offer evidence that in the initial reversible E . . . I complex between XI and adenosine deaminase, the alkylating agent of XI is not positioned as close to a nucleophilic group on the enzyme as occurs when 9-(*p*-bromoacetamidobenzyl)adenine is employed; consequently, the rate of alkylation by XI in the E . . . I complex is lower. However, it is possible that when the alkylating group is in the *meta* position of 9-benzyladenine as in XI, it is alkylating a different amino acid of the enzyme than when the alkylating group is in the *para* position of 9-benzyladenine. Further studies are necessary to differentiate these two suggestions.

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Analysis of Selected Pharmaceuticals by Quantitative Thin-Layer Chromatography

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Thin-layer chromatography was employed to separate the components of 14 selected commercial pharmaceutical mixtures in tablet and capsule form. The components included the amphetamines, certain barbiturates, and several related compounds. A recording photoelectric densitometer with an electronic integrator was utilized to scan and estimate quantitatively the various constituents which were rendered visible by specific reagents. Silica Gel G was the adsorbent used and a dioxane–benzene–25 per cent ammonia mixture (40:40:10 v/v) was found to be a suitable developing solvent for separating the mixtures examined. Most of the drugs studied could be estimated with an experimental error of approximately 5 per cent when applied in concentrations of 25 to 100 mcg.

MULTICOMPONENT tablets and capsules provide two of the most convenient forms for the oral administration of drugs since they are easily mass produced and provide a compact accurate dose in a convenient form. For this reason they constitute the method of administration for over 75% of all drugs prescribed.

The widespread use of these dosage forms has given rise to problems in pharmaceutical analysis. In many cases the concentration of an incorporated drug is extremely small—a milligram or less—and rapid techniques of semimicro analysis must be devised for its assay. As the formulation of the preparation becomes more complex the greater are the assay problems introduced since the components must be separated and estimated individually. For the purpose of quality control, three or more components may have to be isolated and assayed in the product.

Since its introduction by Stahl in 1956 (1) the technique of thin-layer chromatography has been used extensively in many fields. In the past 3 years, however, it has been applied increasingly to pharmaceutical analysis because of its rapidity and the high degree of resolution achieved. Under specified conditions the technique lends itself to quantitative interpretation and can be used for analytical control and toxicological investigations. Direct elution techniques from the adsorbent may be used provided no extracted contaminants interfere with the assay, although colorimetric reagents can be employed to ensure that only specific chemicals are estimated (2). Since there is a correlation between the amount of drug applied to a plate and the area of the developed spot, drugs have been estimated by careful measurement of spot area (3). By this method Morrison and Chatten estimated antihistamines in drug mixtures and measured the spot areas involved manually (4).

The present work was undertaken to develop the technique of densitometric measurement, a method in which spot area is calculated electronically. This method of area measurement is simple to operate and compares favorably with gravimetric or spectrophotometric techniques. Furthermore, in using a photoelectric device, the process of measurement is rapid and convenient. Such an instrument allows analytical procedures to become automated when their reliability has been established and these procedures can then be carried out by semiskilled operators. By employing specific reagents to render the drug or drugs visible on the chromatoplate, the technique nullifies the effect of any impurities present in the adsorbent and ensures that only the drug is estimated. The need for elution or extraction of the drug is thus eliminated (5–7).

The authors report the application of such a technique to pharmaceutical preparations available in Canada and suggest possible sources of error in the method.

EXPERIMENTAL

Materials and Apparatus.—Glass plates (200 × 200 mm.) in glass developing tanks lined with solvent saturated filter paper.

Preparation of the Plates.—The plates were coated with a layer of adsorbent 250 mμ thick according to the method of Stahl (1). The slurry was prepared by mixing 30 Gm. of Silica Gel G with 60 ml. of 25% 1,2-dimethoxyethane in distilled water. This resulted in a smooth even film of adsorbent which was not liable to flake or crack.

Chemicals.—All chemicals and reagents used were analytical reagent grade. The chemical purity of the standards was checked by observing their melting points and comparing them with the literature. As further proof of purity, each standard produced only one spot on chromatographic examination. The drugs used in this investigation were acetylsalicylic acid, salicylic acid, phenacetin, amphetamine sulfate,

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TABLE I.—SPRAY REAGENTS USED FOR DRUG DETECTION

Spray Reagent	Constituents	Color, Spot	Color, Background
50% aqueous sulfuric acid	...	Black–brown	White
Furfural spray	20% furfural in *o*-phosphoric acid	Blue–black	White
Mercury-dithizone	(*a*) Suspend 5 Gm. mercuric oxide in 100 ml. of water and add 20 ml. of conc. H_2SO_4. Cool and dilute to 250 ml. with water. (*b*) 10% diphenylthiocarbazone in chloroform. Spray with (*a*) and then (*b*).	Reddish–brown	Gray
Sodium molybdate	0.1% sodium molybdate in conc. sulfuric acid.	Brown	White
Ferric chloride	(*a*) 10% aqueous ferric chloride, 2 parts. (*b*) 5% aqueous potassium ferricyanide, 1 part. (*c*) Distilled water, 8 parts. Mix and spray immediately.	Blue–mauve	Pale green
Dragendorff's reagent (modified)	Bismuth subnitrate, 3.4 Gm. Glacial acetic acid, 20.0 ml. Potassium iodide, 10.0 Gm. Distilled water, 60.0 ml. Dilute 1 ml. of above with 3 ml. of glacial acetic acid and 6 ml. of distilled water.	Orange–red	Gray
Bratton-Marshall reagent	(*a*) 1 *N* hydrochloric acid. (*b*) 5% sodium nitrite. (*c*) 0.1% solution of *N*-(1-naphthyl) ethylene diamine dihydrochloride. Spray with (*a*) and (*b*). Heat and spray with (*c*).	Red–purple	Gray

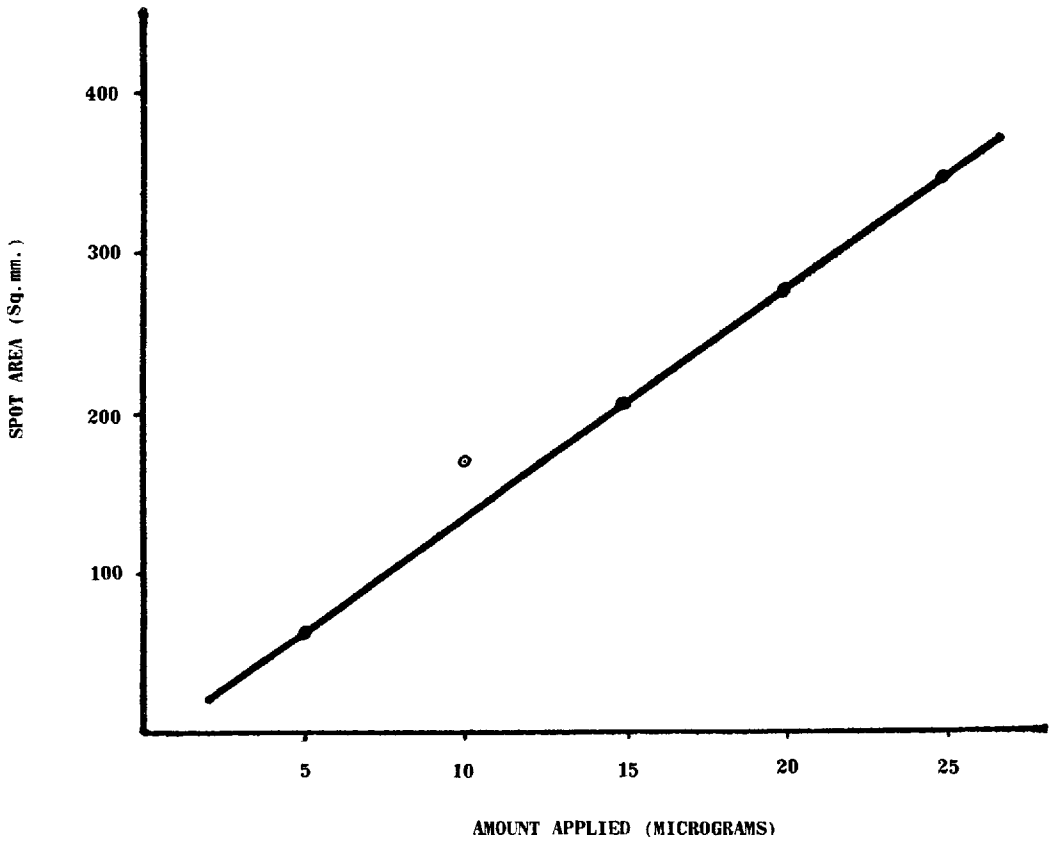

Fig. 1.—Relationship between spot area and spot weight for amphetamine sulfate.

methamphetamine hydrochloride, theophylline, caffeine, ephedrine, phenobarbital, amobarbital, butabarbital, pentobarbital, carbromal, meprobamate, and prochlorperazine maleate.

Developing Solvent.—The developing solvent used in all cases was dioxane–benzene–25% ammonia (40:50:10 v/v).

Spray Reagents.—The compositions of the seven spray reagents used in this investigation are listed in Table I.

Application of Drugs and Development of Chromatogram.—The samples for analysis were applied in ethanolic solution approximately 1 in. from the edge of the plate. Self-filling lambda pipets cali-

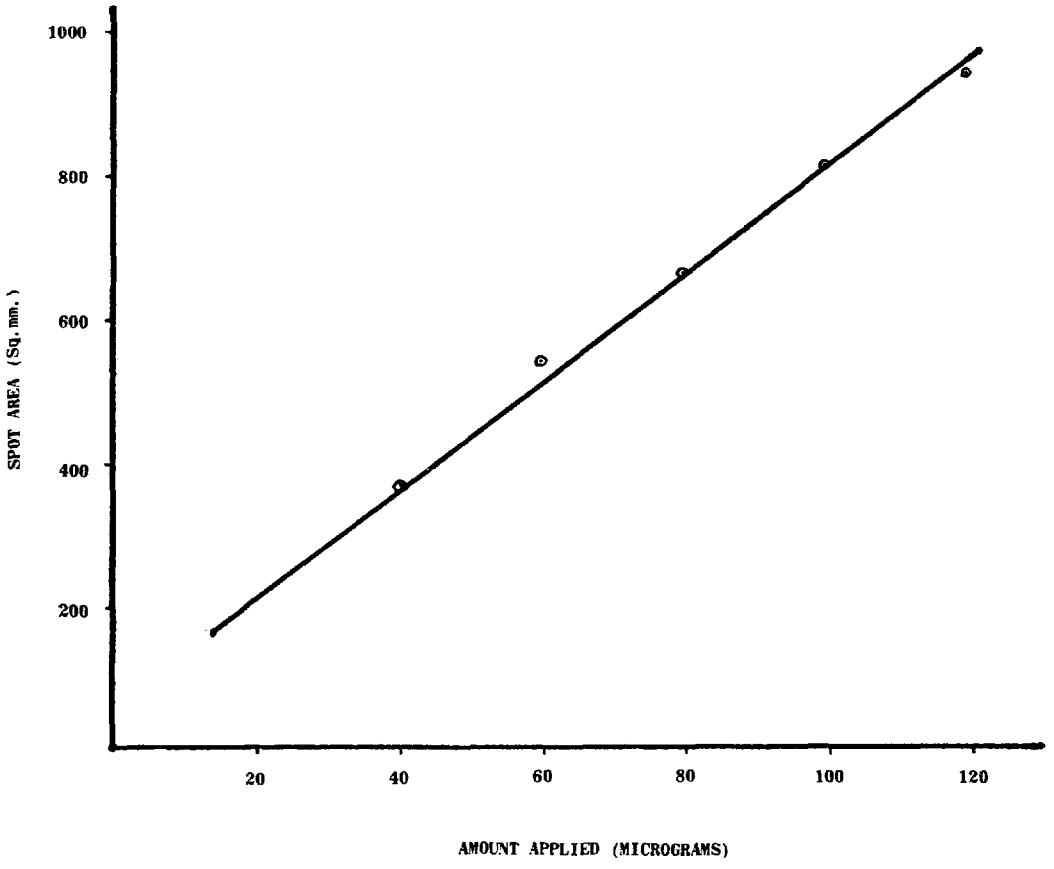

Fig. 2.—Relationship between spot area and spot weight for phenobarbital.

brated to deliver accurately known volumes were used for application, and the spot area was 6–8 mm. in diameter. The plates were developed through a distance of 15 cm., dried at room temperature for 15 min., oven dried for 20 min. at 110°, and sprayed with the appropriate reagent. Reference standards for each drug mixture were applied on the same plate and in concentrations commensurate with the drugs in the sample.

Measurement of Spot Area.—A photovolt densicord (model 542) was used to obtain quantitative results from the developed chromatograms which were positioned directly under the scanning head at a height of approximately 6 mm. The response control selected for the instrument varied with the chemical reagent used to render the drugs visible. The resulting graph plotted by the instrument was rendered quantitative by the densicord electronic integrator which calculated the area under the curve. Results were calculated with reference to the appropriate standard.

Preparation of Standard Curves.—To establish the relationship between spot area and concentration, amphetamine, phenobarbital, and acetylsalicylic acid were spotted in various concentrations in their respective detectable ranges. Following development, the chromatograms were scanned and the integrated areas found for each concentration.

Assay of Simulated Drug Mixtures.—To check the reliability of the method ethanolic solutions of several drugs in concentrations approximating those of various commercial preparations were spotted on plates and developed. The chromatograms were then scanned with the densitometer and compared with relative standards.

Assay of Commercial Preparations.—Each of the preparations was treated individually due to differences in the concentrations of the constituents. The amount of drug applied to the chromatogram varied from one product to another, but this was necessary to ensure that the spot would contain the minimum assayable quantity of the drug. The general procedure for tablets consisted in crushing several weighed tablets in a mortar and dissolving a weighed portion in approximately 15 ml. of ethanol. After shaking for 20 min., the solution was filtered, rinsed with two portions of ethanol, and made up to volume. Assay limits were established by running a series of chromatograms containing several concentrations of the drugs. The appropriate standards were applied to the plate to ensure a total of 10 assays for each constituent. Standards and samples were spotted alternately on the plate to facilitate assay and nullify any variation in the silica gel layer. After development, the plates were sprayed with the appropriate reagent to de-

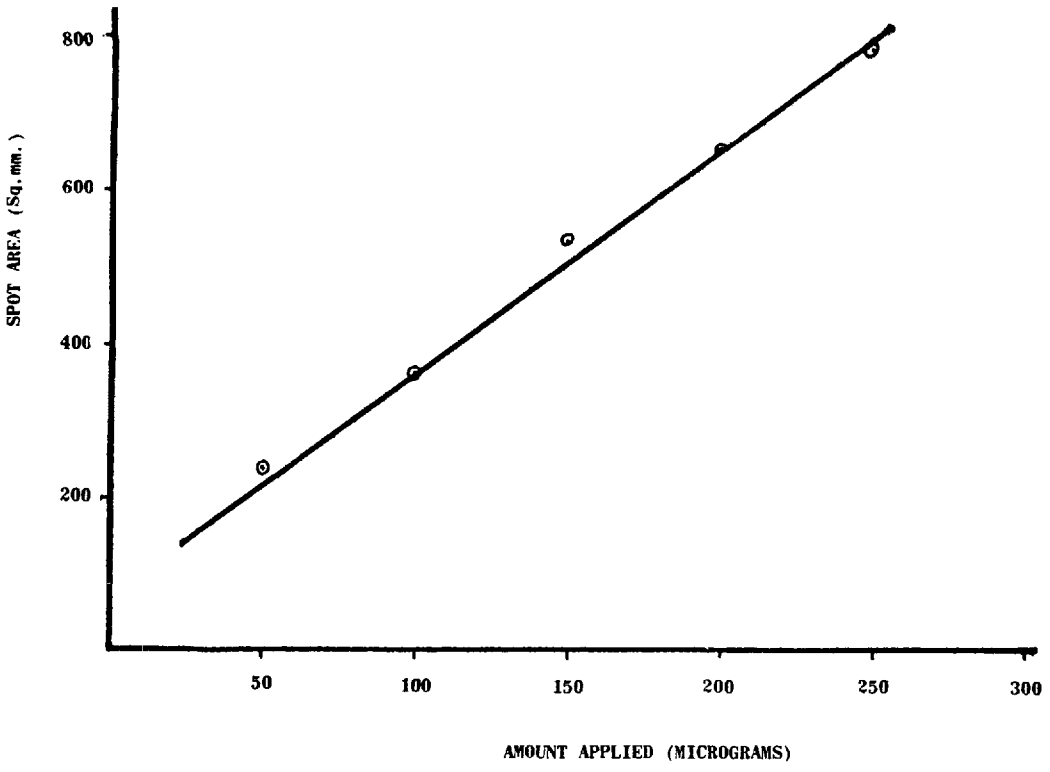

Fig. 3.—Relationship between spot area and spot weight for acetylsalicylic acid.

TABLE II.—DATA SHOWING CORRELATION BETWEEN SPOT WEIGHT AND SPOT AREA

Drug	Amt. Applied, mcg.	Area, Sq. mm.
Amphetamine sulfate	5	60
	10	168
	15	204
	20	276
	25	348
Phenobarbital	40	372
	60	540
	80	660
	100	804
	120	936
Acetylsalicylic acid	50	240
	100	360
	150	528
	200	648
	250	780

velop visible spots. R_f values were recorded and the chromatograms scanned by the densitometer.

RESULTS

Relationship Between Spot Area and Concentration.—By preparing a series of known standards it was possible to demonstrate a linear relationship between spot area and spot content and to reproduce this correlation within certain parameters (Figs. 1–3). This relationship was established for amphetamine sulfate in concentration ranges between 5 and 25 mcg. and for phenobarbital and acetylsalicylic acid in concentrations between 40 and 120 mcg. and between 50 and 250 mcg., respectively. The slight variation in the slopes of the lines can be attributed to several factors such as the intensity of stray light

TABLE III.—QUANTITATIVE RESULTS FOR SIMULATED DRUG MIXTURES

Drug	Amt./Tablet, mcg.	Mean % Recovery	R_f Value	Spray Reagent
Simulated preparation *8*				
d-Amphetamine sulfate	12.0	96.4 ± 2.3	0.44	50% sulfuric acid
Phenobarbital	48.0	97.0 ± 2.4	0.62	
Simulated preparation *1*				
d-Amphetamine sulfate	5.0	99.7 ± 3.7	0.21	50% sulfuric acid
Meprobamate	400.0	98.3 ± 2.0	0.51	Furfural
Simulated preparation *12*				
Amobarbital	32.0	95.4 ± 1.3	0.79	Mercury-dithizone
Phenacetin	160.0	98.7 ± 3.6	0.48	Ferric chloride

TABLE IV.—QUANTITATIVE RESULTS FOR COMMERCIAL PHARMACEUTICALS

Prepn.	Labeled Strength/ Tablet	Mean % Recovery	Min. Detectable Quantity, mcg.	Min. Assayable Quantity, mcg.	Max. Assayable Quantity, mcg.	Spray Reagent	R_f Value
1 (tablets)							
d-Amphetamine sulfate	5.0	99.4 ± 3.4	10	10	20	50% sulfuric acid	0.22
Meprobamate	400.0	98.0 ± 3.3	80	80	1600	Furfural	0.49
2 (tablets)							
d-Amphetamine sulfate	5.0	94.5 ± 2.7	5	10	200	50% sulfuric acid	0.21
Prochlorperazine maleate	2.5	92.6 ± 3.5	5	10	150		0.38
3 (tablets)							
d-Amphetamine sulfate	5.0	94.6 ± 4.8	5	10	200	50% sulfuric acid	0.29
Amobarbital	32.0	93.7 ± 2.6	40	50	300	Mercury-dithizone	0.85
4 (tablets)							
d-Amphetamine sulfate	5.0	97.1 ± 5.2	5	10	200	Sodium molybdate	0.27
Sodium butabarbital	32.0	94.1 ± 1.7	40	50	300	Mercury-dithizone	0.55
5 (tablets)							
d-Amphetamine sulfate	15.0	95.8 ± 5.6	5	10	200	Sodium molybdate	0.31
Amobarbital	100.0	95.9 ± 2.2	40	50	300		0.86
6 (tablets)							
d-Amphetamine phosphate	5.0	96.3 ± 3.9	5	10	200	50% sulfuric acid	0.44
Pentobarbital	32.0	94.2 ± 2.6	40	50	300	Sodium molybdate	0.73
7 (tablets)							
Methamphetamine hydrochloride	15.0	94.0 ± 2.9	5	10	200	50% sulfuric acid	0.20
Phenobarbital	64.8	95.2 ± 5.0	40	50	350		0.85
8 (tablets)							
d-Amphetamine sulfate	12.0	97.9 ± 4.9	5	10	200	50% sulfuric acid	0.46
Phenobarbital	48.0	96.2 ± 3.9	40	50	350		0.60
9 (capsules)							
Carbromal	250.0	96.4 ± 4.3	85	100	300	Furfural	0.73
Pentobarbital	100.0	95.6 ± 3.4	40	50	300	Sodium molybdate	0.59
10 (tablets)							
Acetylsalicylic acid	160.0	98.9 ± 5.8	50	50	350	Ferric chloride	0.00
Phenacetin	160.0	98.5 ± 6.5	50	50	350	Ferric chloride	0.47
dl-Amphetamine	2.5	99.4 ± 3.8	10	10	350	50% sulfuric acid	0.18
11 (tablets)							
Phenobarbital	25.0	97.3 ± 2.7	40	50	300	50% sulfuric acid	0.40
Ephedrine hydrochloride	48.0	91.8 ± 3.3	10	10	200	Dragendorff's	0.18
Theophylline	180.0	No recovery	...	...	...	...	0.00
12 (tablets)							
Amobarbital	32.0	99.1 ± 3.5	40	50	300	Mercury-dithizone	0.72
d-Amphetamine sulfate	5.0	100.6 ± 2.9	5	10	300	50% sulfuric acid	0.23
Acetylsalicylic acid	160.0	96.5 ± 3.3	50	80	400	Ferric chloride	0.00
Phenacetin	160.0	96.4 ± 3.7	50	80	400	Ferric chloride	0.51
13 (tablets)							
Acetylsalicylic acid	200.0	93.8 ± 3.0	50	80	300	Dragendorff's	0.00
Phenacetin	150.0	93.8 ± 3.4	50	80	300	Ferric chloride	0.63
Caffeine citrate	30.0	96.6 ± 2.5	10	30	300	Ferric chloride	0.53
Meprobamate	200.0	96.9 ± 3.5	80	80	1600	Furfural	0.45
Triple-sulfas (tablets)							
Sulfamethazine	167.0	96.6 ± 4.9	0.2	0.5	2.5	Bratton-Marshall	0.75
Sulfadiazine	167.0	94.5 ± 2.7	0.2	0.5	2.5	reagent	0.63
Sulfamerazine	167.0	96.3 ± 3.8	0.2	0.5	2.5		0.71

on the photocell, the detection spray used, and variations in layer thickness (8). The results are shown in Table II.

Results for Simulated Drug Mixtures and Commercial Preparations.—The percentage recoveries obtained from the drug mixtures simulating existing commercial preparations are shown in Table III, and the recoveries from the commercial preparations are shown in Table IV which includes the labeled strength of each preparation and the mean recovery from 10 assays on each preparation. The R_f values and the minimum and maximum assayable quantities are included for each preparation together with the staining reagent used to render the drugs visible.

DISCUSSION

From the results obtained in this study it is suggested that the technique of densitometric measurement is sufficiently accurate for routine pharmaceutical analysis and can be applied to pharmaceutical dosage forms. The average error involved was found to be about 5%, a figure which is similar to that reported in related fields (9–11).

Several experimental factors were found to be critical in obtaining consistent and accurate results. Since the area of the spot can increase during development from 25 to 100%, the size of the initial spot application should be as small and as uniform as possible. An applied spot of 6–8 mm. was found to be satisfactory. Spots which are not uniformly

applied may occupy different areas, and this difference with its consequent error will be registered by the photocell. Similarly, variations in the layer thickness of the silica gel can cause alterations in the light reflected by the background, and this error can be recorded by the densitometer since it is designed to produce results which are based on the difference in photoelectric intensity between the visible spot and the background of the plate. For this reason it is essential to use spray reagents which produce stains specific for individual drugs and which do not color the background to any appreciable extent, otherwise the contrast between spot and background will be diminished. If a satisfactory spray reagent cannot be developed, it is doubtful whether the method could be used for quantitative work.

In using thin-layer densitometry it was essential that the drugs being assayed separated sharply and distinctly as any degree of spot overlap renders area measurement techniques liable to error. To some extent it was found possible to counteract the effects of overlap by spraying the plate with a stain which rendered only a particular drug visible for purposes of assay. Alternatively, two or more plates could be used, one of which was treated to allow estimation of one fraction of the drug mixture while the other plate was developed to estimate any additional components.

The minimum quantity of a drug necessary for detection on a thin-layer plate appeared to depend on the specificity of the spray reagent used to produce the necessary color, and it was observed that these chemical stains varied in their relative sensitivities of detection. For example, with the exception of phenobarbital, the barbiturates did not appear distinctly after charring with sulfuric acid, but when a 2% mercuric sulfate spray was used followed by 0.1% sodium molybdate in concentrated sulfuric acid both amobarbital and butabarbital appeared as distinct brown spots on a gray background and could be assayed satisfactorily. Consequently, the minimum assayable quantity for these drugs using the acid-molybdate spray was 40 mcg. as opposed to 50 mcg. with the sulfuric acid spray. Thus, it was found necessary to vary the amount of solution applied to a plate in such a way that the amount of drug being estimated contained the minimum detectable quantity. For example, in preparation *I* the tablets have a ratio of amphetamine to meprobamate of 1–80 and the amounts of solution applied to the plate in the assay of this preparation must be varied to allow the accurate estimation of each drug.

Due to the very low solubility of prochlorperazine maleate in the extracting solvents the agitation time during its extraction was increased from 30 to 60 min. to ensure its complete removal from the tablet. Solubility was also a factor in the assay of preparation *11* when it was found that the theophylline in the tablets has such a low solubility and was so slowly soluble that quantitative estimation by this method proved unreliable.

Triple-sulfa tablets U.S.P. were included in this study to demonstrate the application of the densitometric technique to systems where distinct stains exist or can easily be found. Using the method reported by Wehrli (12) for the qualitative separation of these sulfonamides, it was found that quantitative evaluation of the tablets could be readily carried out by this method.

It is suggested that if a sensitive spray reagent exists and the correct experimental conditions are observed regarding the parameters of the assay, the densitometric method could be applied in various fields of pharmaceutical analysis to yield quantitative results.

SUMMARY

A thin-layer chromatographic technique using Silica Gel G as the adsorbent has been devised for the separation and analysis of 14 selected pharmaceuticals which included amphetamines, barbiturates, and related compounds. It was demonstrated that a linear relationship exists between spot area and spot content. Quantitative evaluation was achieved without elution from the adsorbent by using a photoelectric densitometer coupled to an electronic integrator which computed the spot areas. The experimental error was found to be approximately 5%. The technique gave quantitative results when the drugs were applied to the plates within certain concentrations. These concentrations varied with individual drugs but were in the range of 25 to 100 mcg. generally. Dioxane–benzene–25% ammonia (40:50:10 v/v) was found satisfactory for resolving the selected pharmaceuticals into their respective components. Results were obtained more rapidly and with greater convenience than by planimetry or visual area measurement. Several experimental factors which influence quantitative recovery are discussed. The technique could be applied routinely in microanalysis and has specific application to pharmaceuticals.

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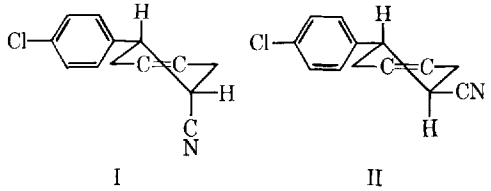
Characterization of Epoxides of *cis*- and *trans*-4-(*p*-Chlorophenyl)-5-cyanocyclohexene from NMR Spectra of Derivatives

By DAVID B. ROLL* and ALAIN C. HUITRIC

The epoxidation of *cis*-4-(*p*-chlorophenyl)-5-cyanocyclohexene with *m*-chloroperbenzoic acid in diethyl ether yielded *trans*-4-(*p*-chlorophenyl)-*trans*-5-cyano-1,2-epoxycyclohexane as the only product. Epoxidation of *trans*-4-(*p*-chlorophenyl)-5-cyanocyclohexene with *m*-chloroperbenzoic acid yielded *cis*-4-(*p*-chlorophenyl)-*trans*-5-cyano-1,2-epoxycyclohexane and *trans*-4-(*p*-chlorophenyl)-*cis*-5-cyano-1,2-epoxycyclohexane in ratio of about 7 to 3, respectively, when carried out in ether but in ratio of about 1 to 1 in 1,2-dichloroethane. The epoxides were characterized from the NMR spectra of the derived diols, mono-, and diacetates by taking advantage of the difference between the spatial 1,3-diaxial deshielding effects of hydroxyl and acetoxy groups.

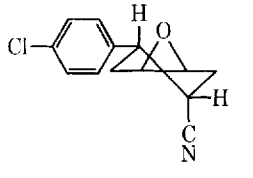
IN THE course of the synthesis of certain agents of possible medicinal interest it was necessary to characterize the epoxidation products of *cis*- and *trans*-4-(*p*-chlorophenyl)-5-cyanocyclohexene, compounds I and II, respectively. Compounds I and II were prepared by the Diels-Alder condensation of butadiene with *cis*- and *trans*-*p*-chloro-β-cyanostyrene (1), respectively. Epoxidation of I with *m*-chloroperbenzoic acid in diethyl ether yielded a single product, while epoxidation of II yielded two products in ratio of about 7 to 3 when carried out in ether but in ratio of 1 to 1 when carried out in 1,2-dichloroethane. The epoxidation of I was not carried out in 1,2-dichloroethane. Analysis was done by gas chromatography. These epoxides have been characterized from the nuclear magnetic resonance spectra of some of their derivatives by taking advantage of the long-range 1,3-diaxial deshielding effects of the hydroxyl and acetoxy functional groups.



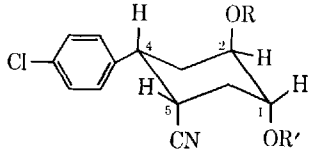
I II

CONFIGURATIONAL ASSIGNMENT OF EPOXIDE DERIVED FROM I

Assignment of configuration of the single epoxide obtained from I was not possible from its NMR spectrum. The epoxide was characterized as the isomer with the epoxy group *trans* to the cyano group, compound IV, from the NMR spectra of the diol (V), the monoacetate (VI), and the diacetate (VII) derived from it. It is interesting to note that of the two possible epoxides only IV was obtained in detectable quantity. From consideration of steric factors it is not surprising that the rate of formation of IV should be faster than that of the isomer with the epoxy group *cis* to the cyano group.



IV



V, R = R′ = H
VI, R = H, R′ = Ac
VII, R = R′ = Ac

The relevant portions of the spectra of V, VI, and VII, measured in pyridine, are given in Fig. 1, A, B, and C, respectively. Analysis of the spectra shows that in pyridine all three derivatives exist predominantly in the chair conformation with the aromatic ring in equatorial orientation.

Spectrum A indicates the equatorial orientation for H-1, H-2, and H-5, and the axial orientation for H-4. The equatorial orientation is established for H-1 at τ 5.65 and H-2 at 5.46 from their narrow signals, half-height width of 6 to 7 c.p.s. The axial orientation is established for H-4 from the sextet at τ 6.23. The sextet indicates a coupling constant of about 13 c.p.s. between H-4 and axial H-3 with additional minor coupling of about 3.5 c.p.s. with equatorial H-3 and H-5. The signal of H-5 at τ 6.73 is partially overlapped with the signal of axial H-3, but the approximate width of the signal indicates that H-5 has the equatorial orientation. The signal centered at τ 7.05, which appears as a main triplet with separation of 13 c.p.s., plus additional minor splitting, is attributed to axial H-3. The main splitting of about 13 c.p.s. results from geminal

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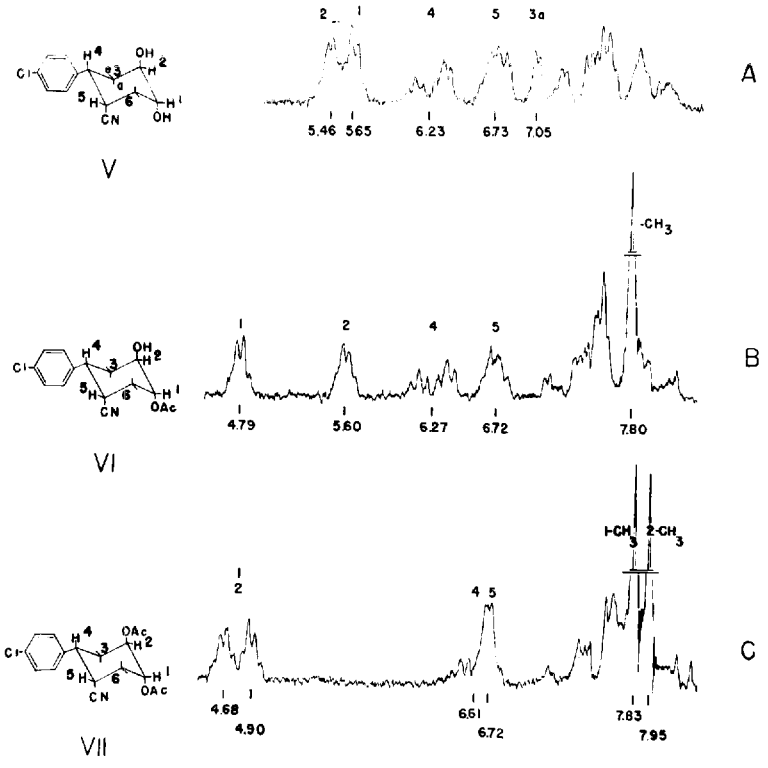


Fig. 1.—NMR spectra of *trans*-2-hydroxy-*cis*-4-(*p*-chlorophenyl)-*cis*-5-cyanocyclohexanol (A), the corresponding 1-acetate (B), and the corresponding 1,2-diacetate (C) at 60 mc. in pyridine at 37°; chemical shifts in τ units; TMS internal reference.

coupling with equatorial H-3 and vicinal coupling with axial H-4. The minor splitting results from coupling with equatorial H-2. The downfield shift of axial H-3 results from long-range deshielding by the axial hydroxyl and cyano groups on C-1 and C-5, respectively. Differentiation of the signals of H-1 and H-2 was done by spin-spin decoupling. Irradiation at τ 5.46 caused the sextet of axial H-3 at τ 7.05 to collapse into a sharp triplet with separations of 13 c.p.s., while irradiation at 5.65 caused no change in this signal.

The given chair conformation is established as the predominant structure of VI from spectrum B in a similar way. The narrow signals of half-height width of about 7 c.p.s. for H-1 at τ 4.79 and H-2 at τ 5.60 indicate the equatorial orientation of these two protons. The axial orientation is established for H-4 from its sextet at τ 6.27 with apparent coupling of 13 c.p.s. with axial H-3 and about 3.5 c.p.s. with equatorial H-3 and H-5. The half-height width of about 8 c.p.s. for the signal of H-5 at τ 6.72 indicates the equatorial orientation of H-5.

In a similar way the narrow signals of half-height width of about 6 to 7 c.p.s. for H-1 and H-2 at τ 4.68 and 4.90, and H-5 at τ 6.72 in spectrum C establish the equatorial orientation of these three protons, and, therefore, also establish the given chair conformation of VII in pyridine. Signals of H-1 and H-2 cannot be differentiated with certainty. The signal of H-4 is partially overlapped by the signal of H-5, but its chemical shift is approximated as 6.61. The acetoxy methyl groups are magnetically nonequivalent. Assignment was made by comparison with spectrum B of VI. The downfield shift of the group on C-1 is attributed to the long-range deshielding effect of the axial cyano group.

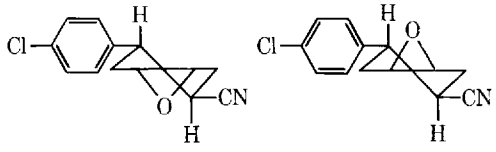
Having established the conformations of V, VI, and VII, the assignment of the configuration of the parent epoxide was possible from the chemical shifts of H-4 of the three derivatives by taking advantage of the spatial 1,3-diaxial deshielding effect of a hydroxyl group on ring hydrogens (2–4) and of the larger deshielding effect of a hydroxyl group compared to the corresponding acetoxy group (3, 4). The chemical shifts of H-4 of the diol V and the monoacetate VI are almost identical, τ 6.23 and 6.27, respectively; but the signal of H-4 appears at about τ 6.61 in the spectrum of the diacetate (VII), about 20 to 23 c.p.s. upfield from that of the monoacetate or diol. This establishes that axial H-4 is deshielded by an axial hydroxyl group on C-2 in the monoacetate (VI) as well as in the diol (V) and in turn establishes that the parent epoxide has the epoxy group *trans* to the cyano group (IV). The established structures of the derivatives indicate that the epoxide opening occurred *via* a transition state with the aromatic group essentially equatorial. A transition state with the aromatic group axial would yield a diol which would differ from structure V by having the *trans* diequatorial hydroxyl groups. The two possible isomeric epoxides would yield identical diaxial diols and diacetates but different monoacetates *via* the transition state with the aromatic group equatorial. If the product of epoxidation had been the *cis*-epoxide, the chemical shift of H-4 should have been almost identical in the mono- and diacetate but significantly different in the monoacetate compared to the diol.

Hydrogenation of I and II yielded *cis*-2-(*p*-chlorophenyl)-1-cyanocyclohexane (III) and *trans*-2-(*p*-chlorophenyl)-1-cyanocyclohexane (VIII), respectively. The NMR spectrum of III measured in

pyridine clearly establishes the *cis* configuration. The signal of H-2 appears as a broad multiplet of about 23 c.p.s. width at τ 7.33. The width of the signal indicates that H-2 has an axial orientation. The signal of H-1 gives a much narrower peak of half-height width of about 7 c.p.s. at τ 6.92, indicating that H-1 has the equatorial orientation. In the spectrum of the *trans* isomer there is overlapping of the broad signals of axial H-2 and H-1 when measured in carbon tetrachloride. The spectrum of VIII was not obtained in pyridine.

CONFIGURATIONAL ASSIGNMENT OF EPOXIDES DERIVED FROM II

The NMR spectra of the two epoxides (IX and X) obtained from II did not provide unequivocal characterization of the two isomers.



IX X

The major component of the epoxidation reaction of II in ether was converted to the diol, monoacetate, and diacetate derivatives, compounds XI, XII, and XIII, respectively, but the spectra of these three derivatives did not allow an unambiguous application of the method used for characterization of epoxide (IV) because of overlapping of signals of H-4 and H-5. In order to obtain reliable chemical shifts for H-4 the three corresponding derivatives deuterated at C-5 were prepared. The deuterium exchange on the cyano-bearing carbon was accomplished by refluxing II in methanol-*d* in the presence of sodium methoxide. Some isomerization occurred during the deuterium exchange process and pure deuterated II was obtained by recrystallization from absolute ethanol. Deuterated II was epoxidized in ether and the major component was converted to the diol, monoacetate, and diacetate derivatives, compounds XI*d*, XII*d*, and XIII*d*, respectively. The pertinent portions of the NMR spectra of the deuterated derivatives measured in pyridine are given in Fig. 2. The effect of deuteration on C-5 is demonstrated by the portions of the spectra of the corresponding nondeuterated compounds shown above the signal of H-4 in each spectrum. In the spectra of the nondeuterated compounds there is partial overlap of the signals of H-4 and H-5. Replacement of H-5 with deuterium also simplifies the signal of H-4 which is now coupled only with the two protons on C-3.

The three spectra show that in pyridine all three derivatives exist predominantly in the chair conformation with the aromatic ring in equatorial orientation. The spectra also establish that the epoxide from which they were derived has the epoxy group *trans* to the cyano group, structure IX.

Spectrum D establishes the equatorial orientation of H-1 and H-2 in XI*d* from their overlapping signals at τ 5.65 which give a narrow peak of half-height width of about 6 to 7 c.p.s. The axial orientation of H-4 is established from the quartet at τ 6.42. The quartet gives apparent coupling constants of 12.0 c.p.s. between H-4 and axial H-3 and about 3.5 to 4 c.p.s. between H-4 and equatorial H-3. The spectrum is consistent with the conformation shown in Fig. 2.

The given conformation of XII*d* is established in the same fashion from the narrow signals (half-height width of 6 to 7 c.p.s.) of H-1, at τ 5.74 and H-2 at τ 4.73 which establish the equatorial orientation of H-1 and H-2, and from the quartet of H-4 at τ 6.74 ($J_{43a} \simeq 12$ c.p.s. and $J_{43e} \simeq 4$ c.p.s.) which established the axial orientation of H-4.

In the spectrum of the diacetate (XIII*d*), spectrum F, the signals of H-1 and H-2 are overlapping to give a narrow signal at τ 4.87. The half-height width of the combined signals is about 7 c.p.s. indicating that H-1 and H-2 have the equatorial orientation. Any significant contribution of the other chair conformation with H-1 and H-2 axial would give wider signals for H-1 and H-2. In this spectrum the signal of II-4 appears as a symmetrical triplet with separation of 8.2 c.p.s. instead of the expected quartet. The important thing to note is that the width of the signal of 16.4 c.p.s. is about identical to the width of the signals of H-4 in spectra D and E. A deceptively simple spectrum, giving a triplet instead of the expected quartet, will occur for the signal of H-4 if averaging of coupling constants J_{43a} and J_{43e} results due to strong coupling effects (5–7). Averaging of coupling constants due to strong coupling effects will become operative when the difference between the chemical shifts of the axial and equatorial protons on C-3 approaches, or become smaller than, the geminal coupling constant between the two protons on C-3. In the limit where the chemical shifts of axial and equatorial H-3 are equal the signal of H-4 will yield a symmetrical triplet with separations equal to $(J_{43a} + J_{43e})/2$ (7). Complications due to strong coupling effects do not cause any change in the width of the signal. Inspection of the upper field portion of spectrum F and comparison with spectra D and E indicates that similar chemical shifts of the two protons on C-3 probably prevails in XIII*d*. In compounds XI*d* and XII*d* the deshielding effect of the C-1 axial OH will cause a downfield shift of the signal of axial H-3 of more than 50 c.p.s. when measured in pyridine at 60 mc. (2). The signal of axial H-3 will, therefore, be downfield from that of equatorial H-3 in XI*d* and XII*d*. This is also indicated to be the case from the up-field portions of spectra D and E. Since the 1,3-diaxial deshielding effect of the acetoxy group is about 20 cycles less than that of the OH group, nearly identical chemical shifts of axial and equatorial H-3 is not unexpected and provides a logical explanation for the observed triplet of H-4. The width of the signal definitely establishes the axial orientation of H-4.

Having established the conformations of XI*d*, XII*d*, and XIII*d*, the configuration of the parent epoxide was possible from the chemical shifts of H-4 in the three derivatives. The chemical shift of H-4 is almost the same in the mono- and diacetate but it is from 19 to 24 cycles to lower field in the diol. This establishes that the acetoxy group is *cis* to axial H-4 in the monoacetate and in turn establishes the configuration of its parent epoxide as the isomer with the epoxy group *trans* to the cyano group, structure IX. The two epoxides obtained from peracid

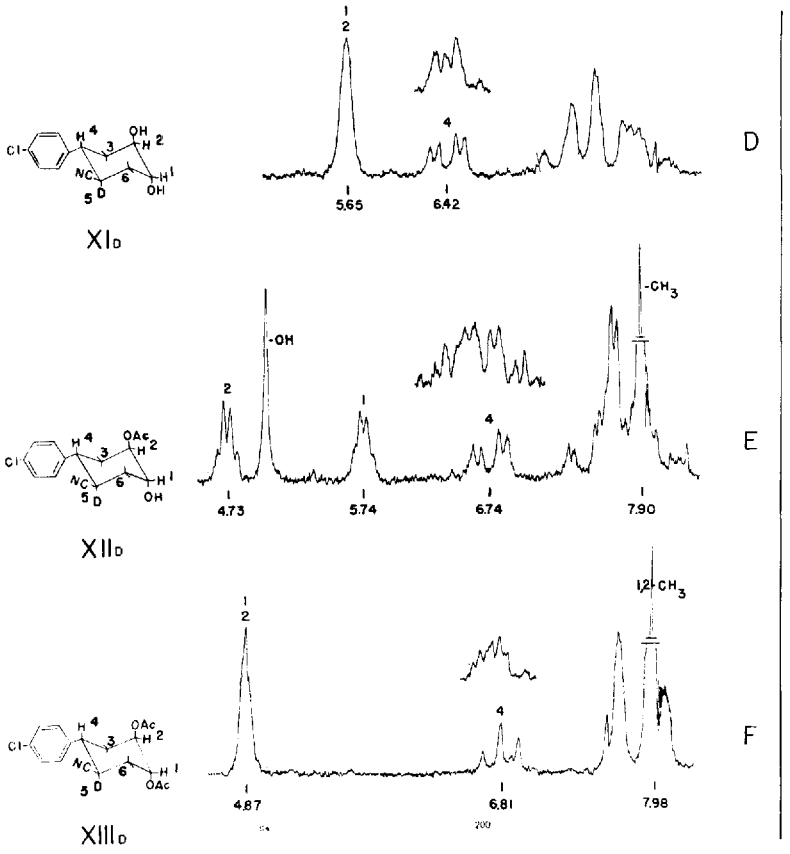


Fig. 2.—NMR spectra of *trans*-2-hydroxy-*cis*-4-(*p*-chlorophenyl)-*trans*-5-cyanocyclohexanol-5-*d* (D), the corresponding 2-acetate (E), and 1,2-diacetate (F); portions of the spectra of the corresponding nondeuterated compounds are shown above signals of H-4; all at 60 mc. in pyridine at 37°; chemical shifts in τ units; TMS internal reference.

oxidation of II yield the same diol (XI) on acid catalyzed hydrolysis. The major product of the epoxidation reaction in ether is, therefore, characterized as structure IX and the minor product has structure X.

cis-2-(*p*-Chlorophenyl)-1-aminomethylcyclohexane (XIV) and the *trans* isomer (XV) were prepared by lithium aluminum hydride reduction of the corresponding cyano compounds (III and VIII). The NMR spectra of these compounds were not very informative. The hydrobromide salts of the two amines elicited strong CNS excitation in mice when injected i.p., but there was little observable difference in potency between the two isomers.

EXPERIMENTAL

Melting points were determined on a Kofler micro hot stage. The NMR spectra were determined with a Varian A-60 spectrometer. Spectra were taken in pyridine, unless otherwise indicated in a concentration of about 150 mg./0.5 ml. with tetramethylsilane as internal reference.

cis-4-(p-Chlorophenyl)-5-cyanocyclohexene (I).—In a typical synthesis 20 Gm. (0.122 mole) of *cis*-*p*-chloro-β-cyanostyrene (1), about 80 ml. of condensed butadiene, 20 ml. of toluene, and a trace of hydroquinone were heated with shaking under a nitrogen atmosphere in a stainless steel bomb for 16 days at a temperature of 138°. Analysis of the reaction mixture by gas chromatography on a 10-ft. column of 20% silicon QF-1 on acid-washed Chromosorb W at 185° showed a ratio of unchanged styrene to product I of about 2 to 1. The dark crude material was passed on a column of 0.05–0.2 mm. silica gel using benzene as solvent, resulting in the removal of much colored material. After removal of the benzene the residue was washed repeatedly with hot isopropyl alcohol. This treatment resulted in the extraction of the product and unreacted styrene from a considerable amount of rubbery polymeric material which was left behind. The isopropyl alcohol was removed and product I was separated from the unchanged styrene by numerous recrystallizations from hexane. Because of the distinctive crystal structure of I the purification could be speeded up by preliminary mechanical separation of crystals. The separation was also accomplished by column chromatography on 0.05–0.2 mm. silica gel using petroleum ether–benzene mixtures as solvent. Final recrystallization was from isopropyl alcohol–benzene mixture, m.p. 48–49°.

Anal.—Calcd. for $C_{13}H_{12}ClN$: C, 71.72; H, 5.56. Found: C, 71.47; H, 5.70.

trans-4-(p-Chlorophenyl)-5-cyanocyclohexene (II).—Compound II was prepared by the same method as I, except that the reaction time was 9 days, the temperature 130°, and the preliminary chromatography was not used in the purification procedure. There was some unchanged styrene present. The product was obtained in 44% yield by recrystallization from hexane. The final crystallization was from a mixture of isopropyl alcohol and benzene, m.p. 132.5–134°.

Anal.—Calcd. for $C_{13}H_{12}ClN$: C, 71.72; H, 5.56; N, 6.44. Found: C, 72.28; H, 5.43; N, 6.45.

cis- and trans-2-(p-Chlorophenyl)-1-cyanocyclohexane (III and VIII).—These compounds were obtained by low pressure catalytic hydrogenation of I and II, respectively, using 10% Pd on carbon in absolute ethanol. The yields were quantitative. An analytical sample of III recrystallized from hexane melted at 103.5–104.5°, whereas VIII recrystallized from the same solvent melted at 107–108°.

Anal.—Calcd. for $C_{13}H_{14}ClN$: C, 71.07; H, 6.42; N, 6.38. Found: (III) C, 71.29; H, 6.41; N, 6.65. (VIII) C, 71.04; H, 6.47; N, 6.20.

trans-4-(p-Chlorophenyl)-trans-5-cyano-1,2-epoxycyclohexane (IV), cis-4-(p-Chlorophenyl)-trans-5-cyano-1,2-epoxycyclohexane (IX), and trans-4-(p-Chlorophenyl)-cis-5-cyano-1,2-epoxycyclohexane (X).—In a typical synthesis, 3.27 Gm. (0.015 mole) of alkene and 7.68 Gm. of 85% *m*-chloroperbenzoic acid (0.38 mole) were dissolved in about 100 ml. of diethyl ether and allowed to stand at room temperature in the dark for 4 days. The reaction mixture was then washed twice with cold 10% sodium hydroxide. The ether solution was washed with water until the washings no longer were basic to litmus and dried with Drierite. The yield of crude product IV was 89%, which when crystallized from benzene–hexane mixture, melted at 109–110.5°. The yield of the mixture of epoxides (IX and X) from epoxidation of II was 86%. These were in a ratio of approximately 7 to 3 as determined by gas chromatography on a 10-ft. column of 20% silicon QF-1 on acid-washed Chromosorb W at 235°. When II was epoxidized in a similar manner as above, but with 1,2-dichloroethane as the solvent, the ratio of the two epoxides was about 1 to 1. Column chromatography of the mixture of epoxides on 0.05–0.2 mm. silica gel using mixtures of purified benzene and petroleum ether, followed by mixtures of benzene and diethyl ether resulted in the separation of substantial quantities of pure IX and only small quantities of X, still somewhat contaminated with IX, as well as a number of fractions with varying ratios of IX and X. Small amounts of pure X could be obtained by crystallizing fractions rich in X from absolute ethanol. The analytical samples of IX and X, when recrystallized from a mixture of benzene–hexane, melted at 128.5–129° and 148–150°, respectively.

Anal.—Calcd. for $C_{13}H_{12}ClNO$: C, 66.81; H, 5.18. Found: (IV) C, 67.12; H, 5.47. (IX) C, 66.94; H, 5.13. (X) C, 66.93; H, 5.21.

trans-2-Hydroxy-cis-4-(p-chlorophenyl)-cis-5-cyanocyclohexyl Acetate (VI) and trans-2-Acetoxy-cis-4-(p-chlorophenyl)-trans-5-cyanocyclohexanol (XII).—In the synthesis of VI, 1 Gm. (0.0043 mole) of IV was dissolved in 6 ml. of glacial acetic acid and refluxed for 14 hr. The solution was added to 100 ml. of cold water, extracted 3 times with ether, the ether washed with 5% NaOH, and dried over Drierite. After drying, the residue was recrystallized from benzene–hexane mixture yielding 645 mg. (51%) of VI which melted at 149–151°. The rest of the material did not crystallize and was shown by comparison of NMR spectra to be a mixture of the monoacetate (VI) and the diacetate (VII). In the synthesis of XII, to avoid the possible formation of diacetate (XIII), 0.75 Gm. (0.0032 mole) of IX was dissolved in 5 ml. of glacial acetic acid containing 10% w/v of potassium acetate and allowed to stand at room temperature for 24 hr. The reaction mixture was worked up in the same manner as the synthesis of VI above. Crystallization from benzene–hexane mixture yielded 685 mg. (73%) of XI, m.p. 148.5–150°.

Anal.—Calcd. for $C_{15}H_{16}ClNO_3$: C, 61.33; H, 5.49. Found: (VI) C, 61.08; H, 5.65. (XII) C, 61.19; H, 5.77.

trans-2-Hydroxy-cis-4-(p-chlorophenyl)-cis-5-cyanocyclohexanol (V) and trans-2-Hydroxy-cis-4-(p-chlorophenyl)-trans-5-cyanocyclohexanol (XI).—In the synthesis of V, 300 mg. (0.0013 mole) of IV was dissolved in 3 ml. of purified dioxane and 1.5 ml. of distilled water and a drop of concentrated sulfuric acid added. In the case of XI the same procedure was followed except either epoxide IX or a mixture of epoxides IX and X was used, since the glycol (XI) is the product of the hydrolysis of either IX or X. The reaction mixture was allowed to stand at room temperature for 24 hr., suspended in 20 ml. of water, extracted with ether, the ether dried with Drierite, and the solvent removed. The yields of unpurified crystalline product from typical syntheses of V and XI were 232 mg. (71%) and 245 mg. (75%), respectively. Recrystallization of both from benzene–acetone mixture yielded pure V, m.p. 189–190.5°, and pure XI, m.p. 175.5–177°.

Anal.—Calcd. for $C_{13}H_{14}ClNO_2$: C, 62.03; H, 5.61; N, 5.56. Found: (V) C, 62.26; H, 5.76. (XI) C, 61.71; H, 5.62; N, 5.31.

trans-2-Acetoxy-cis-4-(p-chlorophenyl)-cis-5-cyanocyclohexyl Acetate (VII) and trans-2-Acetoxy-cis-4-(p-chlorophenyl)-trans-5-cyanocyclohexyl Acetate (XIII).—The monoacetate VI or XII was placed in 2 ml. each of pyridine and acetic anhydride and allowed to stand at room temperature for 24 hr. The reaction mixture was dissolved in ether and extracted with cold 5% HCl and the ether layer dried over Drierite. In the case of VII, after removal of solvent, the yield of crude product was quantitative. Recrystallization from benzene–hexane mixture yielded pure VII, m.p. 127–128.5°. In the case of XIII, recrystallization from benzene gave a crystalline material melting at 58–61°. The NMR spectrum of this material indicated the presence of a considerable amount of benzene. Heating under vacuum at 80° resulted in removal of the benzene. On solidification, XIII melted over a wide range, 82–106°. The analysis was correct and the NMR spectrum consistent with the expected product.

Anal.—Calcd. for $C_{17}H_{18}ClNO_4$: C, 61.08; H, 5.40. Found: (VII) C, 61.35; H, 5.48. (XIII) C, 61.26; H, 5.16.

cis-2-(p-Chlorophenyl)-1-aminomethylcyclohexane Hydrobromide (XIV) and trans-2-(p-Chlorophenyl)-1-aminomethylcyclohexane Hydrobromide (XV).—A slurry of compound III in ether was added to an excess of $LiAlH_4$ in ether at a rate that maintained a gentle refluxing. The reaction mixture was allowed to stir at room temperature for 1 hr. after the addition was completed. The excess $LiAlH_4$ was destroyed by dropwise addition of ice water. Aqueous 5% NaOH was then added to increase the water layer, the mixture extracted with ether, and the ether solution dried with Drierite. Removal of the solvent gave a quantitative yield of the crude amine, the infrared spectrum of which

showed no —C≡N absorption. The crude amine was dissolved in *n*-hexane and HBr gas bubbled into the solution. The yield of the salt was quantitative. Recrystallization from absolute ethanol yielded pure XIV, m.p. 290–292°.

In the synthesis of XV tetrahydrofuran was used as solvent. Due to low solubility, compound VIII was added as a slurry to an excess of $LiAlH_4$ in tetrahydrofuran. The rest of the reaction and work-up was the same as in the synthesis of XIV above. The yield was quantitative for both the free amine and the salt. Pure XV melted at 237–239° when crystallized from absolute ethanol–benzene mixture.

Anal.—Calcd. for $C_{13}H_{19}BrClN$: C, 51.25; H, 6.29; N, 4.60. Found: (XIV) C, 51.46; H, 6.31; N, 4.44. (XV) C, 51.49; H, 6.24; N, 4.45.

Deuteration on C-5 of II, IX–XIII.—Deuterium exchange on C-5 of II was carried out by refluxing 1.5 Gm. of II in 15 ml. of CH_3OD in the presence of 1 Gm. of sodium methoxide. After cooling, 10 ml. of deuterium oxide was added; the resulting suspension was extracted with anhydrous ether and the ether solution dried with Drierite. After removal of the ether, the NMR spectrum of the product indicated that a considerable amount of isomerization of II to I had occurred during the exchange. Deuterated II was obtained by recrystallization from ethanol. Compound II deuterated at C-5 was submitted to the same sequence of reactions as II in order to obtain compounds IX–XIII deuterated at C-5.

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Hypoiodite Oxidation of 3α-Bromo and 3α-Chloro-2β-hydroxy-5α-androstan-17-one

By R. E. COUNSELL and G. W. ADELSTEIN

Hypoiodite oxidation of 3α-bromo and 3α-chloro-2β-hydroxy-5α-androstan-17-one gave the expected 2β,19-oxide along with lesser amounts of 16-iodinated, 19-hydroxylated, and 19-acetoxylated products. The product composition as well as the yield of each product was found to vary considerably even when the reaction was performed under presumably identical conditions. The structures were assigned on the basis of chemical and spectroscopic evidence. The NMR and other spectroscopic properties of the epimeric 16α- and 16β-iodinated derivatives of 3α-bromo-2β,19-oxido-5α-androstan-17-one are discussed.

INTEREST in 19-substituted steroids as potential anabolic agents led to the investigation of the hypoiodite oxidation of 2β-hydroxyandrostanes as a means of introducing functional groups at the C-19 position (1). During the course of these studies it was noted that a number of minor by-products were formed in addition to the expected oxidation product. This report involves a study of this reaction and structural elucidation of the by-products.

DISCUSSION

The conversion of 2β-hydroxysteroids to 19-functionalized products has been accomplished by: (*a*) oxidation with lead tetraacetate alone (2, 3) or in the presence of iodine (hypoiodite) (4) and (*b*) photolysis of nitrite esters (3, 5). In each case, an alkoxy radical (A) is generated which can interact with the proximal angular methyl group. In the hypoiodite reaction, a furan (B) or an iodofuran (C) can form depending on the mechanism of ring closure. (Scheme I).[1] Although compounds of type C have not been isolated, the characterization of the corresponding hydroxy and acetoxy derivatives among the reaction products has suggested the intermediacy of such an iodinated precursor.

In these studies, hypoiodite oxidation of 3α-bromo-2β-hydroxy-5α-androstan-17-one (I) in refluxing carbon tetrachloride afforded a 36% yield of 3α-bromo-2β,19-oxido-5α-androstan-17-one (III) after chromatography.[2] In addition to this expected product, three other crystalline products were isolated. Two of these products preceded the major product on chromatography and were characterized as the 16β- and 16α-iodinated derivatives (IV and V). These epimers were obtained in 1.4 and 1.2% yield, respectively. The third product, obtained in 10% yield, followed the major product on chromatography and was identified as the hemiacetal (VI).

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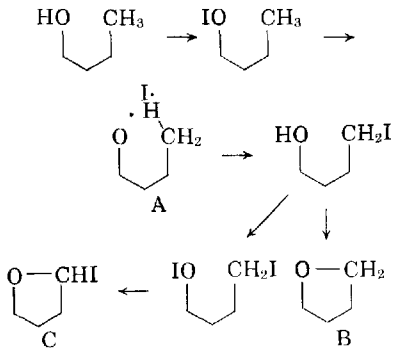


Scheme I

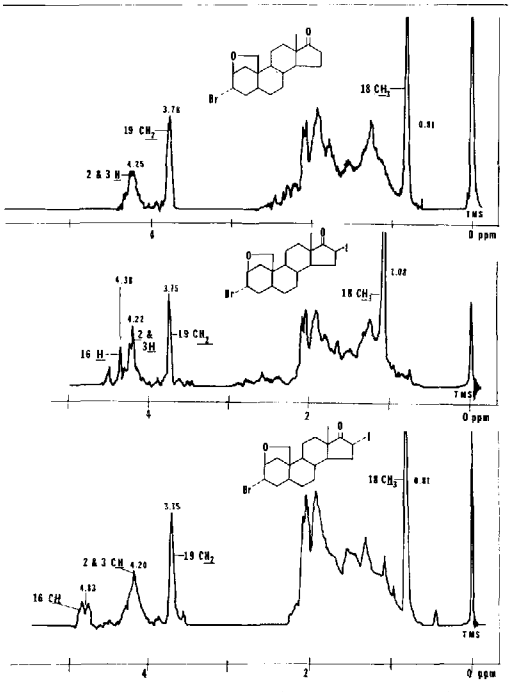


Fig. 1.—NMR spectra of unsubstituted ketone (III) (top) and the epimeric 16β-(IV) (middle), and 16α-iodoketones (V) (bottom).

The hypoiodite oxidation of I was repeated several times under presumably identical conditions using purified reagents and chromatographically pure halohydrins and each time the yield of products and the product composition were found to vary. The 16α-iodo product (V) was isolated in subsequent experiments, but none of the 16β-epimer could be separated. Moreover, in one experiment trituration of the crude reaction product with methanol afforded a high-melting product in 5.5% yield which was subsequently identified as the 19-acetoxy derivative (VII). In this instance none of the corresponding 19-hydroxylated product (VI) could be distinguished after chromatography. In still another experiment, VII was isolated in 5% yield after chromatography of the crude product which would tend to indicate that this ester is not hydrolyzed during chromatography. No explanation can be offered at this time for the variations in product yield and composition.

The structural assignments for the epimeric iodoketones (IV and V) were based on chemical evidence as well as spectral and elemental analysis. The 16α-epimer (V) was synthesized by first converting III to its enol acetate (IX) followed by iodination with iodine and mercuric acetate as described by Mueller and Johns (6). No 16β-isomer (IV) was isolated in this experiment.

The NMR spectra revealed distinct differences between the epimeric iodoketones (see Fig. 1). One epimer displayed a profound downfield shift of 14.5 c.p.s. for the C-18 methyl resonance when compared to the unsubstituted ketone (III). Cross and Beard (7) have noted a similar deshielding of the C-18 methyl protons by 16β-methyl substituted steroids. Moreover, the C-18 methyl resonance is shifted downfield by 19 c.p.s. in 11β-bromo-12-ketosteroids and is essentially unaffected in 11α-bromo-12-ketosteroids (8). On the basis of these findings as well as others indicating that introduction of a new 1:3 nonbonded diaxial interaction with an angular methyl group leads to a noticeable downfield shift in the angular methyl proton frequency (9), the iodine atom in IV was assigned as 16β and that in V is 16α. These assignments also agree with the observed chemical shifts for the 16 proton in each case. The latter appeared as an overlapping doublet at 261 c.p.s. in IV and at 290 c.p.s. in V. Thus, if observations made for steroidal α-haloketones (10) hold for 16-halo-17-ketosteroids, such chemical shifts would indicate that the 16α-hydrogen in IV is pseudoequatorial while the 16β-hydrogen in V is pseudoaxial. A study of the NMR spectra of more readily available 16-iodo-17-ketosteroids is currently in progress.

Although very few studies have been concerned with the ultraviolet absorption of α-iodoketones, Djerassi and co-workers (11) have established that absorption in the 260 mμ region is due to the iodine atom which masks the π-π* absorption band in the 300 mμ region corresponding to the carbonyl

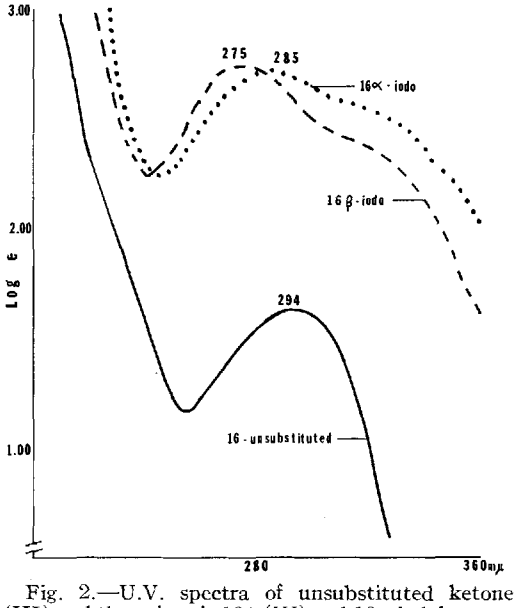
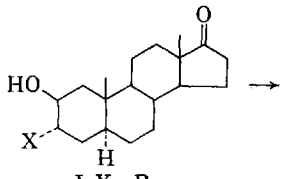
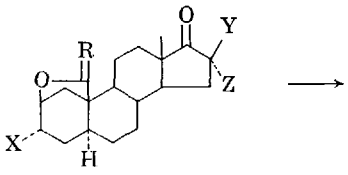


Fig. 2.—U.V. spectra of unsubstituted ketone (III) and the epimeric 16β-(IV) and 16α-iodoketones (V).

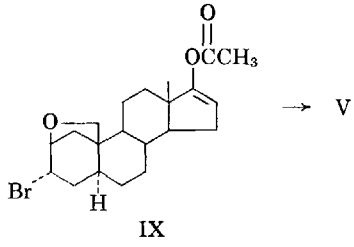


I, X = Br
II, X = Cl



III

III, X = Br, R = H_2, Y = Z = H
IV, X = Br, R = H_2, Y = I, Z = H
V, X = Br, R = H_2, Y = H, Z = I
VI, X = Br, R = H—OH, Y = Z = H
VII, X = Br, R = H—OAc, Y = Z = H
VIII, X = Br, R = O, Y = Z = H
X, X = Cl, R = H_2, Y = Z = H
XI, X = Cl, R = H_2, Y = H, Z = I
XII, X = Cl, R = H—OAc, Y = Z = H



IX

Scheme II

group. They noted, however, that a bathochromic shift of the maximum was apparent for the axially oriented α-iodo-ketosteroids and suggested that this red shift could be used as a spectral criterion for assigning an axial orientation to substituted α-iodocyclohexanones. Ultraviolet analysis of IV and V showed maxima at 275 and 285 mμ, respectively (see Fig. 2). Such results would suggest a pseudoaxial orientation for the iodine atom in V if the above criterion apply. Such an interpretation is contrary to that drawn from the NMR spectra. An attempt to resolve some of these disparities is currently in progress in our laboratories.

In the α-halocyclohexanone series, Jones (12) and Corey (13) were able to distinguish between an axial and equatorial halogen atom by the small or large shift in the carbonyl frequency. In the case reported here, however, no conclusion regarding the conformations of the 16 halogens could be made on the basis of the infrared spectra. Both iodoketones (IV and V) showed carbonyl maxima at 1725 cm.$^{-1}$, the same as for the noniodinated product (III). A similar observation was made by Mueller and Johns in the estrone series (6). These results emphasize the hazards in attempting to translate relationships established in the cyclohexanone series to substituted cyclopentanones.

The structure of VI was readily determined from chemical and spectral evidence. The NMR showed a singlet at 318 c.p.s. which shifted to 390 c.p.s. upon acetylation. This is the expected region for a hemiacetal acetate proton (4, 14) and the downfield shift of 72 c.p.s. corresponds to that observed upon acetylation of secondary alcohols (8). The hemiacetal was readily oxidized with Jones reagent (15) to the corresponding lactone which lacked a proton in the 300–400 c.p.s. region. The configuration of the 19-hydroxyl group remains to be established.

Similar results were obtained upon hypoiodite oxidation of the chlorohydrin (II). The major product was the expected 2β,19-oxide (X) but the corresponding 16α-iodinated (XI) and 19-acetoxylated (XII) derivatives were isolated in low yield by chromatography. Although this reaction has been carried out several times, no 16β-iodinated product has been isolated. (Scheme II.)

EXPERIMENTAL[3]

Hypoiodite Oxidation of 3α-Bromo-2β-hydroxy-5α-androstan-17-one (I).—A mixture of I (21.1 Gm.), lead tetraacetate (78.5 Gm.), and iodine (29.6 Gm.) in carbon tetrachloride (2.15 L.) was stirred under reflux for 8 hr. The reaction mixture was allowed to stand at room temperature for 3 hr. and filtered. The filter cake was washed with methylene chloride until no longer pink. The filtrate was washed with 10% sodium thiosulfate solution (2 × 200 ml.) and water (200 ml.), the layers separated, and the organic phase dried over anhydrous sodium sulfate. Evaporation of the solvent left an orange oil which was dissolved in benzene and adsorbed onto a column of silicic acid (500 Gm.). The column was eluted with benzene, followed by benzene containing increasing concentrations of ethyl acetate. The benzene–ethyl acetate (19:1) eluates afforded two distinct crystalline products. The first product was established to be 3α-bromo-16β-iodo-2β,19-oxido-5α-androstan-17-one (IV, 0.26 Gm.), m.p. 196–198° (from methanol); $\gamma_{max.}$ 1725, 1228, and 1018 cm.$^{-1}$; $\lambda_{max.}$ 275 mμ, log ϵ 2.72; NMR: 63.5 (C-18 methyl), 224 (C-19 proton), 251 (C-2 and C-3 protons, multiplet), and 261 c.p.s. (C-16 proton, overlapping doublet $J_{AX} + J_{BX} = 17$).

Anal.—Calcd. for $C_{19}H_{26}BrIO_2$: C, 46.26; H, 5.31. Found: C, 46.42; H, 5.43.

The other product was characterized as the epimer, 3α-bromo-16α-iodo-2β,19-oxido-5α-androstan-17-one (V, 0.3 Gm.), m.p. 210–212° (from methanol); $\gamma_{max.}$ 1725, 1232, and 645 cm.$^{-1}$; $\lambda_{max.}$ 285 mμ, log ϵ 2.73; NMR: 50 (C-18 methyl), 225 (C-19 proton), 252 (C-2 and C-3 protons, multiplet), and 290 c.p.s. (C-16 proton, overlapping doublet $J_{AX} + J_{BX} = 8$).

Anal.—Calcd. for $C_{19}H_{26}BrIO_2$: C, 46.26; H, 5.31; Br, 16.20; I, 25.73. Found: C, 46.27; H, 5.27; Br, 16.35; I, 25.73.

Further elution with benzene–ethyl acetate (9:1) furnished 3α-bromo-2β,19-oxido-5α-androstan-17-one (III, 7.6 Gm.), m.p. 130–132° (from methanol),

[3] The melting points were taken on a Fisher-Johns apparatus and are corrected. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Infrared spectra were taken in KBr disks with a Perkin-Elmer 337 spectrophotometer. Ultraviolet spectra were recorded on a Beckman DK2A spectrophotometer in 95% ethanol. The NMR spectra were obtained in $CDCl_3$ with a Varian A-60 spectrometer using tetramethylsilane as the internal standard. The lead tetraacetate used in the experiments was obtained from Arapahoe Chemicals, Inc., and recrystallized from benzene prior to use. The silicic acid used in the column chromatography was Baker and Adamson reagent grade.

undepressed by admixture with an authentic sample (1). Elution with benzene–ethyl acetate (1:1) gave 3α-bromo-19-hydroxy-2β,19-oxido-5α-androstan-17-one (VI, 2.1 Gm.), m.p. 168–170° (from methanol–water); $\gamma_{max.}$ 3440, 1715, and 1012 cm.$^{-1}$; NMR: 52 (C-18 methyl), 249–269 (C-2 and C-3 protons, multiplet), and 318 c.p.s. (C-19 proton).[4]

Anal.—Calcd. for $C_{19}H_{27}BrO_3$: C, 59.53; H, 7.10; Br, 20.85. Found: C, 59.61; H, 7.21; Br, 20.92.

19-Acetoxy-3α-bromo-2β,19-oxido-5α-androstan-17-one (VII).—A solution of VI (10 mg.) in acetic anhydride (0.5 ml.) and pyridine (0.5 ml.) was allowed to stand at room temperature for 23 hr. The solution was added slowly to cold water and the product collected by filtration. Recrystallization from methanol afforded pure VII, m.p. 235–237°; $\gamma_{max.}$ 1725, 1219, 1016, and 1009 cm.$^{-1}$; NMR: 50 (C-18 methyl), 130 (acetate methyl), 259–265 (C-2 and C-3 protons, multiplet), and 390 c.p.s. (C-19 proton).

Anal.—Calcd. for $C_{21}H_{29}BrO_4$: C, 59.30; H, 6.87. Found: C, 59.13; H, 6.86.

3α-Bromo-2β,19-oxido-19-oxo-5α-androstan-17-one (VIII).—The hemiacetal (VI, 14 mg.) was dissolved in acetone (2 ml.) and the solution cooled to 0°. An 8 *N* chromic acid solution (15) (0.1 ml.) was added and the mixture stirred for several minutes. The excess oxidant was decomposed with a few drops of isopropyl alcohol. The solution was diluted with water and the crystalline product collected by filtration. The crude product (12 mg.), m.p. 192–194°, was recrystallized from methanol to afford an analytical sample, m.p. 198–200°; $[\alpha]_D^{25}$ + 92°; $\gamma_{max.}$ 1750, 1725, and 715 cm.$^{-1}$; NMR: 57 (C-18 methyl), 275–287 (C-2 proton multiplet), 257–268 c.p.s. (C-3 proton, multiplet).

Anal.—Calcd. for $C_{19}H_{25}BrO_3$: C, 59.85; H, 6.61. Found: C, 59.88; H, 6.56.

3α-Bromo-2β,19-oxido-5α-androst-16-en-17-ol Acetate (IX).—A solution of III (0.5 Gm.) and *p*-toluene sulfonic acid (75 mg.) in isopropenyl acetate (10 ml.) was slowly distilled for 4 hr. and 5 ml. of distillate collected. Another portion of isopropenyl acetate (10 ml.) was added along with sodium bicarbonate (0.5 Gm.). The solvent was removed by distillation and the remaining traces removed *in vacuo*. The residue was extracted with ether which was subsequently washed with an ice-cold saturated salt solution and dried over anhydrous sodium sulfate. The solvent was removed *in vacuo* and the brown residue recrystallized from methanol. This afforded pure IX (0.1 Gm.), m.p. 150–151°.

Anal.—Calcd. for $C_{21}H_{29}BrO_3$: C, 61.61; H, 7.14. Found: C, 61.51; H, 7.20.

Iodination of IX.—A solution of IX (55 mg.) and mercuric acetate (9 mg.) in acetic acid (3 ml.) was cooled in an ice bath. A solution of iodine (50 mg.) in acetic acid (3 ml.) was added dropwise with stirring. The iodine was decolorized after adding a few drops, but then the color persisted. The brown solution was poured into water, and the precipitate collected by filtration. The crude product was washed with 10% sodium thiosulfate solution, water, and air dried. Recrystallization from methanol gave V (20 mg.), m.p. 205–207°, undepressed by admixture with the product isolated above.

Hypoiodite Oxidation of 3α-Chloro-2β-hydroxy-5α-androstan-17-one (II).—Oxidation of II (3.3 Gm.) with lead tetraacetate (13.6 Gm.) and iodine (5.2 Gm.) in refluxing carbon tetrachloride (340 ml.) was carried out as described above. The resulting orange oil was adsorbed onto a column of silicic acid (100 Gm.) and eluted with benzene. This was followed by benzene containing increasing concentrations of ethyl acetate. Fractions obtained by elution with benzene–ethyl acetate (19:1) gave 3α-chloro-16α-iodo-2β,19-oxido-5α-androstan-17-one (0.1 Gm.), m.p. 197–200° dec. Recrystallization from methanol afforded pure XI, m.p. 212–214° dec.; $\gamma_{max.}$ 1725, 1235, and 643 cm.$^{-1}$; NMR: 50 (18-methyl), 224 and 227 (C-19 protons, inner peaks of unresolved quartet), 240–265 (C-2 and C-3 protons), and 283–300 c.p.s. (16β-proton).

Anal.—Calcd. for $C_{19}H_{26}ClIO_2$: C, 50.85; H, 5.84. Found: C, 50.97; H, 5.94.

Further elution with benzene–ethyl acetate (9:1) gave a crude crystalline product (1.7 Gm.), m.p. 120–128°. Recrystallization from methanol gave a high-melting compound (0.2 Gm.), m.p. 235–236°. Recrystallization of this product from methanol gave a pure sample characterized as 19-acetoxy-3α-chloro-2β,19-oxido-5α-androstan-17-one (XII), m.p. 243–244°; $\gamma_{max.}$ 1735, 1710, and 1235 cm.$^{-1}$; NMR: 49 (C-18 methyl), 128 (acetate methyl), 252–257 (C-2 and C-3 protons) and 384 c.p.s. (C-19 proton).

Anal.—Calcd. for $C_{21}H_{29}ClO_4$: C, 66.21; H, 7.67. Found: C, 66.00; H, 7.64.

Concentration of the mother liquor afforded a crystalline product (0.4 Gm.) shown to be identical with authentic 3α-chloro-2β,19-oxido-5α-androstan-17-one (X)[5] described previously (1).

[4] In another experiment involving hypoiodite oxidation of 36.3 Gm. of I, trituration of the crude reaction product with methanol afforded a crystalline product (1.7 Gm.) which upon recrystallization from methanol was found to be the hemiacetal acetate (VII), identical with that obtained by acetylation of VI.

[5] In another experiment, oxidation of II (3.7 Gm.) gave X (1.9 Gm.) and XII (0.2 Gm.), but no XI could be isolated.

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Quantitative Determination of Ethinyl Estradiol by Gas Chromatography, and a Comparison of Gas Chromatographic and U.S.P. Procedures

By O. D. BOUGHTON, RHYS BRYANT, W. J. LUDWIG, and D. L. TIMMA

Ethinyl estradiol was extracted from other tablet or granulation excipients by a modified U.S.P. procedure. It was then quantitatively determined by gas chromatography of its trimethylsilyl ether. Estrone was used as an internal standard. A comparison of this gas chromatographic method with the U.S.P. method indicated that, in general, the precision of the U.S.P. method was superior to that of the gas chromatographic method.

THE ACCURATE analytical determination of estrogenic and progestational substances is of interest to all pharmaceutical manufacturers, especially those concerned with the now well-established oral contraceptives. Analysis of the progestational agent is usually much easier than analysis of the estrogen, because of the necessity for a larger amount of the former in tablets.

The authors' main interest has been in the analysis of the estrogen ethinyl estradiol. The accepted standard method for assay of this material has been that of the U.S.P. XVII (1). This method is time-consuming and is nonspecific in that it may be used for any estrogen characterized by a phenolic steroidal A ring (2).

Subsequently, alternative methods using gas chromatography have been applied to the problem. Schulz (3) reported the estimation of ethinyl estradiol-3-methyl ether in the presence of other steroids by gas chromatography. More recently, Talmage, Penner, and Geller (4) reported a gas chromatographic technique for determination of ethinyl estradiol in sesame oil solutions and solid dosage forms. The ethinyl estradiol appears to have been uncontaminated with other steroids and was estimated as its acetate.

A quantitative gas chromatographic method for determination of ethinyl estradiol in which the active ingredient is ethinyl estradiol alone, or ethinyl estradiol plus 17β-hydroxy-6α-methyl-17-(1-propynyl)-androst-4-en-3-one (dimethisterone) has been developed.

A comparison of the gas chromatographic method *versus* the U.S.P. method to gain insight into the precision of the two methods has also been made. (No comparison of the two methods has been made until now, although Talmage, Penner, and Geller stated (4), with no supporting data, that the U.S.P. method was no more accurate than $\pm 10\%$.)

The method of Talmage *et al.* depends on initial preparation of the 3-acetate of ethinyl estradiol, and subsequent gas chromatography of this compound. In attempting to apply this method to the problems reported here, two distinct disadvantages in the method were discovered. Preparation of the acetate involves the use of acetic anhydride which must subsequently be removed by evaporation at an elevated temperature. Even under the conditions stated in the original paper (evaporation under nitrogen on the steam bath) (4), this step is time consuming and introduces a possible source of error. Second, the authors wished to analyze for at least 50% less ethinyl estradiol than Talmage, Penner, and Geller had analyzed, and for our purposes the acetate-derivative did not give sufficient response on the chromatograph.

In this method the ethinyl estradiol, after extraction, is converted to its trimethylsilyl ether and chromatographed. Advantages of the use of the trimethylsilyl ether are that it is readily prepared, quantitatively, at room temperature, and is sufficiently volatile to give an excellent chromatographic response. (Quantities of ethinyl estradiol as low as 1 mcg. after trimethylsilylation are readily detectable.) Details of the procedure are presented under *Experimental.*

The comparison of the gas chromatographic and U.S.P. methods of analysis took the form of two experiments. In experiment *A*, the authors took a small amount of granulation and further homogenized it using a mortar and pestle. It was then assayed 10 times by both U.S.P. (1) and gas chromatographic (GLC) methods. In experiment *B* the authors allowed conditions to approach those usually found in analysis of a production batch, when the possibility of a certain amount of inhomogeneity cannot be ruled out. Both tablets and granulations were

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analyzed 10 times by each method. The results of these experiments were then analyzed statistically in order to obtain the comparisons required.

GAS CHROMATOGRAPHIC METHOD

Experimental

Apparatus.—Gas chromatograph, F and M model 609, with flame ionization detector. Aerograph model 471 digital integrator.

Column.—Stainless steel column (6 ft. × 1/4 in.) packed with 3.8% SE-30 on Anakrom ABS, 60/70 mesh. The liquid phase was deposited by the filtration technique (5) from a 2% solution of SE-30 in methylene chloride. The column was "no flow" preconditioned (no carrier gas flowing) for 0.5 hr. at 325°. The carrier gas used was nitrogen.

Chromatography Conditions.—Column temperature, 260°; injection port temperature, 285°; detector temperature, 265°; hydrogen flow, 7; air flow, 7; nitrogen flow, 9 (these flows may vary according to the instrument); attenuation, 800×.

Standard Ethinyl Estradiol Solution.—*Solution A*.—A 100.0-mg. quantity of reference standard ethinyl estradiol U.S.P. was dissolved in 100 ml. absolute ethanol.

Solution B.—A 10.0-ml. quantity of solution *A* was diluted to 100 ml. with absolute ethanol. (The ethanolic solutions are stable and require no special precautions.)

Solution C.—A 5.0-ml. quantity of solution *B* was diluted to 100 ml. with chloroform. Twenty milliliters of standard solution *C* contained 0.1 mg. of ethinyl estradiol. Solution *C* was prepared fresh daily.

Internal Standard (Estrone) Solution.—*Solution A*.—A 150.0-mg. quantity of estrone U.S.P. was dissolved in 100 ml. absolute ethanol.

Solution B.—A 5.0-ml. quantity of solution *A* was diluted to 100 ml. with absolute ethanol. (The ethanol solutions are stable and require no special precautions.)

Solution C.—A 10.0-ml. quantity of solution *B* was diluted to 100 ml. with chloroform. Ten milliliters of solution *C* contained 0.075 mg. estrone. Solution *C* was prepared fresh daily.

Etherification Reagents.—In a screw-cap vial was placed a mixture[1] of anhydrous pyridine[2] (4.5 ml.), hexamethyldisilazane[3] (1.5 ml.), and anhydrous trimethylchlorosilane[3] (0.5 ml.). No purification of reagents was necessary. The tube was capped tightly and the contents mixed thoroughly. It is essential to exclude atmospheric or other moisture, which destroys the reagent. The etherification reagent was prepared fresh daily.

Extraction Procedure.—An amount of tablet or granulation equivalent to 0.1 mg. of ethinyl estradiol was placed in a 125-ml. separator containing sulfuric acid (30 ml., 1 *N*), and the suspension extracted with chloroform (3 × 20 ml.). After filtration through a small quantity of cotton, the combined chloroform solution was evaporated to low bulk (about 5 ml.). Petroleum ether (25 ml., b.p. 30–60°) was added to the hot solution. It was cooled to room temperature and transferred to a 125-ml. separator with the aid of several small portions of petroleum ether. The solution was extracted with 10% aqueous sodium hydroxide (3 × 10 ml.). The extract was acidified with dilute sulfuric acid (6 ml., 1:1 v/v) and cooled. The acid solution was extracted with chloroform (2 × 20 ml.), which was passed through a column (10 × 1.2 cm.) of anhydrous sodium sulfate. The column was rinsed with chloroform (1 × 10 ml.). All column eluates were combined, 10.0 ml. of internal standard solution (estrone) was added, and the solution was evaporated to dryness. Chloroform (1–2 ml.) was added to the hot residue. The resulting solution, after cooling, was transferred to a 1-dr. screw-cap vial and evaporated to dryness under a gentle air stream. It was finally dried at 80° for 5 min. *in vacuo*.

Etherification Step.—To the cooled ethinyl estradiol extract in the vial was added 10 drops (0.1 ml.) of etherification reagent. The vial was tightly stoppered and the contents thoroughly mixed. After 30–60 min. excess reagent was evaporated with a gentle air stream (about 15 min.). (The reaction time was varied from 15 min. to 60 min. with no effect. The reaction is obviously complete in less than 15 min. A lapse-time of 30–60 min. was chosen arbitrarily to allow simultaneous assay of a number of samples.) Immediately prior to chromatography, chloroform (10 drops) was added and the resulting solution mixed thoroughly. This solution (5–6 μl.) was injected into the chromatograph. Duplicate injections of each sample are to be preferred.

Standard.—A standard was carried through the procedure with each set of samples. Twenty milliliters of standard ethinyl estradiol solution was shaken with dilute sulfuric acid (30 ml., 1 *N*). The chloroform solution was drained through cotton, and the sulfuric acid extracted with chloroform (2 × 20 ml.). The combined chloroform solution was then treated as for the samples.

Calculation.—The peaks due to estrone and ethinyl estradiol have retention times of approximately 4.5 min. and 7 min., respectively. The ratio (R) of the peak areas is given by:

$$R = \frac{\text{area of ethinyl estradiol peak}}{\text{area of estrone peak}}$$

For tablets containing 0.1 mg. ethinyl estradiol:

$$\text{mg. of ethinyl estradiol} = \frac{R \text{ for sample}}{R \text{ for standard}} \times 0.100$$

For granulations containing 0.04% ethinyl estradiol:

$$\%\ \text{ethinyl estradiol} = \frac{R \text{ for sample}}{R \text{ for standard}} \times 0.0400$$

RESULTS

Reproducibility of the Etherification Step.—Four aliquots of a standard solution of ethinyl estradiol, after addition of estrone, were etherified and each aliquot chromatographed in duplicate.

[1] This mixture is available commercially from Applied Science Laboratories, Inc.

[2] Analytical reagent grade pyridine supplied by Mallinckrodt was used. It was dried with molecular sieve, type 4A.

[3] Hexamethyldisilazane and trimethylchlorosilane were obtained from Peninsular Chemresearch, Inc.

TABLE I.—ETHERIFICATION STEP

	R	Av. R
Std. 1	1.755	1.740
Std. 1	1.725	
Std. 2	1.695	1.720
Std. 2	1.745	
Std. 3	1.655	1.635
Std. 3	1.615	
Std. 4	1.650	1.685
Std. 4	1.720	

TABLE II.—STANDARD THROUGH EXTRACTION PROCEDURE[a]

	R	Av. R	% Recovery
Sample 1	1.710	1.685	99.4
Sample 1	1.660		
Sample 2	1.690	1.710	101
Sample 2	1.730		
Sample 3	1.720	1.670	98.5
Sample 3	1.620		
Sample 4	1.695	1.730	102
Sample 4	1.765		
Sample 5	1.670	1.680	99.1
Sample 5	1.690		

Av. recovery = 100%, $\sigma = \pm 1.5$

[a] All samples were aliquots of the same standard ethinyl estradiol solution.

Table I incorporates the results. The average R value was 1.695, $\sigma = \pm 0.014$.

Reproducibility of the Extraction.—This was checked (*a*) by taking a known quantity of ethinyl estradiol through the extraction procedure; (*b*) by adding a known quantity of ethinyl estradiol to the tablet placebo (containing all excipients including dimethisterone, but without the ethinyl estradiol) and taking this through the procedure; and (*c*) by taking the placebo through the procedure, and then adding a known quantity of ethinyl estradiol. The samples were then etherified and chromatographed in the normal manner. The results were compared with the average R value (1.695), obtained by chromatography of the standards which had not been taken through the method, to give the percentage recovery. (See Tables II–IV.)

Reproducibility of the Method.—Table V summarizes the results obtained when 4 separate aliquots of the same granulation (containing only ethinyl estradiol as active ingredient) were taken completely through the method. The average percentage of ethinyl estradiol was 0.0404, $\sigma = \pm 0.0025$.

Table VI gives similar results for 5 aliquots of another granulation, which contained both ethinyl estradiol and dimethisterone. In this case, the percentage of ethinyl estradiol was found to be 0.0412, $\sigma = \pm 0.0018$.

General Applicability.— Some results of the applicability of the method to tablets and granulations are included in Table VII (EE = formulations containing ethinyl estradiol; MEE = formulations containing both dimethisterone and ethinyl estradiol).

COMPARISON OF THE METHODS

Experiment *A*

Samples.—Two granulations were used, one containing ethinyl estradiol as sole active ingredient (EE granulation); the other containing both dimethisterone and ethinyl estradiol (DMEE granulation).

Preparation of Samples.—A small sample (11 Gm.) of the EE granulation was finely ground in a mortar. (The sample size was restricted in order to ensure as high a degree of homogeneity as

TABLE III.—STANDARD PLUS PLACEBO THROUGH EXTRACTION PROCEDURE[a]

	R	Av. R	% Recovery
Sample 1	1.785	1.765	104
Sample 1	1.745		
Sample 2	1.680	1.710	101
Sample 2	1.740		
Sample 3	1.900	1.890	112
Sample 3	1.880		
Sample 4	1.705	1.715	101
Sample 4	1.720		
Sample 5	1.680	1.675	98.7
Sample 5	1.670		

Av. recovery = 103%, $\sigma = \pm 5.2$

[a] All samples were aliquots of the same standard solution.

TABLE IV.—PLACEBO THROUGH PROCEDURE, THEN ADDITION OF STANDARD[a]

	R	Av. R	% Recovery
Sample 1	1.735	1.695	100
Sample 1	1.655		
Sample 2	1.830	1.850	109
Sample 2	1.870		

Av. recovery = 104%

[a] Samples were aliquots of the same standard solution.

TABLE V.—METHOD APPLIED TO ALIQUOTS OF ONE GRANULATION[a]

	Theory	% Ethinyl Estradiol
Sample 1	0.040%	0.0377
Sample 2	0.040	0.0410
Sample 3	0.040	0.0390
Sample 4	0.040	0.0436

Av. % ethinyl estradiol = 0.0404, $\sigma = \pm 0.0025$

[a] Containing only ethinyl estradiol as active ingredient.

TABLE VI.—METHOD APPLIED TO ALIQUOTS OF ONE GRANULATION[a]

	Theory	% Ethinyl Estradiol
Sample 1	0.040%	0.0396
Sample 2	0.040	0.0406
Sample 3	0.040	0.0414
Sample 4	0.040	0.0443
Sample 5	0.040	0.0404

Av. % ethinyl estradiol = 0.0412, $\sigma = \pm 0.0018$

[a] Containing both ethinyl estradiol and dimethisterone.

TABLE VII.—RESULTS

	Theory	Found
EE tablet 1	0.100 mg.	0.093 mg.
EE tablet 2	0.100 mg.	0.091 mg.
EE tablet 3	0.100 mg.	0.094 mg.
EE tablet 4	0.100 mg.	0.108 mg.
DMEE tablet 1	0.100 mg.	0.102 mg.
DMEE tablet 2	0.100 mg.	0.106 mg.
DMEE tablet 3	0.100 mg.	0.094 mg.
DMEE tablet 4	0.100 mg.	0.097 mg.
DMEE tablet 5	0.100 mg.	0.098 mg.
DMEE tablet 6	0.100 mg.	0.088 mg.
DMEE tablet 7	0.100 mg.	0.100 mg.
DMEE granulation 1	0.040%	0.0399%
DMEE granulation 2	0.040%	0.0417%
DMEE granulation 3	0.040%	0.0398%
DMEE granulation 4	0.040%	0.0403%

TABLE VIII.—PERCENTAGE ETHINYL ESTRADIOL IN GRANULATIONS

EE Granulation		DMEE Granulation	
U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
0.0383%	0.0431%	0.0404%	0.0382%
0.0369	0.0410	0.0408	0.0371
0.0408	0.0351	0.0414	0.0327
0.0422	0.0386	0.0445	0.0382
0.0449	0.0366	0.0400	0.0399
0.0417	0.0351	0.0382	0.0387
0.0425	0.0346	0.0383	0.0336
0.0376	0.0431	0.0387	0.0387
0.0377	0.0406	0.0413	0.0391
0.0397	0.0406	0.0458	0.0377

possible.) All 10 assays by the U.S.P. method and all 10 assays by the gas chromatographic method were done on this 11-Gm. sample.

An 11-Gm. sample of DMEE granulation was treated similarly, and all 20 assays performed on it.

Method of Assay.—*a.*—The U.S.P. method was carried out by a single operator experienced with the method using 0.500 Gm. of sample for each assay. Not more than four assays (two EE and two DMEE) were undertaken on the same day.

b.—The gas chromatographic method was performed by a single operator experienced with the method using 0.250 Gm. of sample for each assay. Again, not more than four assays were done on a single day.

Results.—The results obtained in experiment *A* are shown in Table VIII. (Theoretically, granulations should contain 0.040% ethinyl estradiol.)

Statistical Analysis.—The results of the statistical calculations are included in Table IX.

Experiment *B*

Samples.—Four samples were used: 1, EE granulation; 2, DMEE granulation; 3, EE tablets; and 4, DMEE tablets.

Preparation of Samples.—*a.*—From the batch of EE tablets, 200 tablets were retained as the stock of tablets. From this stock each day, 20 tablets were taken and pulverized in a mortar; 0.500 Gm. of this sample was used for the U.S.P. method, and 0.250 Gm. of the sample was used for the gas chromatographic method. A separate lot of 20 tablets was used each day, and 0.500 and 0.250-Gm. samples removed from it.

TABLE IX.—STATISTICAL COMPARISON OF THE TWO METHODS

	EE Granulation		DMEE Granulation	
	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
Av. value, %	0.04023	0.03884	0.04094	0.03738
Variance (σ^2)	6.86×10^{-6}	10.89×10^{-6}	6.35×10^{-6}	5.57×10^{-6}
S.D. (σ)	±0.0026	±0.0033	±0.0025	±0.0024
S.D. as %	±6.5	±8.5	±6.1	±6.4

TABLE X.—COMPARISON OF THE U.S.P. AND GAS CHROMATOGRAPHIC METHODS

	EE Granulation		DMEE Granulation		EE Tablet		DMEE Tablet	
Day	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
1	0.0359	0.0431	0.0409	0.0405	0.108	0.108	0.106	0.104
2	0.0390	0.0342	0.0390	0.0438	0.108	0.111	0.106	0.111
3	0.0353	0.0352	0.0450	0.0374	0.099	0.096	0.101	0.100
4	0.0392	0.0358	0.0412	0.0401	0.100	0.103	0.105	0.107
5	0.0366	0.0341	0.0362	0.0418	0.100	0.090	0.102	0.104
6	0.0386	0.0388	0.0404	0.0411	0.108	0.105	0.119	0.110
7	0.0405	0.0348	0.0394	0.0492	0.104	0.110	0.105	0.112
8	0.0391	0.0371	0.0410	0.0575	0.098	0.116	0.100	0.131
9	0.0398	0.0380	0.0410	0.0397	0.109	0.102	0.098	0.107
10	0.0382	0.0412	0.0388	0.0450	0.100	0.105	0.100	0.106

TABLE XI.—STATISTICAL COMPARISON OF THE METHODS FOR GRANULATIONS

	EE Granulation		DMEE Granulation	
	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
Av. value, %	0.03822	0.03723	0.04029	0.04361
Variance (σ^2)	2.97×10^{-6}	9.37×10^{-6}	5.09×10^{-6}	34.6×10^{-6}
S.D. (σ)	±0.0017	±0.0030	±0.0023	±0.0059
S.D. as %	±4.5%	±8.1%	±5.7%	±13.5%

Table XII.—Statistical Comparison of the Methods for Tablets

	EE Granulation		DMEE Granulation	
	U.S.P. Method	GLC Method	U.S.P. Method	GLC Method
Av. value, mg.	0.1034	0.1046	0.1042	0.1092
Variance (σ^2)	19.8×10^{-6}	56.4×10^{-6}	35.1×10^{-6}	71.8×10^{-6}
S.D. (σ)	±0.0045	±0.0075	±0.0059	±0.0085
S.D. as %	±4.4%	±7.2%	±5.7%	±7.8%

A stock of DMEE tablets was treated in exactly the same way.

b. Granulations.—From the batch of EE granulation, 160 Gm. was removed. It was passed through a sample splitter to give 2 × 80-Gm. portions. Each 80-Gm. portion was passed separately through the sample splitter to yield 4 × 40-Gm. portions. Each 40-Gm. portion was similarly split, and so on, with each subsequent 20-Gm. and 10-Gm. portion until 32 × 5-Gm. portions were obtained. Of these 32 × 5-Gm. portions, 10 × 5 Gm. were used for the current experiment.

Each day one of the 5-Gm. portions was taken and again pulverized using a mortar and pestle; 0.500 Gm. was taken for the U.S.P. assay, and 0.250 Gm. was taken for the gas chromatographic assay.

The DMEE granulation was treated in the same way.

Method of Assay.—The U.S.P. method was performed by a single operator experienced with the method. Each day for a total of 10 days, one each of the EE tablet, DMEE tablet, EE granulation, and DMEE granulation was assayed.

The gas chromatographic method was performed by a single experienced operator. Each day, for a total of 10 days, one each of the EE and DMEE tablets, and EE and DMEE granulations was assayed.

Results.—The results obtained in experiment *B* are summarized in Table X. Granulations are quoted as percentages; tablets as mg./tablet. (Theoretical content of granulations is 0.040%; and of tablets 0.100 mg.)

Statistical Analysis.—The average values obtained and the variance (σ^2) and standard deviations (σ) of the methods are reported in Tables XI (for granulations) and XII (for tablets).

DISCUSSION

The Gas Chromatographic Method.—Dimethisterone had a retention time of 12 min. and in no way interfered with the other steroid peaks. It was therefore unnecessary to extract the ethinyl estradiol completely from dimethisterone, as is required by the U.S.P. XVII procedure.

The use of a short column of sodium sulfate helped to remove any trace of acid which might be occluded in the chloroform and subsequently interfere with the etherification step.

Comparison of the Methods.—In all cases, the standard deviation (as per cent) is lower for the U.S.P. method than for the gas chromatographic method. While it is also obvious that the average value obtained differs between the methods, statistically (using the *t* test) only in the case of the DMEE granulation (experiment *A*) is this significant. Also, only in the case of the DMEE granulation (experiment *B*), and possibly the EE granulation (experiment *B*), can any significance be attached to the difference in variances (*F* test).

SUMMARY

The Gas Chromatographic Method.—Ethinyl estradiol was extracted into chloroform from an acid suspension and separated from dimethisterone by alkaline extraction. With estrone as an internal standard, the ethinyl estradiol was converted to its trimethylsilyl ether and this was chromatographed on a SE-30 column.

Evaluation of the results contained in Table VII shows that, based on an expected value of 0.0400% ethinyl estradiol, the average value found was 0.039%, σ = ±0.002. Based on an expected value of 0.100 mg./250 mg. tablet, the average found value was 0.098 mg., σ = ±0.002.

The Comparison of Methods.—Under the conditions of our comparisons, the U.S.P. method in all cases showed a standard deviation lower than that of the gas chromatographic method. The over-all average standard deviation for the U.S.P. method was ±5.5% and for the GLC method ±8.6%. Although the U.S.P. method would appear to give a better precision, in most cases the difference in variances is not statistically significant.

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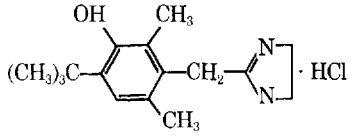
(2) Dale, E., and Greene, J. W., Jr., *Am. J. Med. Sci.*, **250**, 700(1965).
(3) Schulz, E. P., *J. Pharm. Sci.*, **54**, 144(1965).
(4) Talmage, J. M., Penner, M. H., and Geller, M., *ibid.*, **54**, 1194(1965).
(5) Whittier, M. B., Mikkelsen, L., and Spencer, S. F., "Factors Affecting the Efficiency of Gas Chromatographic Analysis of Steroids," presented to the American Chemical Society, Southwest regional meeting, December 1962.

Qualitative and Quantitative Tests for Oxymetazoline Hydrochloride

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

6-TERT-BUTYL-3-(2-IMIDAZOLIN-2-YLMETHYL)-2,4-DIMETHYL-PHENOL HYDROCHLORIDE; $C_{16}H_{24}N_2O \cdot HCl$; mol. wt. 296.84. The structural formula of oxymetazoline hydrochloride may be represented as



Physical Properties.—Oxymetazoline hydrochloride occurs as a white to nearly white, fine crystalline powder, m.p. about 300° dec., U.S.P. class I. It is soluble in water, freely soluble in alcohol, and insoluble in ether and in chloroform.

Identity Tests.—Dissolve about 2 mg. of oxymetazoline hydrochloride in 1 ml. of water, add 0.5 ml. of sodium nitroprusside solution (1 in 100), 2 drops of sodium hydroxide solution (15 in 100), mix, and allow to stand 10 min. Add 1 ml. of sodium bicarbonate solution (5 in 100) and allow to stand 10 min.: a violet color is produced.

A 1 in 20,000 solution of oxymetazoline hydrochloride in water exhibits an ultraviolet maximum at about 279 mμ [absorptivity (a) about 6.0] and a minimum at about 252 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of oxymetazoline hydrochloride in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Dissolve about 50 mg. of oxymetazoline hydrochloride in 3 ml. of water, add ammonia T.S. until basic, and filter. Acidify the filtrate with diluted nitric acid and add 1 ml. of silver nitrate T.S.: a white precipitate forms, which is insoluble in diluted nitric acid, but soluble in ammonia T.S. (presence of chloride).

Purity Tests.—Dry about 1 Gm. of oxymetazoline hydrochloride, accurately weighed, at 105° to con-

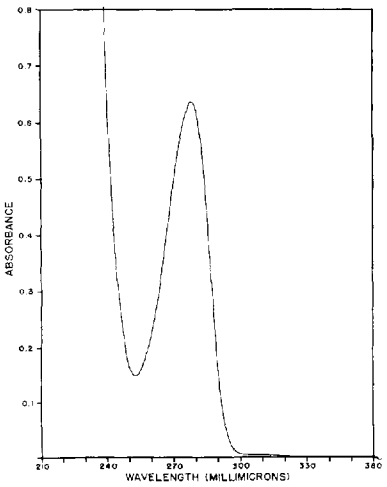


Fig. 1.—Ultraviolet absorption spectrum of oxymetazoline hydrochloride in water (100 mcg./ml.); Beckman model DK-2A spectrophotometer.

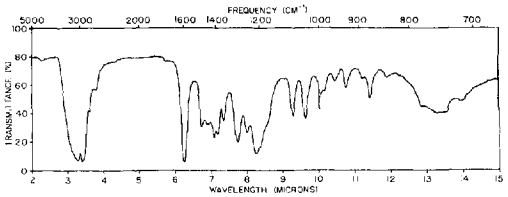


Fig. 2.—Infrared spectrum of oxymetazoline hydrochloride in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

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The Schering Corp., Bloomfield, N. J., has cooperated by furnishing samples and data to aid in the development and preparation of this monograph.

stant weight (about 3 hr.): it loses not more than 0.5% of its weight.

Char about 1 Gm. of oxymetazoline hydrochloride, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of oxymetazoline hydrochloride in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U.S.P. heavy metals test, method I: the heavy metals limit for oxymetazoline hydrochloride is 10 p.p.m.

Assay.—Transfer about 500 mg. of oxymetazoline hydrochloride, accurately weighed, to a tall-form 200-ml. beaker and dissolve in 50 ml. of glacial acetic acid. Add 10 ml. of mercuric acetate T.S. and titrate potentiometrically with 0.1 *N* acetous perchloric acid. Alternatively, add 2 drops of crystal violet T.S. and titrate to an emerald-green end point.[1] Each milliliter of 0.1 *N* perchloric acid is equivalent to 29.68 mg. of $C_{16}H_{24}N_2O.HCl$. The amount of oxymetazoline hydrochloride found is not less than 98.5% and not more than 101.5%.

DOSAGE FORMS OF OXYMETAZOLINE HYDROCHLORIDE

Oxymetazoline Hydrochloride Nasal Solution

Identity Tests.—Transfer 2 ml. of oxymetazoline hydrochloride nasal solution to a test tube containing 2 ml. of alcohol, add 0.5 ml. of sodium nitroprusside solution (1 in 100), 2 drops of sodium hydroxide solution (15 in 100), mix, and allow to stand 10 min. Add 1 ml. of sodium bicarbonate solution (5 in 100), adjust the pH to about 8 with diluted hydrochloric acid, and allow to stand 10 min.: a violet color is produced.

Assay.—Transfer to a separator an accurately measured volume of oxymetazoline hydrochloride nasal solution equivalent to 5 mg. of oxymetazoline hydrochloride. Add 25 ml. of saturated sodium borate solution and extract with four 25-ml. portions of chloroform, combining the extracts in a 250-ml. separator. Extract the chloroform phase with two 20-ml. portions of 0.5 *N* hydrochloric acid, combine the acid extracts in a 100-ml. volumetric flask, dilute with the acid to volume, and mix. Concomitantly determine the absorbance of this solution and of a standard solution of oxymetazoline hydrochloride, in the same medium, at a concentration of about 50 mcg./ml., in 1-cm. cells, at the maximum at about 279 mμ, with a suitable spectrophotometer, using 0.5 *N* hydrochloric acid as the blank. Calculate the quantity, in mg., of $C_{16}H_{24}N_2O \cdot HCl$ in each ml. of the nasal solution taken, by the formula $0.1 \times (C/V) \times (A_u/A_s)$, in which C is the exact concentration of the standard solution, in mcg./ml., V is the volume, in ml., of the nasal solution taken, A_u is the absorbance of the sample solution, and A_s is the absorbance of the oxymetazoline hydrochloride standard solution. The amount of oxymetazoline hydrochloride found is not less than 95.0% and not more than 115.0% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., has been used wherever feasible.

Oxymetazoline hydrochloride,[2] synthesized by Fruhstorfer and Mueller-Calgan (1), is a nasal decongestant for the treatment of a wide variety of allergic and infectious disorders, including rhinitis, nasopharyngitis, and sinusitis.

Identity Tests.—The absorbance maximum for oxymetazoline hydrochloride in water or acid solution is about 279 mμ. A bathochromic displacement, typical for phenols, is noted by an absorbance maximum at about 302 mμ in 0.5 *N* NaOH with a calculated absorptivity of about 15.3.

Quantitative Methods.—The potentiometric nonaqueous titration for oxymetazoline hydrochloride was conducted using glass *versus* calomel electrodes. The calomel electrode was modified by replacing the aqueous potassium chloride salt bridge with 0.02 *N* lithium chloride in glacial acetic acid. The indicator end point is sharp and corresponds to a 100–150-mv. break in the potentiometric titration curve obtained under identical conditions. An average value of 99.9 ± 0.4%[3] was obtained for oxymetazoline hydrochloride by this titrimetric method.

Analysis of commercial oxymetazoline hydrochloride nasal solution by the spectrophotometric method gave an average value of 110.7 ± 0.9%[3] of the labeled amount. The suitability of the procedure was verified by the quantitative recovery of a standard oxymetazoline hydrochloride solution carried through the extractive steps as included for the nasal solution.

REFERENCE

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[1] If the indicator method is used, perform a blank titration and make any necessary correction.

[2] Marketed as Afrin by the Schering Corp., Bloomfield, N. J.

[3] Maximum deviation from the mean value.

Compressed Coated Tablets II

Influence of Size Distribution of Coating Granulation on Weight Uniformity of Tablets

By LEON LACHMAN, HANNA D. SYLWESTROWICZ, and PETER P. SPEISER*

Compressed coated tablets were prepared on a Manesty machine from four granulations of the same formulation, each having a granule of different size as its major component. The effect of granulation size on the weight variability of tablets was determined. The coating granulation sizes which give minimum and maximum weight variability are illustrated and are shown to be reproducible. The relationship that exists between weight variation and core centration is discussed.

IT IS EVIDENT from the numerous compressed coated tablets on the market today that the principle of coating tablets by compression has met wide acceptance in the pharmaceutical industry.

Literature reports (1–9) appearing on the subject of dry coating are concerned with (*a*) the physical properties required for core and coating granulation to give suitable compressed coated tablets, (*b*) coating and core formulations adequate for use in compression coating, (*c*) formulations concerned with disguising a bitter taste, eliminating discoloration, and improving the stability of an active ingredient, (*d*) formulations for imparting sustained-action properties to a drug, and (*e*) forming enteric-coated tablets. However, studies relative to the factors influencing the quality of the compressed coated tablets appear to be lacking. Accordingly, investigations along these lines were initiated in our laboratories. An initial report (10) from these studies was concerned with the factors influencing core centration and methods of evaluating same.

In this report, it will be shown that coating granulation size influences the weight variability of compressed coated tablets prepared on the Manesty Dry Cota machine. The relationship that exists between the weight variation results and the core centration data presented in an earlier report is discussed. The utility of these evaluation techniques to determine the coating granulation size for a particular formula compressed coated tablet to give optimal centration and weight uniformity is illustrated.

Received May 2, 1963, from the Research Department, Ciba Pharmaceutical Co., Summit, N. J., and Ciba Ltd., Basle, Switzerland.

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Previous paper: Lachman, L., Speiser, P. P., and Sylwestrowicz, H. D., *J. Pharm. Sci.*, **52**, 379(1963).

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EXPERIMENTAL

The Manesty Dry Cota shown in Fig. 1 basically consists of two rotary tablet presses coupled by a single drive shaft and a special transfer device in such a manner that core tablets can be compressed and coated in one continuous cycle.

Core Granulation.—The formula and procedure of preparation of this core granulation have been described in a previous study (10). The formula is as follows:

Material and Formula	
Lactose U.S.P.	10.000 Kg.
Wheat starch	2.750
Aerosil compositum[1]	0.750
Gelatin U.S.P.	0.250
Stearic acid, spray dried	0.625
Charcoal	0.125
Talcum U.S.P.	0.500
Purified water	*q.s.*

The sieve analysis for a representative sample of this formulation is given in Table I.

Coating Granulation.—The formula and procedure of preparation of this coating granulation have been described in an earlier report (10). The formula is as follows:

Material and Formula	
Lactose U.S.P.	16.000 Kg.
Aerosil compositum	1.500
Gelatin U.S.P.	0.500
Wheat starch	2.500
Arrowroot starch	2.500
Talcum U.S.P.	1.000
Stearic acid, spray dried	1.000
Purified water	*q.s.*

The above granulation was fractionated through screens to give four granulations of different size

[1] Composed of 85% colloidal silica and 15% hydrolyzed starch.

Fig. 1.—Photograph illustrating the Manesty Dry-Cota machine.

distribution, each one having a different size granule as its major component. The sieve analyses for these four granulations are presented in Table II. In an earlier study concerned with core centration, granulation fraction 12 was numbered 8, and granulation fraction 20 was numbered 35.

Preparation of Compressed Coated Tablets on Manesty Dry Cota.—The compressed coated tablets were prepared on the Manesty Dry Cota model 350 at a rate of 18,000 tablets/hr. The cores weighed 150 mg., were 7 mm. in diameter (0.276 in.), and had a radius of curvature of 13 mm. The coated tablets weighed 400 mg., were 10 mm. in diameter (0.394 in.), and had a radius of curvature of 18 mm.

Influence of Coating Granulation Size Distribution on Weight Uniformity.—By varying the size distribution of the coating granulation and maintaining the size of the core granulation constant, the influence of coating granulation on tablet weight uniformity was investigated. Tablets were compressed for 1 hr. with each of the four granulations. The Manesty machine was regulated for tablet weight uniformity at the start of the hour's run. Then 100 tablets were collected initially and every 10 min. thereafter during the hour without adjusting the machine. This resulted in a collection of seven samples of 100 tablets each for each of the four granulations. This experiment was repeated one-half year later to determine the reproducibility of the data. In the first experiment, 15 tablets were taken at random from each of the seven samples of 100 tablets; and for the second experiment, 10 tablets were taken at random from each of the second set of seven samples of 100 tablets. The tablets in each of these subsamples were weighed individually on an analytical balance and the weights analyzed statistically. Therefore, the set of data from the first experiment consisted of 28 samples of 15 tablets, and the set of data from the second experiment consisted of 28 samples of 10 tablets.

Table I.—Sieve Analysis of Core Granulation

Sieve No.	On Screen, %
8	60.5
12	34.5
20	4.0
30	0.5
50	0.5

Statistical Analysis of Data.—For the samples of tablets taken at the seven time periods for the four granulations in each experiment, the means and standard deviations of tablet weights were computed. The sample means at the various time periods will be hereafter called "time-means."

To permit a comparison of the variabilities "between time-means" and the variabilities "within time-means" of the tablets produced from the four coating granulations, a simple manner of rating these variabilities is provided.

An *Index of Variability* was calculated to describe the variations that exist "between time-means," and an *average variance* for 1 hr. of machine operation was used to describe the variability which exists "within time-means." Both measures of variability are based on an analysis of variance on individual tablet weights of each granulation. The indices are provided by "between time-means" variance components, and the average variances are provided by residual terms of analysis of variance (Table V). The homogeneity of sample variances (seven time samples of two experiments = 14 variances for each granulation) was checked by Bartlett's test (11).

RESULTS AND DISCUSSION

It should be noted that the estimations of tablet weight variability prepared from the four different granulation fractions of the same formulation was based on data obtained from compressed coated tablets. The variance of coated tablets equals the variance of core plus the variance of coating plus twice the covariance of core and coating expressed as follows:

$$V_t = V_c + V_{ct} + 2\,Cov_{c \times ct} \qquad \text{(Eq. 1)}$$

where V_t = variance of coated tablets; V_c = variance of core; V_{ct} = variance of coating; and $Cov_{c \times ct}$ = covariance of core and coating.

A preliminary study on 50 core tablets prepared from the core granulation used for all the compressed coated tablets in the first experiment indicated that

Table II.—Sieve Analysis of the Four Coating Granulations

Granulation Fraction	On Screen, %					Through Screen, %
	No. 8	No. 12	No. 20	No. 30	No. 50	No. 100
12	8.0	(68.5)	15.5	2.0	5.0	1.0
20	0.0	26.0	(43.5)	6.0	18.5	6.0
50	0.0	0.2	13.5	9.0	(56.0)	21.3
100	0.0	0.0	0.0	0.1	15.5	(84.4)

TABLE III.—MEANS OF TABLET WEIGHTS FOR THE SEVEN TIME PERIODS FOR TWO EXPERIMENTS

Granulation	Expt.	Sample Size	Tablet Wt. Means at Time Periods, min. 0	10	20	30	40	50	60
12	I	10	396.6	415.2	422.8	411.6	402.1	421.5	421.5
	II	15	398.8	419.5	427.4	416.8	407.8	421.2	425.6
20	I	10	393.4	404.6	407.5	407.6	406.8	407.3	398.2
	II	15	393.9	408.5	412.4	408.9	412.0	412.6	399.4
50	I	10	410.6	404.1	405.0	403.3	408.2	402.5	409.2
	II	15	414.3	405.1	409.7	407.7	411.2	406.3	411.5
100	I	10	385.3	395.2	396.5	388.9	380.8	393.2	400.1
	II	15	395.2	396.0	401.9	391.2	384.5	395.7	400.7

TABLE IV.—STANDARD DEVIATIONS OF THE MEANS OF TABLET WEIGHTS FOR THE SEVEN TIME PERIODS OF TWO EXPERIMENTS

Granulation	Expt.	Sample Size	S. D. of Tablet Wt. at Time Periods, min. 0	10	20	30	40	50	60
12	I	10	7.89	2.49	5.29	5.10	5.02	4.72	2.42
	II	15	7.47	4.11	4.31	4.25	3.67	4.34	2.43
20	I	10	2.59	2.22	2.64	2.12	2.53	1.95	2.25
	II	15	2.41	2.79	2.31	2.53	2.78	1.91	3.25
50	I	10	2.50	3.63	2.79	4.57	3.82	3.44	1.62
	II	15	1.76	6.02	3.46	4.60	4.68	3.60	3.11
100	I	10	5.52	15.93	5.91	11.34	4.96	1.87	4.53
	II	15	9.38	15.95	10.15	6.10	4.24	7.04	4.16

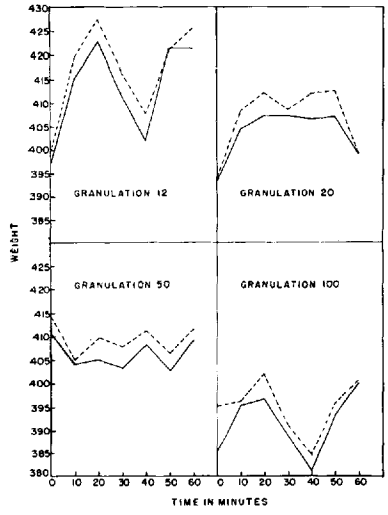

Fig. 2.—Means of tablet weights at 10-min. intervals for 1 hr. tablet press operation. Key: ————, experiment I; ----------, experiment II.

the variance of core (V_c) is very small relative to the variance of coated tablets (V_t). The absolute value of covariance of core and coating ($Cov_{c \times ct}$) is at most the square root of the product of variance of core (V_c) and variance of coating (V_{ct}) (Eq. 2)

$$| Cov_{c \times ct} | \leq \sqrt{V_c}\,\sqrt{V_{ct}} \qquad \text{(Eq. 2)}$$

and is small relative to the variance of the coated tablets (V_t).

From Eq. 1, it can be seen that if V_c and $Cov_{c \times ct}$ are small, the difference, $V_t - V_{ct}$, is necessarily very small.

Therefore, in this study, core variance corrections were not made, and the analysis was performed on the assumption that variance of coating equals variance of coated tablets ($V_{ct} = V_t$). This assumption is further justified by the fact that the authors are more interested in relative values of the four granulations "estimates of variances" than in their absolute values.

The computed means and standard deviations for each sample of 10 or 15 tablets of the two experiments are summarized in Tables III and IV.

To depict more clearly the variability of the tablet weights for the two experiments over the 60-min. time period of the compression operation, the means and standard deviations are plotted in Figs. 2 and 3, respectively. It is interesting to note that each

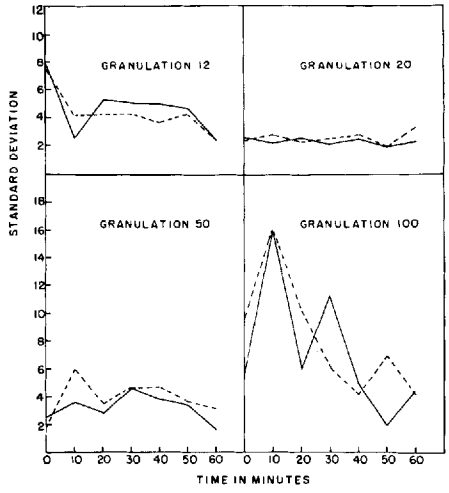

Fig. 3.—Standard deviation of tablet weights sampled at 10-min. intervals for 1 hr. tablet press operation. Key: ————, experiment I; ----------, experiment II.

TABLE V.—ANALYSIS OF VARIANCE ON INDIVIDUAL TABLET WEIGHTS FOR THE FOUR GRANULATIONS

Source of Variation	Granulation 12 S.S.	d.f.	M.S.	20 S.S.	d.f.	M.S.	50 S.S.	d.f.	M.S.	100 S.S.	d.f.	M.S.
Total	9755.48	149		3897.17	149		3490.00	149		16546.47	149	
Between time-means	6749.68	5	1349.94[a]	2443.79	5	488.76[a]	829.88	5	165.98[a]	5304.27	5	1060.85[a]
Between experiments[c]	561.69	1	561.69	479.60	1	479.60	367.35	1	367.35	236.13	1	236.13
Interaction	138.61	5	27.72	107.71	5	21.54[b]	59.07	5	11.81	98.64	5	19.73
Residual	2305.50	138	16.71	866.07	138	6.28	2233.70	138	16.19	10907.43	138	79.04
Indices (The estimates of between time-means variance components)	$I_{12} = \frac{1349.94-27.72}{25} = 53$			$I_{20} = \frac{488.76-21.54}{25} = 19$			$I_{50} = \frac{165.98-11.81}{25} = 6$			$I_{100} = \frac{1060.85-19.73}{25} = 42$		
Average variances[d] (Within time-means provided by residual)	$V_{12} = 17$			$V_{20} = 6$			$V_{50} = 16$			$V_{100} = 79$		

[a] Significant at $p = <0.001$. [b] Significant at $p = 0.01$. [c] "Between experiments" mean squares are not tested for significance since they reflect initial adjustment of tablet press. "Interaction" mean squares are not significant for granulations 12, 50, and 100, indicating that time variation of means does not differ significantly from experiment to experiment, *e.g.*, these time patterns are systematic. "Interaction" mean squares for granulation 20 are significant. However, the "between time-means" mean square is much larger than the "interaction" mean square so that although the time pattern does differ between experiments, the common part of the pattern is much greater than the difference between experiments (Fig. 2, granulation 20). The variances of 12 samples for granulation 100 are not homogeneous across an hour run. However, the samples with heterogeneous variances were included in the analysis of variance so that the variability "within time-means" is measured by the average variance for the whole run of the machine. The ratio of "between time-means" mean square and "residual" mean square is sufficiently large so that the significance of the former is not in doubt.

granulation has a typical pattern of variability for time-means and a different pattern for standard deviations which was similar for both experiments, even though they were performed about 6 months apart. It is evident from the plots in Fig. 2 that granulation 12 shows the greatest scatter of mean tablet weights over the time period of compression and granulation 50 the least. However, the standard deviation of the mean tablet weights is the smallest for granulation 20 and largest for granulation 100 as seen from the plots in Fig. 3.

The results obtained from Bartlett's test showed that for granulation 12 and 50, the zero time variances differ significantly (are heterogeneous) when compared with the remaining 12 samples' variances from 10 to 60 min. Granulation 20 showed homogeneous variances for the mean weights of the 0- to 60-min. samples. Granulation 100, however, showed heterogeneous variances for the mean tablet weights for the 0-, 10-, and 20-min. samples as compared with the other time periods. As a result of these findings, the zero time data were eliminated from further analysis since it was felt that the tablet press was not yet standardized at the zero time sampling. Although the 10- and 20-min. samples of granulation 100 exhibited heterogeneous variances, they were retained for the analysis of variance. The justification for this is given with Table V which shows the analysis of variance and the calculations of indices (I), as well as average variances (V) for each granulation.

It was found that granulation 12 had an $I = 53$, granulation 20 an $I = 19$, granulation 50 an $I = 6$, and granulation 100 an $I = 42$. The higher the index, the larger the variability of the tablet weight time-means during the 1-hr. compression period. The granulations exhibit the following order of increase of variability of time-means over the 1 hr. duration of tablet manufacture: $50 < 20 < 100 < 12$. It is interesting to note that the granulations of the medium particle size range show the smallest indices. The average variances for the four granulations are $V_{12} = 17$, $V_{20} = 6$, $V_{50} = 16$, and $V_{100} = 79$. It is evident from these data that the following increasing order of variability exists within the mean tablet weights for the four granulations: $20 < 50 < 12 < 100$. The average variances for the four granulations demonstrate that granulation 100 exhibits the worst variability.

Comparison of Weight Uniformity and Core Centration Data.—In order to permit this evaluation, the tablets used in this study and in a previous one on core centration (10) were from the same batch. When the results obtained in this study are compared with the core centration data, it is found that the tablets prepared from coating granulation fraction 20 also exhibited optimal core centration.

SUMMARY

The influence of coating granulation size distribution of the same formulation on the weight variability of compressed coated tablets prepared on a Manesty machine was studied, and simple measures of tablet weight variability were presented. The following summarizes the findings.

1. The size distribution of the coating granulation substantially affects the weight uniformity of the compressed coated tablets.

2. For each granulation, there is a pattern of time variation of means which is systematic for the experiments.

3. Granulations 12 and 100 cause the largest variability in tablet weight means during the 1-hr. period of tablet press operation. In addition, granulation 100 has the largest variability of all the granulations when scatter of tablet weight within a sample of 10 or 15 tablets is taken under consideration.

4. Granulations 20 and 50 cause small scatter of means of tablet weights; and in addition, granulation 20 shows particularly low variability of tablet weights within samples of 10 or 15 tablets.

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Lined and Unlined Rubber Stoppers for Multiple-Dose Vial Solutions II

Effect of Teflon Lining on Preservative Sorption and Leaching of Extractives

By LEON LACHMAN, WAYNE A. PAULI, PRAVIN B. SHETH, and MIRIAM PAGLIERY

This report demonstrates the protective action of Teflon linings on the sorption and leaching characteristics of polyurethan and natural rubber stoppers. This lining was effective in essentially eliminating extractives from appearing in water, 50 per cent polyethylene glycol 300, 50 per cent *N,N*-dimethylacetamide, 10 per cent ethanol, and 2 per cent benzyl alcohol when these solvents were in contact with the closures for 6 hr. at 115°. Sorption of the preservative, *p*-chloro-β-phenylethyl alcohol, from aqueous solution was effectively retarded by the Teflon lining on the stoppers. Reduced protection against sorption and leaching was found when Teflon lined closures, which previously underwent multiple puncture with a 20-gauge hypodermic needle, were used.

WITHIN recent years, the influence of rubber closures on the contents of multiple-dose vial solutions has received considerable attention (1, 2). It has been shown that both leaching of rubber extractives from rubber closures into solution and sorption of materials from solution by rubber stoppers can be detrimental to multiple-dose injectable preparations. Since many of the materials extracted from closures are reactive chemicals, they could cause stability (3–5) or toxicity and pyrogenicity (6, 7) problems with the vial contents, as well as interfere with assay methods (2), making it difficult or impossible to quantitatively identify active ingredients. The loss of antibacterial preservatives from multiple-dose vials resulting from sorption into rubber closures and/or reaction with rubber extractives is recognized as a serious problem. Since these agents are added to multiple-dose injectable preparations to insure bacteriostasis for the life of the product, any significant loss of the antimicrobial agent from solution can seriously undermine sterility maintenance of the product. Various attempts have been made to retard sorption of materials from vial solutions and to reduce the amount of extractives leached from closures into solutions. Certain rubber stopper manufacturers have attempted to eliminate these incompatibilities between vial solutions and closures by application of a lacquer lining to the inner surface of their closures. This lining appears to be essentially noneffective in retarding both sorption and leaching effects (8). A recent report from these laboratories (9) contains an evaluation of the protective action of an epoxy lining on rubber stoppers of varying composition. Although this lining was found to afford partial protection against leaching, no protective action against sorption was observed.

This study was initiated to determine the extent of protection afforded by Teflon linings, described by Hopkins (10), on polyurethan and natural rubber stoppers against sorption and leaching. The sorption characteristics of the lined and unlined elastomer closures were tested

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with an aqueous buffered solution of the preservative, *p*-chloro-β-phenylethyl alcohol, stored in multiple-dose vials. The leaching tendencies of water, 10% ethanol, 50% polyethylene glycol 300, 50% *N,N*-dimethylacetamide, and 2% benzyl alcohol on the closures were evaluated. Identification of the extractives leached from the stoppers was attempted.

EXPERIMENTAL

Materials.—*p* - Chloro - β - phenylethyl alcohol (Ciba), b.p. 80–83°, at 1.07 mm.; thiazole-type accelerator, thiuram-type accelerator, dithiocarbamate reaction product, urethan monomer glycol-amine curing agent, and an organo-metallic catalyst were supplied by the rubber manufacturer.

Polyethylene glycol 300 (Union Carbide); *N,N*-dimethylacetamide (E. I. du Pont); benzyl alcohol, reagent grade (Fisher Scientific Co.), double distilled at Ciba; 0.275 *M* citric acid–sodium phosphate buffer of pH 4.0; polyurethan and natural rubber stoppers Teflon lined and unlined, 13 mm. (West Co.); U.S.P. type 1, 10-ml. clear ampuls and vials (Kimble Glass Co.); three-piece aluminum caps for vials (West Co.), No. 13-30 were also utilized.

Equipment.—Beckman model DB recording spectrophotometer, Beckman model G pH meter, and microdistillation apparatus, as previously described in an earlier report (2), were employed.

Preparation of Ampuls, Vials, and Stoppers.—These units were washed and dried in accordance with the procedures described in a previous publication from this laboratory (1).

Sorption of Preservative from Solution.—To evaluate the effectiveness of Teflon lining against preservative sorption, lined and unlined closures of the same basic rubber formulations were used. The preservative solution used in the evaluation, 0.3% *p*-chloro-β-phenylethyl alcohol was prepared on a weight to volume basis with water for injection buffered to a pH of 4.0. Before being filled into 10-ml. clear glass ampuls and vials, the preservative solution was filtered through a medium porosity sintered-glass filter. Ampul closure was accomplished by the customary pull sealing technique with an oxygen-gas flame. The vials containing the preservative solution were then divided into four equal parts and stoppered with Teflon lined and unlined polyurethan and natural rubber stoppers. The stoppered vials were sealed with three-piece aluminum caps using a Fermpress hand crimper. The ampuls and vials containing the preservative solution were then placed into constant-temperature cabinets regulated at 25, 50, and 60 ± 1.5°. Half of the vials were stored upright and half inverted; and at designated time intervals, samples were withdrawn and evaluated for residual preservative content.

To investigate the effect of multiple puncture of lined closures on preservative sorption, Teflon lined natural rubber stoppers were punctured 5 times with a 20-gauge hypodermic needle. Ten-milliliter clear vials containing the 0.3% buffered preservative solution were stoppered using these closures and then capped as described previously. Half of the vials were stored upright and half inverted at the same conditions employed for the other vials.

Sealed 10-ml. clear ampuls of the preservative solution served as controls at the various storage conditions.

Rubber Closure Extractives.—To determine the effectiveness of the Teflon lining on the two different closures against leaching of unreacted materials and reaction products into the vial solution, several solvents commonly found in parenteral formulations were employed. These solvents, including water, 10% ethanol, 50% polyethylene glycol 300, 50% *N,N*-dimethylacetamide, and 2% benzyl alcohol, were filled into 10-ml. clear glass vials and stoppered with the unlined and Teflon lined polyurethan and natural rubber closures. Multipunctured Teflon lined natural rubber stoppers were also employed to evaluate the possible loss of protection resulting from the multiple perforations in the Teflon film. The stoppered vials were then sealed with three-piece aluminum caps again using the hand crimper described previously. After autoclaving the vials in an inverted position at 115° (10 p.s.i.) for 6 hr., the solutions in the vials were scanned spectrophotometrically to obtain ultraviolet absorption curves of the rubber extractives.

Identification of Extractives.—For the various organic additives and their possible reaction products of the two rubber compositions used in this study, saturated solutions were prepared in the five solvent systems and scanned spectrophotometrically to obtain their characteristic ultraviolet absorption spectra. This made possible a qualitative identification of the materials extracted from the polyurethan and natural rubber closures.

Analytical Method.—The assay method employed for *p*-chloro-β-phenylethyl alcohol has been described in an earlier publication from these laboratories (1).

RESULTS AND DISCUSSION

The protective action of a Teflon lining on polyurethan and natural rubber stoppers against sorption of the preservative, *p*-chloro-β-phenylethyl alcohol, from solution and leaching of extractives from the stoppers by several solvents was investigated. The composition and per cent rubber content of these stoppers are presented in Table I.

Sorption Studies.—The sorption tendencies of Teflon lined, multipunctured Teflon lined, and unlined rubber stoppers were evaluated with vial solutions of *p*-chloro-β-phenylethyl alcohol buffered to a pH of 4.0. The protective action of the Teflon lin-

TABLE I.—CLOSURE COMPOSITION

Natural Rubber	Polyurethan Rubber
Natural crepe[a]	Urethan monomer[b]
Calcined clay	Amine-glycol curing agent
Barium sulfate	Organo-metallic catalyst
Zinc oxide	Titanium dioxide
Iron oxide	
Stearic acid	
Paraffin wax	
Thiazole-type accelerator	
Thiuram-type accelerator	

[a] Rubber content: 56% of total composition. [b] Rubber content: 95% of total composition.

TABLE II.—EFFECTIVENESS OF TEFLON LINING ON NATURAL RUBBER STOPPERS AGAINST SORPTION OF *p*-CHLORO-β-PHENYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time,	Temp., 25°							Temp., 50°							Temp., 60°						
	Upright			Inverted				Upright			Inverted				Upright			Inverted			
Wk.	L[a]	PU	P	L	PU	P	A	L	PU	P	L	PU	P	A	L	PU	P	L	PU	P	A
0	...	...	...	...	...	...	100	...	...	...	...	...	...	...	...	...	...	...	...	...	...
2	99	98	96	99	95	90	100	99	91	90	99	90	79	98	100	90	84	99	89	78	98
4	100	98	95	100	95	88	99	100	91	83	98	84	77	99	100	88	78	100	84	75	99
8	99	98	88	100	94	81	101	99	84	74	99	81	70	101	99	77	63	100	81	63	92
12	102	95	91	102	91	82	102	102	80	72	102	80	71	102	102	74	64	108	74	63	102

[a] L = Teflon lined; PU = multipunctured Teflon lined; P = plain; A = ampul.

TABLE III.—EFFECTIVENESS OF TEFLON LINING ON POLYURETHAN RUBBER STOPPERS AGAINST SORPTION OF *p*-CHLORO-β-PHENTYLETHYL ALCOHOL FROM VIAL SOLUTIONS REPRESENTED AS PER CENT RESIDUAL PRESERVATIVE

Time,	Temp., 25°					Temp., 50°					Temp., 60°				
	Upright		Inverted			Upright		Inverted			Upright		Inverted		
Wk.	L[a]	P	L	P	A	L	P	L	P	A	L	P	L	P	A
0	...	...	...	...	100	...	...	...	...	...	...	...	...	...	...
2	99	85	98	51	97	99	74	97	37	98	99	69	98	38	98
4	96	63	97	42	97	96	51	96	29	97	96	45	97	30	97
8	97	61	96	38	95	97	39	97	26	97	101	24	96	23	97
12	100	78	99	36	99	100	34	99	23	99	97	20	101	19	101

[a] L = Teflon lined; P = plain; A = ampul.

TABLE IV.—ABSORBANCE VALUES OF EXTRACTIVES LEACHED BY SEVERAL SOLVENTS IN 10-ml. VIALS FROM TEFLON LINED AND UNLINED RUBBER STOPPERS AFTER AUTOCLAVING THE VIALS IN AN INVERTED POSITION AT 115° FOR 6 hr.

Solvent	Natural				Polyurethan		
	Wavelength, mμ	Absorbance			Wavelength, mμ	Absorbance	
		P[a]	PU	L		P	L
Water	310	0.74	0.02	0.01	276	0.07	0.01
	230	0.83	0.07	0.02			
Ethanol, 10%	308	0.86	0.03	0.01	274	0.17	0.01
	228	0.91	0.11	0.02			
Benzyl alcohol, 2%	312	0.77	0.03	0.01	280	0.14	0.02
Polyethylene glycol 300, 50%	324	0.66	0.13	0.02	270	0.85	0.12
	248	0.71	0.40	0.27	250	0.88	0.21
N,*N*-Dimethylacetamide, 50%	326	1.95	...	0.01	278	1.55	0.01

[a] P = plain; PU = multipunctured Teflon lined; L = Teflon lined.

ing on natural rubber closures against sorption of the preservative from solution can readily be seen from the data in Table II. At all temperature conditions throughout the 12-week period, no decrease in preservative content is noted for any of the vials with lined closures. The observed loss of *p*-chloro-β-phenylethyl alcohol from vials with plain closures appears to level off at around the 8-week period. This plateau could indicate the presence of an equilibrium condition. It is interesting to note that only for the unlined closures do vial solutions stored in an inverted position show greater loss of preservative content than those stored upright.

To evaluate the effectiveness of the Teflon lining after the stopper was punctured several times by a hypodermic needle, Teflon lined natural rubber closures were punctured 5 times with a 20-gauge hypodermic needle and employed as closures for vials containing the preservative solution. These vials were stored at the same conditions and for the same time period as the other group. Results show that even when perforated several times by a hypodermic needle, the Teflon lining still affords some protection against sorption when compared with loss of preservative for the unlined closures.

The data in Table III summarize the sorption tendencies of lined and unlined polyurethan closures for *p*-chloro-β-phenylethyl alcohol at the various temperature conditions. Excellent protection was afforded by the Teflon lining against sorption at all storage conditions throughout the 12-week evaluation. Again, only for the unlined closures do the vial solutions stored in an inverted position show a greater loss of preservative than those stored upright.

Since there was no diminution of preservative concentration in the ampul solutions, the loss in the vial solutions stoppered with natural and polyurethan closures must be attributed to closures.

Although no physical changes in the natural rubber closures were noted at the various elevated temperature conditions employed, both the lined and unlined polyurethan closures deformed markedly at these same temperatures. In fact, the unlined closures were so badly deformed that the loss data of preservative in the vials could be due to loss around the closure and out to the atmosphere in addition to that sorbed by the closure.

Leaching of Extractives.—Several solvents commonly used in injectable formulations were employed to evaluate the leaching of extractives from

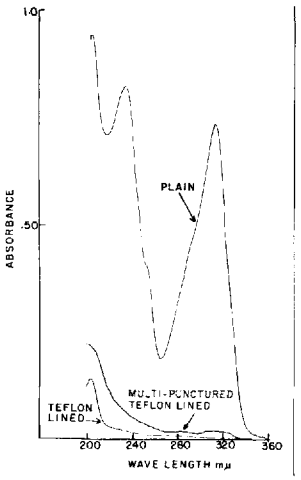

Fig. 1. — Ultraviolet absorption curve of leached extractives. Rubber, natural; solvent, water; storage condition, 6 hr. at 115°, vial inverted.

Teflon lined and unlined stoppers when used on multiple-dose vials. Table IV lists the ultraviolet maxima and corresponding absorbances representing extractives from the polyurethan and natural rubber closures appearing in the various solvents. It can readily be seen that for each of the rubber compositions employed and in each of the solvent systems, the Teflon lining afforded considerable protection against leaching of extractables from the closures as evidenced by the extremely low absorbance values obtained when lined closures were used. Considerable protection against leaching was also provided using the multipunctured Teflon lined closures. Representative ultraviolet absorption spectra for the extractives found in the vial solutions containing water and stoppered with lined, multipunctured lined, and unlined natural rubber stoppers are given in Fig. 1.

It is interesting to note from the data in Table IV that of the five solvents investigated with the lined closures, only 50% polyethylene glycol 300 appeared to be successful in leaching out a significant amount of extractives from both polyurethan and natural rubber. To explore whether decomposition of the polyethylene glycol 300, when exposed to the extreme conditions of the evaluation, gave degradation products which also showed ultraviolet absorption, samples of this solvent were sealed in clear glass ampuls and heated for the same period at the same temperature as the vials. The ultraviolet absorption spectra of the 50% polyethylene glycol 300 solution heated in ampuls and an untreated control are given in Fig. 2. The appearance of an absorption peak at approximately 250 mμ for the heated glycol solution indicates that the decomposition of the glycol most likely contributes to the relatively high absorbance values found with this solvent from vials using Teflon lined closures.

Identification of Extractives.—Materials of the rubber compositions studied, which would tend to give absorption in the ultraviolet region, were obtained from the rubber stopper manufacturer and ultraviolet absorption spectra taken in the solvents studied. This information was employed to aid in the identification of the extractives leached from the polyurethan and natural rubber stoppers. The absorption data for these various ingredients are summarized in Table V. With this information, positive identification of each of the various extractables was made possible.

For the natural rubber stoppers, the material responsible for the absorption at 308–326 mμ and 228–230 mμ is believed to be the thiazole-type accelerator. The solvent decomposition products and/or the dithiocarbamate reaction product could account for the absorption at 248 mμ in 50% polyethylene glycol 300. In the case of the polyurethan closures, the absorption at 270–280 mμ can be ascribed primarily to the organo-metallic catalyst employed with the possibility of minor contribution from the urethan monomer. The observed peak found for 50% polyethylene glycol 300 of approximately 250 mμ can be attributed to solvent decomposition and not to extractives from the rubber. Even though slight ultraviolet absorption was observed for the glycol-amine curing agent, the absorption spectra of this agent in the various solvent systems employed were rather diffuse with no definable maxima.

SUMMARY

Teflon lined and unlined polyurethan and natural rubber closures were evaluated for their sorption and leaching characteristics. Multiple-dose vials containing an aqueous buffered solution of the preservative, *p*-chloro-β-phenylethyl alcohol, were stoppered with the lined and unlined rubber closures for the sorption studies. Several solvents commonly used in injectable formulations were employed to evaluate the leaching of extractives from the lined and unlined closures when used on multiple-dose vials. The solvents used were water, 10% ethanol, 2% benzyl alcohol, 50% *N,N*-dimethylacetamide, and 50% polyethylene glycol 300. The following results were obtained.

1. The Teflon lining was found to afford excellent protection against sorption of the preservative into either the polyurethan or natural rubber stoppers.
2. The lining was found to be effective in preventing the leaching of extractives from the rubber closures by the various solvents employed.
3. The extractives leached by the different sol

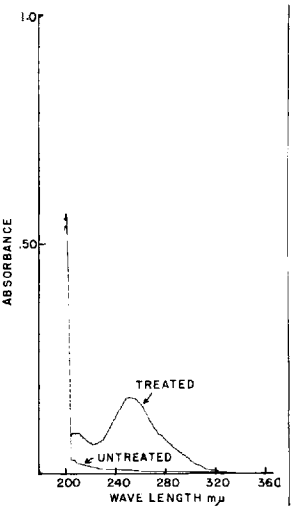

Fig. 2. — Ultraviolet absorption spectra of a 50% polyethylene glycol 300 solution after autoclaving in sealed ampuls for 6 hr. at 115°, 10 p.s.i., and an untreated control.

TABLE V.—ULTRAVIOLET ABSORPTION MAXIMA OF RUBBER CLOSURE INGREDIENTS

Rubber Stopper	Wavelength, mμ				
	Water	10% Ethanol	2% Benzyl Alcohol	50% *N,N*-Dimethylacetamide	50% Polyethylene Glycol 300
Natural					
Thiazole-type accelerator	312	312	312	322	322
	227	230	...	...	228
Thiuram-type accelerator	274	274	278	276	278
Dithiocarbamate reaction product	274	276	...	274	274
	...	250	...	257	253
Polyurethan					
Urethan monomer	...	...	...	288	287
Organo-metallic catalyst	272	274	272	274	274

vents from both the unlined polyurethan and natural rubber closures were qualitatively identified.

4. Teflon lined natural rubber stoppers, which were perforated several times by a hypodermic needle, still afforded some protection against sorption and considerable protection against leaching.

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Notes

Phosphorus-Nitrogen Compounds V. Some Guanidine and 2-Aminopyrimidine Derivatives

By LINDLEY A. CATES and NOEL M. FERGUSON

Five amidino(thio)phosphoramidates and six 2-pyrimidyl(thio)phosphoramidates were prepared for screening in four cancer test systems. Three of these derivatives were also tested against *Plasmodium berghei*. None of the compounds exhibited significant antineoplastic or antimalarial activity.

THE SYNTHESIS of phosphoramidopyrimidines was undertaken to further ascertain the effect of phosphorus bonding on the cytotoxic properties of heterocyclic amines. The preparation of similar pyridine-containing compounds was described in an earlier report in this series (1).

The rationale for antineoplastic activity by these derivatives originates from an hypothesis concerning the mechanism of action of cyclophosphamide. According to this theory, cyclophosphamide exists as an inactive transport form *in vivo* until the P-N bond is cleaved by the enzymatic action of phosphamidases or phosphatases to yield the alkylating ethylenimmonium ion (2). Thus, replacement of the bis(2-chloroethyl)amino group by an aminopyrimidine moiety might give rise to an antimetabolic activity of a more specific nature than would be realized by administration of the parent compound. More recent evidence (3), however, suggests that the conversion of cyclophosphamide *in vivo* into a cytostatically effective activation product is the result of a metabolic process dependent on the presence of oxygen and not the result of simple hydrolysis. At this time it can be stated that no direct proof of the postulated biochemical transformations of cyclophosphamide and related congeners is available.

Since guanidine and some of its derivatives are known to possess antiprotozoal properties (4) three of the amidino(thio)phosphoramidate intermedi-

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Previous paper: Cates, L. A., and Ferguson, N. M., *J. Pharm. Sci.*, **54**, 465(1965).

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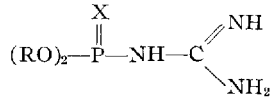
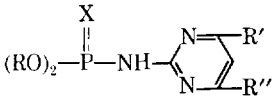
TABLE I.—AMIDINO(THIO)PHOSPHORAMIDATES



Compd.	R	X	M.p., °C.[a]	Formula	Anal., %[b] Calcd.	Found
I	Methyl	S	108–110	$C_3H_{10}N_3O_2PS$	C, 19.7 H, 5.5 N, 22.9	C, 19.8 H, 5.6 N, 23.3
II	Ethyl	S	87–88	$C_5H_{14}N_3O_2PS$	C, 28.4 H, 6.7 N, 19.9	C, 28.6 H, 6.8 N, 19.9
III	Ethyl	O	127–129	$C_5H_{14}N_3O_3P$	C, 30.8 H, 7.2 N, 21.5	C, 30.9 H, 7.4 N, 21.8
IV	*n*-Propyl	S	73–74	$C_7H_{18}N_3O_2PS$	C, 35.1 H, 7.6 N, 17.6	C, 35.2 H, 7.8 N, 17.4
V	*n*-Butyl	O	83–84	$C_9H_{22}N_3O_3P$	C, 43.0 H, 8.8 N, 16.7	C, 43.2 H, 8.8 N, 16.6
VI[c]	Phenyl	O	119	$C_{13}H_{14}N_3O_3P$	C, 53.6 H, 4.8 N, 14.4	C, 53.4 H, 4.8 N, 14.5

[a] All melting points were determined on a Fisher-Johns apparatus and are uncorrected. [b] Elemental analyses were run on a Fisher CHN analyzer. [c] Previously prepared by different methods [reported m.p. 118° (6, 8)].

TABLE II.—2-PYRIMIDYL(THIO)PHOSPHORAMIDATES



Compd.	R	X	R′	R″	M.p., °C.[a]	Formula	Anal., %[b] Calcd.	Found
VII	Phenyl	O	H	H	137–139	$C_{16}H_{14}N_3O_3P \cdot H_2O$	C, 55.6 H, 4.7 N, 12.2	C, 55.6 H, 4.7 N, 12.0
VIII	Phenyl	O	CH_3	CH_3	152–153	$C_{18}H_{18}N_3O_3P \cdot H_2O$	C, 57.9 H, 5.4 N, 11.3	C, 58.1 H, 5.4 N, 11.3
IX	Ethyl	S	OH	OH	149–150	$C_8H_{14}N_3O_4PS$	C, 34.4 H, 5.0 N, 15.0	C, 34.3 H, 5.1 N, 14.4
X	*n*-Propyl	S	OH	OH	108–109	$C_{10}H_{18}N_3O_4PS$	C, 39.1 H, 5.9 N, 13.7	C, 39.5 H, 6.0 N, 13.5
XI	*n*-Propyl	S	OH	NH_2	154–156	$C_{10}H_{19}N_4O_3PS$	C, 39.2 H, 6.2 N, 18.3	C, 39.5 H, 6.3 N, 18.3
XII	*n*-Propyl	S	OH	CH_3	127–128	$C_{11}H_{20}N_3O_3PS$	C, 43.3 H, 6.6 N, 13.8	C, 43.3 H, 6.7 N, 13.9

[a] All melting points were determined on a Fisher-Johns apparatus and are uncorrected. [b] Elemental analyses were run on a Fisher CHN analyzer.

ates, which contain the guanidino moiety, were incidentally tested for antimalarial activity.

The pyrimidine derivatives were prepared by two different methods: phosphorylation and condensation. Two of the products were synthesized by direct acylation using diphenyl phosphorochloridate, whereas several attempts at phosphorylation of halo- and hydroxy-substituted pyrimidines employing alkyl and aryl phosphorochloridates proved unsuccessful. This failure is attributed to the relatively high pK_b values of these pyrimidines. A recent report (5) indicates that halo-substituted 2-aminopyrimidines will, however, yield phosphoramidic dichlorides and phosphorimidic trichlorides

when the more reactive phosphorus pentachloride is employed.

As an alternate route pyrimidine synthetic methods involving double condensation between cyanoacetic, malonic and β-keto esters and substituted guanidines were employed. The amidinophosphoramidates and amidinophosphoramidothionates required in these condensations were obtained by the reaction between phosphorochloridates or phosphorochloridothionates and guanidine. The latter reactant was prepared *in situ* using guanidine hydrochloride and alcoholic potassium hydroxide. The synthesis of amidinophosphoramidates is somewhat controversial in that Shvachkin and Prokof'ev (6) reported that the preparation of diphenylphosphorylguanidine from phosphoryl chloride, phenol, and guanidine (7) could not be duplicated, and they synthesized the product using diphenylphosphorochloridate and guanidine. This procedure was also reported by Cramer and Vollmar (8), as well as an alternate method involving the treatment of guanidine hydrochloride in aqueous sodium hydroxide with diphenylphosphorochloridate in benzene. The guanidine hydrochloride–alcoholic potassium hydroxide process reported in this paper is considered to be a convenient and economical method for the synthesis of phosphoryl(thio)guanidines. Of the guanidine derivatives prepared, the ethyl and *n*-propyl amidinophosphoramidothionates were selected for pyrimidine condensation.

EXPERIMENTAL

Chemistry

Amidinophosphoramidates and Amidinophosphoramidothionates (Table I).—A solution of potassium hydroxide (0.2 mole) in 100 ml. of absolute ethanol was added in portions to guanidine HCl (0.2 mole) in 100 ml. of absolute ethanol at 0° with stirring. This temperature was maintained and the appropriate phosphorochloridate or phosphorochloridothionate (0.1 mole) was introduced dropwise with stirring. The reaction mixture was allowed to remain overnight and then spin evaporated over a steam bath to yield a white mass or oil. The residue was dissolved in hot benzene and ether was added to precipitate guanidine hydrochloride. The filtrate was again spin evaporated to yield a white mass or oil. Compounds I, II, and III were isolated by crystallizing from hot benzene. Compounds IV and V were obtained in pure form by washing an ethereal solution of the residue with 10% sodium hydroxide solution and water, drying over anhydrous calcium sulfate, and concentrating the ether filtrate. Known compound VI was purified according to a previously described method (6). All products were white crystalline solids.

2-Pyrimidylphosphoramidates (Table II).—A mixture of 2-aminopyrimidine or 2-amino-4,6-dimethylpyrimidine (0.2 mole) and diphenyl phosphorochloridate (0.1 mole) in 500 ml. of reagent dioxane was refluxed for 0.5 hr. and allowed to remain overnight. The reaction mixture was spin evaporated to yield a brown mass which was washed with petroleum ether and water and the residue dissolved in hot ethanol. After treating with activated charcoal, clouding with water, and cooling, the product formed as a white crystalline solid.

2-Pyrimidylphosphoramidothionates (Table II.)—The appropriate amidinophosphoramidothionate (0.05 mole) was refluxed for 1 hr. with sodium (0.25 mole), which was previously reacted with 300 ml. of absolute ethanol, and the reaction mixture was allowed to set overnight. A fivefold excess of diethyl malonate, ethyl cyanoacetate, or ethyl acetoacetate was added, the mixture refluxed for 2 hr., and allowed to remain overnight. The reaction mixture was concentrated by spin evaporation and any precipitate dissolved by addition of the least amount of water. Following filtration the solution was adjusted to pH 8 using 5% hydrochloric acid and a Corning pH meter. The resulting precipitate was collected and crystallized from ethanol–water.

Infrared Spectra

All starting materials and products were examined by means of a Beckman IR-8 spectrophotometer using a Nujol mull. The new guanidine derivatives showed the following characteristic absorptions, ν in cm.$^{-1}$: 3200 (NH), 1020–1060 (POCalkyl), and 1240 (P=O in compounds III and V). Some of the principal absorptions given by the pyrimidine derivatives were, ν in the cm.$^{-1}$: 1520–1580 (C=C, C=N in all compounds), 1240 (P=O in compounds VII and VIII), 1080–1090 and 1200 (POC aryl in compounds VII and VIII), 1020–1055 (POC alkyl in compounds IX–XII), and 3350 (NH_2 in compound XI). The lack of a free amino group (except, of course, in compound XI) indicates the correctness of the proposed structures, eliminating an alternative course of the condensations. No assignment of P–N absorption was attempted since this band, usually designated around 715 cm.$^{-1}$, falls in the skeletal structure absorption range. Derkach (9) reported the infrared spectra of some related phosphoramidates and assigned the 893–910 cm.$^{-1}$ range to P–N. This assignment may not be correct since a similar strong peak was noted in the compounds reported here but only in the case of the phenyl esters. The reliability of assigning P–N absorption in the region of 715 cm.$^{-1}$ has also been questioned by Bellamy (10) and Thomas (11).

Screening Data

Samples of the new compounds were submitted for antitumor testing against sarcoma 180, Lewis lung carcinoma, lymphoid leukemia L-1210, and KB cell culture.[1] Results of that testing which have been completed indicate that only compound I has a per cent T/C (ratio of tumor weight of test animals to control animals; expressed as per cent) of less than 50 when screened against sarcoma 180 and that at a dose of 500 mg./Kg. The most toxic member of the series was compound V which gave no survivors at a dose of 100 mg./Kg. The pyrimidine derivatives exhibited relatively low acute toxicities having lethal doses in excess of 250 mg./Kg. Five mice were infected with a lethal dose of *Plasmodium berghei* 3 days prior to administration of compounds I, III, and V in doses of 40, 160, and 640 mg./Kg.[2] At the higher dose level compound III (most active) extended the survival time by only 35%. Compounds I and III showed low acute toxicities and the relatively high toxicity of compound V was confirmed in these tests.

[1] Testing results furnished by the Cancer Chemotherapy National Service Center, Bethesda, Md.

[2] Testing results furnished by the Walter Reed Army Medical Center, Washington, D. C.

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Tracer and Radioactivation Studies on Tartar Emetic Impurities

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The problem of contamination of tartar emetic with arsenic and lead was examined using the radioactive isotopes, ^{76}As and ^{209}Pb. Neutron activation and radiochemical separation methods have been applied for the determination of arsenic and lead concentrations throughout the different stages of tartar emetic synthesis. It has been shown that, while most of arsenic is eliminated during the synthesis process, the major portion of lead remains in the final product. It was also found that the two impurities can be eliminated by successive washing of the starting materials with 0.2 *M* nitric acid and water. Tartar emetic synthesized from purified starting materials conforms to the requirements in pharmacopeias.

ONE OF THE most important problems of the pharmaceutical industry is the production of synthetic drugs in a suitably pure condition. The classical methods of tracing impurities are either misleading, tedious, or inaccurate. Antimony potassium tartrate (tartar emetic) is the most commonly employed drug in the treatment of bilharziasis, schistosomiasis, leishmaniasis, filariasis, ascariasis, and other tropical diseases (1). It is one of the early drugs found to be active against trypanosomes (2). The drug is widely used in Africa, Asia, and Latin America because of the simplicity and easiness of its synthesis and acceptance among patients and physicians. It is still the drug of choice in the treatment of bilharziasis in Egypt as well as other countries where infections occur (3). Cases of toxicity and inconvenience of intravenous injection of this drug are reported, however, and have been attributed, at least partly to the presence of toxic impurities such as arsenic and lead (3, 4).

Most pharmacopeias in which tartar emetic is mentioned require certain specifications for arsenic and lead limits for the sake of its medical safety. These limits vary in the case of arsenic to not more than 200 p.p.m. in the U.S.P. (5), 10 p.p.m. in the E. P. (6), 8 p.p.m. in the B.P. (7), and in case of lead to not more than 10 p.p.m. in the E.P. and 5 p.p.m. in the B.P.

The quantitative determination of the two metals, particularly arsenic, at such low concentrations in the presence of antimony cannot be performed with accuracy by the conventional analytical methods (8–11). The utilization of radioactive isotopes in solving some production problems and in working up technical operations in the pharmaceutical industry has found its way only recently (12). Some studies have been published on the manufacture of pure tartar emetic (3, 13), but none of the authors have made use of radioactive isotope techniques.

The purpose of the present paper was to find an economical method to eliminate arsenic and lead and to produce tartar emetic in pure form. For tracing these two metals, the radioactive isotopes, ^{76}As and ^{209}Pb, have been used, while their concentrations in the starting materials and in the final products have been determined by a neutron radioactivation procedure.

EXPERIMENTAL

Materials.—Unless otherwise stated, all chemicals employed were analytical grade reagents. The radioactive isotopes ^{76}As and ^{209}Pb were prepared by irradiating spectrographically pure arsenic trioxide and lead. Arsenic trioxide was then dissolved in dilute hydrochloric acid, while lead was dissolved in dilute nitric acid. Standard and carrier solutions of arsenic and lead were prepared by dissolving inactive arsenic trioxide and lead in a similar manner.

Apparatus.—The instruments used were an EKCO scintillation assembly for counting the γ radiation of ^{76}As and a Philips counting instrument with an end window G.M. tube for measuring ^{209}Pb β-radiations.

Irradiations.—Samples to be irradiated were wrapped in thin aluminum sheets and were enclosed in aluminum cans which were then irradiated at a flux of about 1.3×10^{13} neutrons/cm.^{2}/sec. in the U.A.-RR-1, 2 MW reactor. For the radioactivation analysis of arsenic and lead, every sample was placed together with the standards in one can and exposures were of about 5 hr. duration.

Procedure.—All experiments on the study of arsenic and lead uptake were performed in 100-ml. conical flasks. For evaluating arsenic uptake on

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TABLE I.—ARSENIC UPTAKE OF TARTAR EMETIC[a]

Expt.	Synthesis Conditions	Original Arsenic, p.p.m.	Arsenic Content in Tartar Emetic, p.p.m.
1[b]	Reaction mixture (paste) was left for 24 hr., then boiled for 15 min.	1000	8.2
2		1000	6.6
3		1100	9.6
4		1250	12.0
5		1500	13.0
6[c]		1500	39.0
7[d]		1500	18.0
8	Reaction mixture was refluxed for 1 hr.	1000	7.0
9		1500	13.0

[a] The values given are the average of two or three experiments. [b] Solution of tartar emetic was cooled in refrigerator. [c] EDTA was added to the reaction mixture. [d] 8-Hydroxyquinoline was added to the reaction mixture.

TABLE II.—LEAD UPTAKE OF TARTAR EMETIC[a]

Original Lead Added, p.p.m.	Lead Content in Tartar Emetic, p.p.m.
~200	~130
~200	~110

[a] The values given are the average of two or three experiments.

TABLE III.—VARIATION OF ARSENIC UPTAKE WITH ITS AMOUNT BEFORE RECRYSTALLIZATION[a]

Expt.	Amt. of Arsenic Added to Tartar Emetic, p.p.m.	Arsenic Content of Synthesized Tartar Emetic Crystals, p.p.m.
1	6.7	0.12
2	16.7	0.20
3	33.3	0.27
4	53	0.75
5	127	1.9
6	163	3.6
7[b]	163	4.9
8	203	6.0
9[b]	203	7.5
10	1000	19.2

[a] The values given are the average of four experiments. [b] Cooled in refrigerator.

tartar emetic, in the coarse of its synthesis, the following procedure was followed: 1.5 Gm. of antimony trioxide, 1.8 Gm. of acid potassium tartrate, both in a finely powdered form, were thoroughly mixed with a few drops of distilled water and a known amount of arsenic containing its radioactive isotope. The paste was left for 24 hr., boiled with 12 ml. of distilled water for 15 min., filtered while hot, and the clear filtrate was left to crystallize at room temperature or in a refrigerator. The crystals of tartar emetic thus obtained, after being filtered off and washed with ice cold water, were dried. The arsenic content of the filtrate and crystals were determined by measuring the radioactivity of ^{76}As. In some other series of experiments a mixture of antimony trioxide, acid potassium tartrate, and arsenic was refluxed with 12 ml. of distilled water for 1 hr., filtered while hot, and the procedure was completed as mentioned before. The study of tartar emetic purification from arsenic by recrystallization was carried out as follows: 3 Gm. of tartar emetic was dissolved in 10 ml. of hot distilled water to which was added a known amount of arsenic. The mixture was then cooled, and tartar emetic was examined for its arsenic content as in the above two series.

The lead contamination of tartar emetic was investigated by refluxing the reaction mixture to which is added a known amount of lead, and in the same manner as in the case of arsenic. The purification of tartar emetic from lead was studied also as in the case of arsenic. Lead which is added in the above two cases, however, was prepared by mixing a known volume of lead solution in nitric acid with 0.5 ml. of concentrated hydrochloric acid. Lead chloride thus precipitated was washed once with ice cold water before being used in the experiment.

The purification of antimony trioxide (Sb_2O_3) was studied by stirring 5 Gm. of crude Sb_2O_3 with different washing solutions. The Sb_2O_3 was then filtered off and examined for the arsenic and lead content.

Radioactivation analysis of arsenic and lead was done by methods similar to those published in the literature (14, 15) except for some modifications to fit with the given samples. For arsenic, a double distillation process was necessary to separate it from antimony, while the preliminary separation of lead from antimony was done by extraction (16).

Radiochemical Purity.—This was checked by measuring the spectra (arsenic) or from the decay curves (lead) of the corresponding radioactive species.

RESULTS AND DISCUSSION

Arsenic and Lead Uptake on Tartar Emetic During Its Synthesis.—The experimental results of arsenic uptake on tartar emetic throughout the process of its synthesis (Table I) show that this uptake increases with the increase of its amount

TABLE IV.—VARIATION OF LEAD UPTAKE ON TARTAR EMETIC WITH THE CONDITION OF EXPERIMENT[a]

Condition of Expt.	Original Lead Added, p.p.m.	Lead Uptake on Tartar Emetic Crystals, p.p.m.
Cooled at room temp.	~200	~ 132
Cooled in refrigerator	~200	~ 138

[a] The values given are the average of two experiments.

TABLE V.—PURIFICATION OF ANTIMONY TRIOXIDE[a]

Washing Soln.	Before Washing		After Washing	
	As, p.p.m.	Pb, p.p.m.	As, p.p.m.	Pb, p.p.m.
Water	450	60	75	58
Water	75	58	1	58
10% ammonium acetate	450	60	380	55
5% sodium carbonate	450	60	425	60
0.1 *M* nitric acid	450	60	...	5
0.1 *M* nitric acid	...	5	...	1
0.2 *M* nitric acid	450	60	...	1

[a] The values given are the average of two or three experiments.

originally present in the reaction mixture. The uptake, however, is always less than 1%. It is clear, also, that when tartar emetic is left to crystallize gradually at room temperature, the arsenic uptake on the crystals is less than in case of enhancing crystallization by cooling the solution. The addition of some complexing agents such as ethylenediaminetetraacetic acid (EDTA) and 8-hydroxyquinoline to the reaction mixture increases the arsenic contamination of tartar emetic. The contamination, however, is the same for the synthesis of the drug by the two methods, *i.e.*, with or without refluxing the reaction mixture.

The uptake of lead, on the other hand, was found to be about 60% of its amount originally added to the reaction mixture (Table II).

Purification of Tartar Emetic by Crystallization.—Table III presents the experimental results of the recrystallization of tartar emetic in the presence of different amounts of arsenic. It is evident that the uptake of arsenic is about 2–3% of its original amount. Cooling the solution increases the arsenic contamination. The percentage uptake, however, is higher than in the case of tartar emetic synthesis. This is most probably due to the uptake of some arsenic onto the unreacted residue.

In some separate experiments, it has been found that the addition of a little amount of hydrochloric acid decreases the arsenic uptake onto the crystals.

The uptake of lead is given in Table IV. It is clear that, while most arsenic can be removed from tartar emetic by recrystallization, the major part of lead remains in the crystals.

The preliminary investigation has shown that the two impurities are mainly present in antimony trioxide, hence, the authors' efforts were directed toward the study of its purification. Water and other washing solutions have been tried for eliminating arsenic and lead from antimony trioxide. The results are presented in Table V.

Washing antimony trioxide with water and 0.2 *M* nitric acid is evidently sufficient to eradicate the arsenic and lead impurities, while losses are negligible. Moreover, this is simpler than eliminating arsenic as arsine using reducing amalgam or complexing lead by a chelating agent (13). Acid potassium tartrate containing arsenic and lead can be sufficiently purified by washing in a similar manner.

RECOMMENDED PROCEDURE AND CONCLUSION

The foregoing data show the different possibilities of eliminating arsenic and lead impurities from tartar emetic. In light of the above discussion, the recommended procedure for the synthesis of highly pure tartar emetic is summarized as follows.

Antimony trioxide is washed once with 0.2 *M* nitric acid and then twice with water. The volume in liters of nitric acid and of water must be fivefold the weight in kilograms of antimony trioxide. Every washing should be followed by filtering the slurry produced. Antimony trioxide is then thoroughly mixed with the calculated amount of similarly washed acid potassium tartrate to obtain a homogeneous paste, and left for 24 hr. The paste is then boiled for 15 min. with distilled water, filtered while hot, and the filtrate is left to cool. The crystals of tartar emetic thus obtained are washed with cold water and then dried. This procedure, while retaining a better yield, is more economic. The product obtained is almost free from arsenic and lead.

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Desoxycholic Acid Enhancement of Orally Administered Reserpine

By MARVIN H. MALONE, HOWARD I. HOCHMAN*, and KARL A. NIEFORTH

Quantities of 1:2, 1:4, 1:8, 1:16, and 1:32 molar amounts of reserpine : desoxycholic acid were dissolved in alcohol and/or chloroform, chilled to −5°, and solvents removed under reduced pressure. When administered orally to mice, the presence of desoxycholic acid hastened blepharoptotic activity and increased blepharoptotic potency of reserpine. Maximum enhancement was observed with 1:16 reserpine–desoxycholic acid where 1 mg. of reserpine as the combination was equivalent to 3.8–2.5 mg. of reserpine base using observations 2 and 24 hr. after dosage, respectively. An equivalent physical mixture prepared by trituration was significantly more potent than reserpine base but much less potent than the prepared combination. Desoxycholic acid alone was without blepharoptotic activity.

THE ENHANCED absorption of various medicinal agents upon concomitant administration with desoxycholic acid may be attributed to its ability to reduce interfacial tension and/or to form inclusion or clathrate compounds. Previous work concerning this increased absorption usually has been restricted to the effect of desoxycholic acid on the absorption of the fat-soluble vitamins and closely related analogs where the effect was thought to be due to the surface action of desoxycholic acid. This report is concerned with an increase in the blepharoptotic activity of reserpine when given in intimate combination with desoxycholic acid.

The general role of bile salts in absorption, the ability of desoxycholic acid to form inclusion compounds (choleic acids), and the relative inefficiency of reserpine absorption prompted this study of the effects of combining reserpine and this acid prior to oral administration. Evidence of an interaction between reserpine and desoxycholic acid was reported by Lach and Pauli (1) after the completion of the work reported here. The interaction was demonstrated by an increase in the solubility of reserpine in hydroalcoholic solutions of desoxycholic acid. Simple interfacial effects of desoxycholic acid were not thought to be the sole contributing factor for this increased solubility, and it was suggested that the interaction might result from a combination of micellar solubilization and inclusion formation.

EXPERIMENTAL

Preparation of Reserpine–Desoxycholic Acid Combinations.—The usual method for the formation of desoxycholic acid inclusion compounds (dissolving both desoxycholic acid and the guest component in ethanol and allowing the inclusion compound to crystallize) was not used in this instance due to the poor solubility of reserpine in anhydrous alcohol.

Method A.—The calculated quantity of purified desoxycholic acid (Nutritional Biochemicals Corp., lot 2185) was dissolved in 5 ml. of commercial grade absolute ethanol in a 20-ml. round-bottom flask. The desired amount of reserpine (C grade, Calbiochem, lot 502858) was added and dissolved with slight warming. The flask and its contents were chilled in an ice–salt bath to −5° and the solvent removed under reduced pressure and trapped in a cold finger cooled with a dry ice–acetone bath. The contents of the flask were protected from light as completely as possible. The residues were dried for 12 hr. in a vacuum to remove the last trace of alcohol.

Method B.—This method was essentially the same as above except that 5 ml. of chloroform was used to dissolve the reserpine, and this solution was added to the desoxycholic acid dissolved in 10 ml. of absolute alcohol. The resultant solution was then treated as described under *Method A*.

Method C.—A physical mixture of desoxycholic acid and reserpine was prepared by intimately triturating the two dry powders without previous dissolution in any solvent. Table I summarizes the physical properties of all the test mixtures.

Blepharoptotic Assay.—White mice were obtained from E. G. Steinhilber, Oshkosh, Wis., and maintained in this laboratory on Purina laboratory chow and water *ad libitum* for at least 4 days prior to test. All animals were taken off food 10 hr. prior to dosing and placed back on food after recording the +6 hr. observations. Free access to water was allowed throughout the entire test period. The mice were dosed orally using precision grade syringes and cut-off, blunted, polished 20-gauge hypodermic needles. A constant dosage volume of 30 ml./Kg. was maintained for all injections using 0.25% aqueous agar as the dosing vehicle. Test drugs and combinations were suspended by trituration. All test solution suspensions were coded and the animals dosed in a random test pattern using a 3 × 3 assay format with further randomization between the two sexes of mice and the two dosing technicians. Prior to ptotic scoring, each mouse was manually aroused and placed on a screen facing the scorer at the scorer's eye level. Ptosis for each eye was rated following the scale used by Rubin *et al.* (2): 4 = complete, 3 = 3/4, 2 = 1/2, 1 = 1/4 closure of the eyelids. Nonptotic responses were scored as 0. All scores of 4 were checked to insure that the lid was not encrusted shut by eye secretions. The individual metameter was the sum of the ptotic scores for both eyes so that the graded test response could vary from 0–8 per animal.

RESULTS AND DISCUSSION

As shown in Table II, eight balanced log dose-response blepharoptotic assays were conducted assaying reserpine (in combination with desoxycholic acid) against reserpine base as a standard. For the assay calculations the reserpine content of

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TABLE I.—PREPARATION OF RESERPINE–DESOXYCHOLIC ACID AND RESERPINE PLUS DESOXYCHOLIC ACID TEST MATERIALS

Identification	Exact molar Ratios	Prepn., Method	Quantities, mg. Reserpine	Quantities, mg. Acid	Melting Range, ° C.
1:2	1:2.06	*B*	59.0	80.0	80–110
1:4	1:4.02	*B*	62.0	161.5	98–125
1:8	1:7.44	*A*	16.2	77.9	114–125
1:16	1:16.31	*A*	29.7	312.6	114–145
1:32	1:31.89	*A*	15.2	311.7	145–148
1 + 16	1 + 17.16	*C*	28.6	316.4	...

TABLE II.—APPARENT ORAL POTENCIES OF RESERPINE IN VARIOUS DESOXYCHOLIC ACID COMBINATIONS AS COMPARED TO RESERPINE BASE ALONE

Identification	hr. After Dosage 2	4	6	10	24
0:0	...	...	...	...	...
1:0	0.781 (0.536–1.138)[a]	1.000 (0.836–1.196)	0.957 (0.793–1.155)	0.928 (0.741–1.162)	1.078 (0.776–1.496)
1:2	2.293 (1.460–3.602)	1.924 (1.482–2.499)	1.658 (1.326–2.073)	1.587 (1.282–1.966)	2.144 (1.672–2.747)
1:4	2.642 (2.182–3.199)	2.542 (1.885–3.428)	2.428 (1.959–3.010)	2.2811 (1.859–2.799)	2.692 (2.135–3.394)
1:8	2.828 (1.858–4.305)	2.349 (1.607–3.433)	2.732 (1.739–4.291)	1.948 (1.393–2.724)	2.056 (1.084–3.901)
1:16	3.779 (2.870–4.974)	3.201 (2.221–4.615)	3.262 (2.645–4.022)	2.594 (2.072–3.247)	2.487 (1.767–3.499)
1:32	3.352 (2.403–4.674)	2.650 (2.075–3.384)	2.471 (1.930–3.165)	2.650 (2.074–3.386)	1.966[b] (1.143–3.382)
1 + 16	1.382 (0.990–1.928)	1.882 (1.393–2.542)	1.876 (1.355–2.596)	1.719 (1.157–2.553)	...[c]

[a] Range of figures within parentheses indicate the calculated 95% confidence limits for the potency. [b] Significant departure from parallelism (P = 0.01–0.05) in 3 × 3 assay; best graphical estimate of potency = 2.49 × reserpine base. [c] Recovery was sufficient so that neither statistical nor graphical estimates of potency could be determined with validity.

the combinations was handled using the "exact" ratios shown in Table I, although the approximate molar ratios are used in this text to identify the various combinations. Doses of 3, 6, and 12 mg./Kg. were used for the reserpine standard (C grade, Calbiochem, lot 502858) and for the reserpine of the 1:2, 1:4, and 1:8 reserpine–desoxycholic acid combinations. Doses of 1.5, 3, and 6 mg./Kg. and 0.75, 1.5, and 3 mg./Kg. were used for the reserpine of the 1:16 and 1:32 combinations, respectively. Where enhancement of the reserpine-like activity did not allow 3 × 3 calculations, a 2 × 2 assay was calculated matching two dosages of the standard with two doses of the combination in the same linear portion of the dose-response curve. Significant departure from parallelism was noted in only one calculation. The statistical treatment for the 3 × 3 (120 mice, 20 animals/dosage level) and 2 × 2 (80 mice) assays involved analysis of variance, factorial analysis, and calculation of potency and its 95% confidence limits using the techniques of Bliss and Calhoun (3). An average λ value (s/b) of 0.27 was obtained for the 32 valid assay calculations reported in Table II, which value agrees well with a λ of 0.29 reported earlier for this assay technique (4) using a different mouse stock, reserpine acetate as the standard, and water as the dosage vehicle.

As shown in Fig. 1, the 1:2 to 1:16 reserpine–desoxycholic acid combinations show a progressive increase in the apparent potency of reserpine with the 1:16 reserpine–desoxycholic acid combination

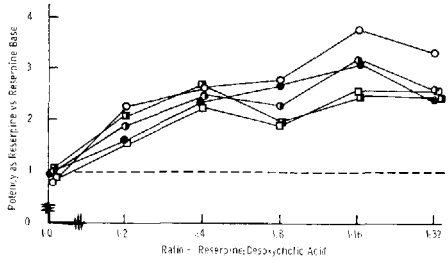

Fig. 1.—Variation of ptotic potency of reserpine when administered orally in the form of various molar ratio coprecipitates with desoxycholic acid. Key: ○, +2 hr.; ◑, +4 hr.; ●, +6 hr.; □, +10 hr.; ◧, +24 hr.

producing the maximum enhancement of activity. The decrease in activity shown by the 1:32 combination would appear to indicate that the maximum attainable activity (1:16) has been physically diluted by excess desoxycholic acid.

As illustrated in Fig. 2, the potency of 1:0 reserpine–desoxycholic acid does not significantly deviate from the theoretical value of 1.0, while maximum distortion of the apparent potency of the 1:2–1:32 combinations was apparent at +2 hr. after oral administration. This appears to indicate that desoxycholic acid increases the speed of absorp-

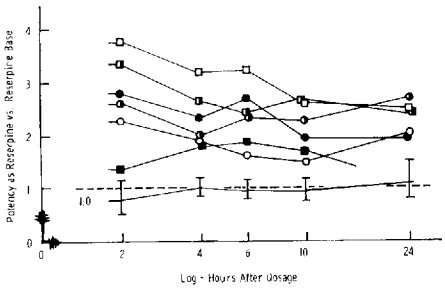

Fig. 2.—Variation with time of the oral potencies of various reserpine–desoxycholic acid molar coprecipitates as compared to reserpine base as a standard. Key: □, 1:16; ◨, 1:32; ●, 1:8; ◑, 1:4; ○, 1:2; ■, 1 + 16; I, 1:0.

tion of reserpine as well as increasing the potency. Considering the 95% confidence limits of potency reported in Table II, reserpine administered as the 1:16 combination behaves equivalent to at least 1.8 mg. of reserpine base (+24 hr.) and possibly equivalent to as much as 5.0 mg. of reserpine (+2 hr.).

A molar equivalent physical mixture of reserpine and desoxycholic acid (1 + 16) produced a slower onset of the period of maximum enhancement (+4–6 hr.), and the potencies were significantly less ($P \leq 0.001$) than those reported for the 1:16 intimate combination. In all cases where calculation was possible, the 1 + 16 mixture was significantly more potent than reserpine base alone (observed P: 0.025–0.05 at +2 hr. and ≤ 0.001 at +4, 6, and 10 hr.).

While there is debate as to whether palpebral ptosis is a peripheral or central manifestation of reserpine-like activity (4–6), this characteristic symptom does indicate absorption of reserpine from the gastrointestinal tract. Coprecipitates of reserpine and desoxycholic acid both increase the potency of reserpine and produce a more rapid onset of reserpine-like activity when administered orally. No attempt has been made here to define the exact physical/chemical nature of the reserpine–desoxycholic acid combination.

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Utilization of Hydrophilic Gums for the Control of Drug Release from Tablet Formulations I. Disintegration and Dissolution Behavior

By H. E. HUBER, L. B. DALE*, and G. L. CHRISTENSON

Tablet formulations and data to illustrate rate of drug dissolution and tablet volume decay during *in vitro* disintegration tests are presented. It was found that tablets prepared by compression of hydrophilic gums, excipients, and drug in specified ratios result in prolonged release of drug. Assay of simulated gastric and intestinal fluids from *in vitro* tests show the drug to be released at essentially a uniform rate after an initial hydration phase. The mechanism of prolonged release is proposed as a combination of drug diffusion from, and attrition of, a dynamically changing gel barrier at the tablet periphery.

ORAL CONTROLLED release dosage forms have been recognized as a therapeutically significant advance in dosage form design, whereby a more uniform and prolonged tissue concentration of drug substance may be achieved. The methods used for obtaining prolonged action have been reviewed by Ballard and Nelson (1) and Parrott (2) and various systems are described, whereby an initial therapeutic dose is released followed by a continual release of additional drug substance over a prolonged period of time.

In 1962, a system was developed by The Wm. S. Merrell Co. (3) describing a novel approach for the control of drug substance release rate from tablet formulations. The method described involves mixing a medicinal agent or agents with certain nondigestible, hydrophilic gums and compressing the mixture into tablets. When such a tablet is exposed to water or digestive fluids, a rapid release of drug substance from the dosage form to the dissolution medium is initially observed. However, hydration and gelation of gum at the tablet–liquid interface also occurs to form a viscous gel barrier. The remaining drug substance is then released at a much slower rate that apparently depends on diffusion from and/or attrition of the gel barrier. The nature of the phenomenon is illustrated by Fig. 1, which shows, in cross section, the appearance of such a tablet after exposure to solvent. It can be seen that the intact tablet core is surrounded by a gel barrier layer of significant size.

The present communication is concerned with studies that were conducted to obtain preliminary

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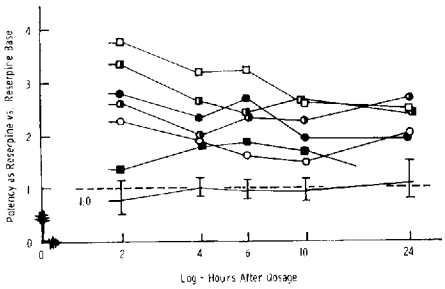

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Fig. 1.—A bisected quinidine sulfate tablet after 1 hr. in simulated gastric fluid.

TABLE I.—INFLUENCE OF GUM CONTENT ON DISINTEGRATION TIME

Drug/gum ratio	10/1	2/1	1/1	1/4	1/16
Disintegration time, min.	105	250	450	570	650

information on the dissolution and disintegration behavior of such tablets and the influence of formulation variables on their behavior.

EXPERIMENTAL

Influence of Gum Content on Disintegration Behavior.—Six batches of tablets containing nicotinic acid and hydroxypropylmethylcellulose 4000 cps. in the ratio of 10:1, 2:1, 1:1, 1:4, and 1:16 were prepared as follows. The powders were mixed and 1% zinc stearate added as lubricant. The mixes were compressed on a $^3/_8$-in. standard cup punch to yield tablets weighing approximately 250 mg. each. Six tablets from each batch were placed in the basket of a U.S.P. modified Stoll-Gershberg apparatus. The basket was lowered into an 800-ml. beaker containing 600 ml. of simulated gastric fluid maintained at 37° ± 0.5° and the apparatus operated in the manner prescribed by the U.S.P. At the end of 1 hr. the basket was transferred to an 800-ml. beaker containing 600 ml. of simulated intestinal fluid, and the operation was continued until disintegration was complete. The time required for complete disintegration was noted.

In Vitro Drug Release Characteristics.—The *in vitro* release characteristics of two formulations were investigated.

Formula *1*	Per Tablet, mg.
Doxylamine succinate	20
Pheniramine maleate	20
Pyrilamine maleate	20
Sodium carboxymethylcellulose	640
Magnesium trisilicate	40
Stearic acid	10

The sodium carboxymethylcellulose was slugged, granulated, and mixed with the remaining powders. Magnesium trisilicate was added to act as an absorbant for the eutectic formed by the antihistamines. The mixture was compressed into $^7/_{16}$-in. tablets at 750 mg. each and a Stokes (Monsanto) hardness of approximately 16 Kg. These antihistamine tablets[1] were placed in the Stoll-Gershberg apparatus and treated as described, except that simulated intestinal fluids were changed at the end of each 2-hr. period. At various time intervals, aliquots of fluid were withdrawn, treated with sodium hydroxide solution, and extracted with 3 vol. of ether. The combined ether extracts were re-extracted with 0.1 *N* HCl and assayed spectrophotometrically for pyrilamine content. Tablets from two different batches were subjected to this study. Three determinations were made on each batch.

[1] Marketed as Tridecamine tablets, by National Drug Co., Division of Richardson-Merrell, Inc., Philadelphia, Pa.

Formula *2*	Per Tablet, mg.
Quinidine sulfate	300
Hydroxypropylmethylcellulose	150
Magnesium stearate	9

The powders were granulated with water and the granulation was ground to a coarse powder. Lubricant was added and the mix was compressed on $^7/_{16}$-in. punches to yield tablets weighing approximately 459 mg. each and a Pfizer hardness of approximately 12 Kg.

Five of these tablets were individually subjected to the release test described for antihistamine tablets. Here, however, the baskets were transferred to fresh solution at the end of each hour. An aliquot of each solution was pipeted into a volumetric flask and made to volume with 0.1 *N* HCl. The solutions were filtered and assayed spectrophotometrically for quinidine sulfate content.

Volume Decay of Tablets.—In order to demonstrate the change in volume during disintegration, quinidine sulfate tablets (disk shaped) were measured, prior to disintegration, with an Ames micrometer gauge. They were disintegrated as previously described for 1, 2, 3, 4, and 5-hr., respectively, removed from the fluids, allowed to dry, and again measured. Tablet volumes were calculated from the formula $\pi r^2 h$. A displacement method for volume determination was also done to verify the micrometer procedure. Volumes were in close agreement with the micrometer procedure.

RESULTS

Influence of Gum Content on Disintegration Behavior.—Tablets made with hydroxypropylmethylcellulose and nicotinic acid had disintegration times proportional to the per cent gum. As the gum content was increased, the disintegration time was extended. The results are given in Table I.

In Vitro Drug Release Characteristics.—Drug substance release patterns from the antihistamine tablets and quinidine sulfate tablets are shown in Figs. 2 and 3. It can be seen that in both cases, an initial rapid release of drug substance occurred. For example, approximately 26% of the pyrilamine content was released from the antihistamine formulation in 1 hr., while approximately 32% of the quinidine sulfate was released in the same time period. After the initial rapid release phase, a much slower constant rate of release occurred until about the 7th hour. It was observed at this time that the tablets were completely hydrated and proceeded to dissolve.

Volume Decay of Tablets.—Tablet volume decay data, obtained for quinidine sulfate tablets, are shown in Fig. 4. An initial rapid decay is noted during the initial period of tablet hydration in gastric fluid followed by an interim change in rate of decay when transferred to intestinal fluid. Linear decay of the dry tablet core takes place

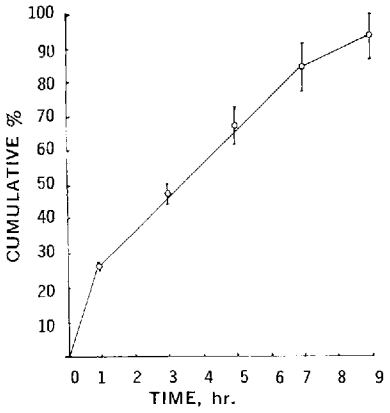

Fig. 2.—*In vitro* dissolution rate for antihistamine tablets. Average and standard deviation of cumulative per cent drug released for six baskets of six tablets.

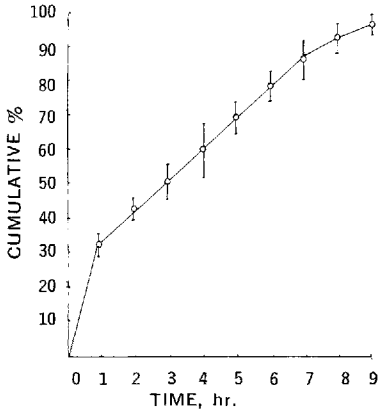

Fig. 3.—*In vitro* dissolution rate for quinidine sulfate tablets. Average and standard deviation of cumulative per cent drug released for five individual tablets.

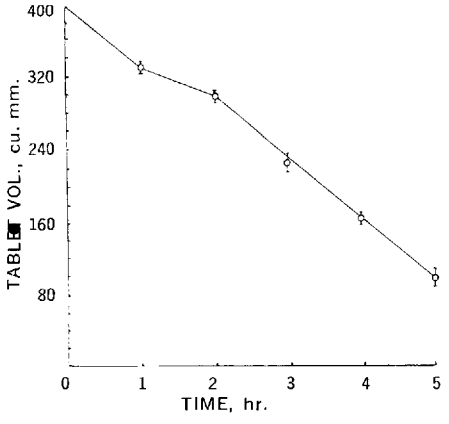

Fig. 4.—*In vitro* volume decay for quinidine sulfate tablets. Average and standard deviations for each of five baskets of six tablets run 1, 2, 3, 4, and 5 hr., respectively.

over the remaining time period until hydration is complete at approximately 7 hr. The 5-hr. sample was the last point at which a large enough dry core was available for accurate measurement.

DISCUSSION

The studies which have been described do not permit a complete elucidation of the exact mechanism involved in this approach to control of drug substance release from tablet formulations. However, the data do support the theory that hydration and gelation of the gum at the tablet–liquid interface constitute an important step in the mechanism. The existence of a gel barrier would be expected to retard drug substance release by limiting the exposure of solid drug to the dissolution liquid. Attrition of and/or diffusion from the gel barrier with concomitant formation of fresh gel could explain the constancy of release rate observed over a considerable time period.

As illustrated by the experiments with the nicotinic acid formulations, the percentage of gum in the formulation has a marked influence on the disintegration and dissolution behavior of the tablet. It should be recognized, however, that this behavior will differ, in quantitative terms, with different drugs and different gums. It has been the author's experience that the most useful gums are those which hydrate readily and rapidly at body temperature. Two examples are sodium carboxymethylcellulose and hydroxypropylmethylcellulose. The antihistamine and quinidine formulations illustrate the type of behavior observed when these gums are used. The dissolution rate patterns here are especially interesting in that a constant rate of release occurred over a considerable time period. This is in contrast to many other systems designed for controlled release where it has been found that release rate decreases as the drug substance content in the dosage form reservoir decreases.

Although no direct correlations between *in vitro* and *in vivo* behaviors of specific formulations have been attempted, it is interesting to note that Halpin (4) in his clinical investigation of the antihistamine formulation reported that the clinical response extended over a 12-hr. period. He also reported a rapid onset of activity. His observations were confirmed by Hansel (5), who reported an antihistaminic effect lasting for 12 hr. Jones *et al.* (6) using an intradermal wheal test, found that the preparation suppressed wheal size for a 12- to 14-hr. period.

The approach that has been described appears to offer attractive possibilities for sustaining the release of drug substances administered orally. The availability of many different gums, the utilization of other excipients, and the application of techniques such as multiple compression of tablets offer the possibility of varying qualitative and quantitative aspects of release rate patterns over a wide range.

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Effect of Glyceryl Trinitrate on Extracellular Fluid Space of Aortic Strips

By O. J. LORENZETTI*, ARTHUR TYE, and JOHN W. NELSON

Extracellular fluid space (ECF) was determined in untreated rabbit aortic tissue and in tissue exposed to 0.1 per cent glyceryl trinitrate. The ECF space determinations were made in tissues bathed in a normal Krebs solution, calcium-free Krebs solution, magnesium-free Krebs solution, and calcium–magnesium-free Krebs solutions. The glyceryl trinitrate increased the ECF space in all solutions except the magnesium-free solution.

THE INCREASING importance of the concentration and distribution of ions in tissue during the state of contraction and relaxation necessitates the determination of distribution of tissue water (1, 2). Of importance in studying some effects of glyceryl trinitrate on smooth muscle relaxation was the determination of the extracellular fluid space (ECF).

The direct methods used to determine the distribution of water between extracellular and intracellular compartments are based on the measurement of the volume of distribution of an impermeable solute molecule, *e.g.*, urea, mannitol, inulin, and radio-iodinated serum albumin. Although these substances penetrate the tissue at various rates dependent on their molecular size, the use of any one substance will give an estimate of the relative distribution of the water in the extracellular fluid space.

For the authors' measurements the ECF space was determined by use of inulin, using the anthrone color reaction for spectrophotometric analysis of the inulin concentration (3). The anthrone color reaction is highly sensitive for determinations of small quantities of inulin, as low as 5 mcg./ml. of solution.

The purpose of this paper is to provide data on the measurement of the ECF space of the rabbit aorta exposed to various ionic media, before and after treatment with glyceryl trinitrate.

MATERIALS AND METHODS

Male albino rabbits weighing 2–3 Kg. were killed by a blow to the back of the neck. The thorax and abdomen were opened, and the descending aorta from the arch extending down 9 cm. was isolated from surrounding tissue. The thoracic aorta was mounted directly onto a glass rod and kept moist with Krebs–bicarbonate solution at room temperature.

The aortic strips were cut by rotating the rod against a fixed scalpel blade according to the method described by Furchgott (4). All of the aortic strips conformed to the following dimensions: 2.0 ± 0.55 mm. wide, 2.0 ± 0.3 cm. long, and 450 ±50 μ thick as noted under a microscope. Throughout this procedure the tissue was kept moist with Krebs–bicarbonate solution and gassed with 95% oxygen and 5% carbon dioxide.

The isolated aortic tissue was placed individually into 12-ml. weighing bottles containing 5 ml. of 1.0% w/v inulin in modified Krebs solution. Inulin was substituted for glucose in all Krebs solutions. Three solutions with various concentrations of electrolytes were employed in addition to the normal Krebs solution. The composition of these modified solutions is shown in Table I. The osmotic concentration of the solutions was between 314.5 and 317.5 milliosmoles. The ionic strength ranged from 0.168 to 0.174. The pH was adjusted to 7.36 ± 0.08. Some aortic muscle strips were exposed to 0.1% glyceryl trinitrate and were called treated strips.

TABLE I.—COMPOSITION OF BATH MEDIA FOR AORTIC STRIPS IN mmoles/L.[a]

	Krebs-HCO_3	Calcium-Free	Magnesium-Free	Calcium and Magnesium-Free
NaCl	118.5	120.0	120.0	128.0
KCl	4.8	4.8	4.8	4.8
$CaCl_2 \cdot 2H_2O$	1.9	...	1.9	...
KH_2PO_4	1.2	1.2	1.2	1.2
NaH_2PO_4	...	...	1.2	...
$MgSO_4 \cdot 7H_2O$	1.2	1.2	...	...
$NaHCO_3$	25.0	25.0	25.0	25.0
Dextrose	10.0	10.0	10.0	10.0

[a] Ionic strength, 0.168 to 0.174; osmolarity, 314.5 to 317.5; pH, 7.28 to 7.44.

Both treated and untreated aortic strips were allowed to equilibrate for 12 hr. The tissue was then removed, blotted, and placed in 5 ml. of distilled water contained in another weighing bottle. At the end of a 12-hr. period in the distilled water, the tissue was removed from its container, was blotted, and was weighed. The tissue was then carefully homogenized in 2 ml. of distilled water using a 10-ml. hand homogenizer. The homogenate was centrifuged at 6000 r.p.m.'s for 5 min., and the supernatant was decanted and analyzed for inulin as described below.

Inulin Assay.—Two samples of the distilled water–inulin solution were removed, placed in 22 × 175-mm. Pyrex test tubes, and cooled in a cold water bath to 8 ± 1° for 10 min. Then 2 ml. of freshly prepared anthrone reagent was added directly to the solution in the test tube and mixed by swirling. The anthrone reagent consisted of 0.02% w/v anthrone and 0.1% w/v thiourea (to prevent oxidation of the active enol tautomer of anthrone) in 96% sulfuric acid. The anthrone reagent was precooled to 8 ± 1°. Then the samples were immediately heated for 6 min. at 90 ± 3° on a water bath. The tubes were cooled for 5 min. in an ice bath to 8° and were allowed to come to room temperature. The resulting turquoise-green solutions were read on a Beckman spectrophotometer at 620 mμ within 20 min.

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of their removal from the water bath, against a distilled water–tissue blank. The blanks gave a reading of 0.97 ± 0.04% ECF per wet weight of tissue, as determined on 20 tissue blanks taken at random. The inulin concentration was read from a standard curve, constructed from determinations on inulin in distilled water. The inulin space is equivalent to the extracellular fluid space and was expressed as the mg. of inulin per 100 mg. of aortic tissue, wet weight, divided by the mg. $ml.^{-1}$ of inulin in the

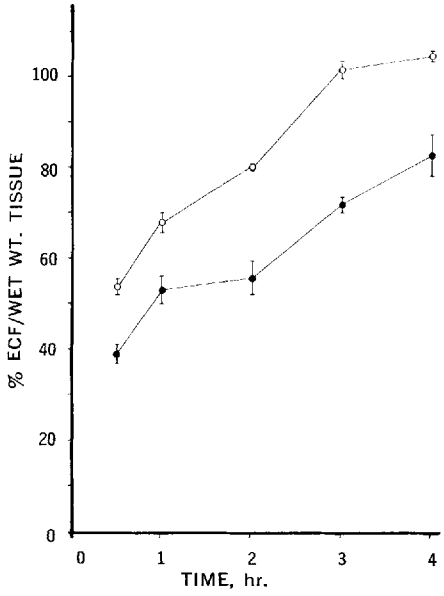

Fig. 1.—Extracellular fluid space of aortic strips in Krebs solution. Key: ●, untreated; ○, glyceryl trinitrate (1 mg.) added; I, standard deviation.

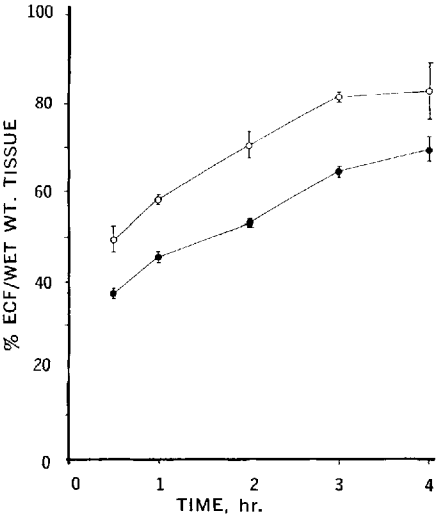

Fig. 2.—Extracellular fluid space of aortic strips in calcium-free Krebs solution. Key: ●, untreated; ○, glyceryl trinitrate (1 mg.) added; I, standard deviation.

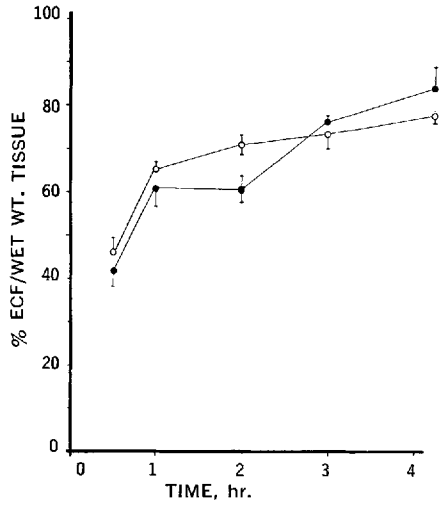

Fig. 3.—Extracellular fluid space of aortic strips in magnesium-free Krebs solution. Key: ●, untreated; ○, glyceryl trinitrate (1 mg.) added; I, standard deviation.

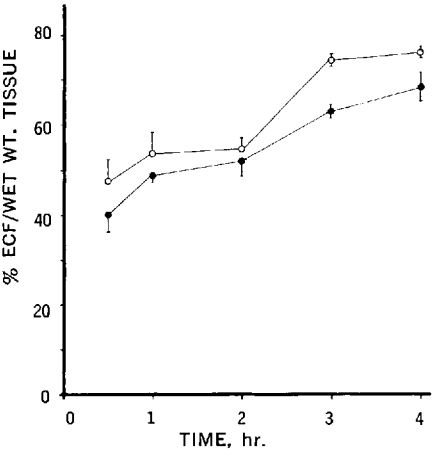

Fig. 4.—Extracellular fluid space of aortic strips in calcium and magnesium-free Krebs solution. Key: ●, untreated; ○, glyceryl trinitrate (1 mg.) added; I, standard deviation.

ECF times 100 to give per cent ECF per wet weight of tissue (5).

In the tissues exposed to 0.1% glyceryl trinitrate, it was found that the nitrates interfered with the assay. The solutions obtained from tissue treated with glyceryl trinitrate were passed through an anion exchange resin,[1] contained in a 3-cm. (i.d.) × 30-cm. column. The resulting glyceryl trinitrate-free solution was then assayed for inulin as described.

RESULTS

The rate of permeability indicated by a plot of the per cent of extracellular fluid space per 100 mg. tissue wet weight *versus* time (Figs. 1–4) shows that

[1] Marketed as Amberlite IR-45 by Mallinckrodt, St. Louis, Mo.

saturation of the inulin spaces occurs in approximately 4 hr.

The results for the normal Krebs solution in Fig. 1 show that the presence of glyceryl trinitrate increases the permeability of the cell membrane in the tissue as represented by the increase in ECF. The increase was significant from the first to the fourth hour. The values obtained were high relative to those reported in the literature by the use of other methods. It was not intended that the measurement would be an exact value of the extracellular fluid space since the incomplete penetration of inulin into the connective tissue has not been considered. Nichols and co-workers have shown that the ECF space measurements obtained with chloride are better estimations, since chloride penetrates much more rapidly than inulin (6).

The muscle exposed to glyceryl trinitrate in a calcium-free medium also shows a significant increase in the extracellular fluid (Fig. 2). The results indicate that absence of calcium does not affect the increased ECF space induced by the presence of glyceryl trinitrate.

The magnesium-free Krebs media produced an inhibition of the ability of glyceryl trinitrate to increase the ECF space of the smooth muscle over most of the 4-hr. period (Fig. 3). The Krebs media without calcium or magnesium showed no change in the ECF space for the first 2 hr., then a significant increase in ECF in the last 2 hr. as seen in the unmodified Krebs medium (Fig. 4).

The approximately $\pm 5\%$ of the calculated mean in the ECF space determination reflects some variability among the multiple samples. That may be attributed to an actual section-to-section variation within the aorta, with respect to the inulin space or endogenous carbohydrates.

DISCUSSION

Although the velocity of inulin distribution is not affected by incubation of the muscle with glyceryl trinitrate, the apparent volume of distribution of inulin is increased. This indicates an increased permeability of the tissue in the presence of nitrate and nitrite. The increased permeability was not observed in the absence of magnesium with calcium present.

In all the bathing solutions, an early rapid phase and a late slow phase was noted before equilibrium was attained. This could be attributed to different water compartments, but recent studies have indicated that there are more than the two classic water compartments (7) indicated in this study. Possibly, the phases noted could be characteristic of the tissue investigated, indicative of latent penetration of inulin into more complex extracellular structures as suggested by Page (8) for heart muscle.

Saturation of the inulin space occurred in 3 to 4 hr. in these studies. Even after this prolonged time, nitrates and nitrites can still exert an effect on the cell membrane (9).

SUMMARY

Glyceryl trinitrate increases the extracellular fluid space of rabbit aortic tissue in normal Krebs and calcium–magnesium-free Krebs solutions. Only the magnesium-free Krebs solution failed to produce an elevation of the extracellular space of aortic tissue after treatment with glyceryl trinitrate.

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Interactions of Surfactants with Lipoproteins

By H. TAKRURI and B. ECANOW

The interactions of benzalkonium chloride and sodium lauryl sulfate with α- and β-serum lipoproteins have been studied. Both fractions, below their isoelectric points, form insoluble complexes with the anionic surfactant. At higher concentrations of the surfactant the insoluble complexes are resolubilized completely. Above the isoelectric point, the lipoprotein fractions exhibit the same phenomenon with benzalkonium chloride. The charge and the hydrophilic nature of the macromolecules are the major factors in these interactions. The formation of insoluble complexes and the resolubilization of these complexes are modified considerably by the addition of urea to the systems.

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Abstracted from a thesis submitted by Harun Takruri to the Graduate Faculty, University of Illinois at the Medical Center, Chicago, in partial fulfillment of Master of Science degree requirements.

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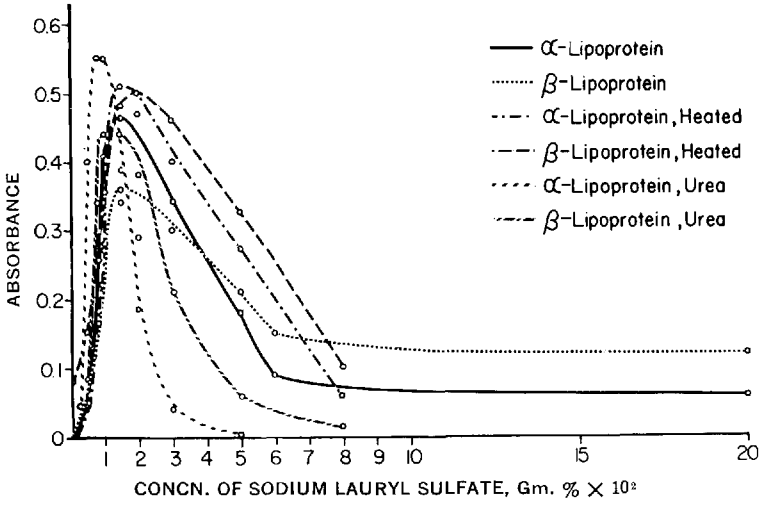

Fig. 1.—Turbidity of various lipoprotein systems as a function of the concentration of sodium lauryl sulfate.

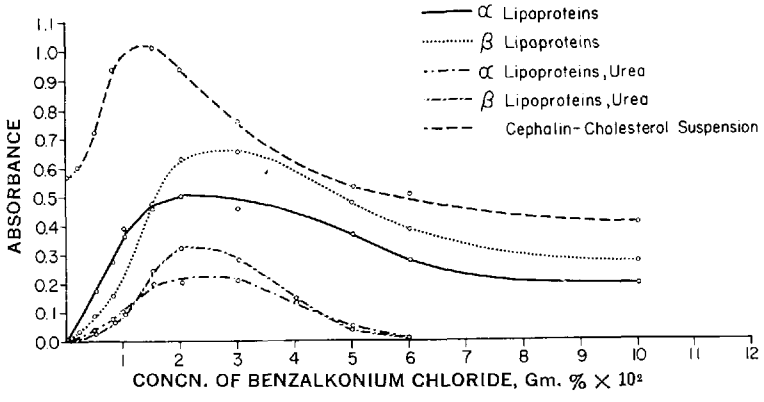

Fig. 2.—Turbidity of various lipoprotein systems as a function of the concentration of benzalkonium chloride.

The lipoproteins have been studied recently in connection with their possible role in the pathogenesis of atherosclerosis and other diseases. Certain subfractions of β lipoprotein were found to be definitely linked to plaque formation (4, 5). The effects of nonionic surfactants on lipoproteins were studied *in vivo* and *in vitro* (6–8). The effects of ionic surfactants on the electrophoretic mobility of these fractions were also studied (1). β Lipoproteins have the property of forming several complexes, soluble and insoluble, with heparin and other polysaccharide sulfates. These interactions depend on several factors, including pH, ionic strength, and metal ions. These interactions are utilized in the separation and characterization of β lipoproteins. Cornwell and Kruger (3) reviewed these interactions in detail.

This paper is a report on a study of the interactions of an anionic and a cationic surfactant with α and β lipoproteins carrying charges opposite to that of the surfactant. The influence of urea on these interactions was also studied.

EXPERIMENTAL

Reagents

Human α lipoprotein, fraction IV-1 (Nutritional Biochemical Corp.), human β lipoprotein, fraction III-0 (Nutritional Biochemical Corp.), bovine α lipoprotein fraction IV-1 (Pentex), cephalin-cholesterol reagent (Wilson), sodium lauryl sulfate (Fisher), benzalkonium chloride solution, 17% (Winthrop Laboratories).

Procedure

Interaction with Sodium Lauryl Sulfate.—A stock solution of 1% sodium lauryl sulfate in distilled water was prepared. Dilutions were made of this stock solution by adding a specified volume to 200-ml. portions of 0.04 *M* sodium chloride solution. One milliliter of 0.1 *N* hydrochloric acid was added to each solution to give a uniform pH of all the solutions. The solutions of the lipoproteins were prepared by dissolving 250 mg. of human β lipoprotein or bovine α lipoprotein in 50 ml. of 0.04 *M* saline. These solutions were dialyzed for 4 hr. at room temperature against a large volume of 0.04 *M* saline. After dialysis, 1.5 ml. of 0.1 *N* HCl was added to each solution and each was made up to 60 ml.

Three milliliters of the lipoprotein solution were added to 22 ml. of the various dilute solutions of sodium lauryl sulfate. This gives a concentration of 50 mg. % of the lipoprotein. This concentration was found to be the optimum concentration for

the determination of turbidity. After the surfactant–lipoprotein solutions were vigorously shaken, they were left to stand for 30–60 min. before the measurements were performed. The interaction was instantaneous and the only observation that required time was the sedimentation of the floccules. The turbidity was measured in a Coleman junior spectrophotometer. The measurements were performed at 450 mμ. The choice of this wavelength was arbitrary. In the study of urea-containing systems, the lipoprotein solutions were subjected to the action of 6 *M* urea for 1 hr. before they were added to the surfactant solutions.

Interactions with Benzalkonium Chloride.—Human lipoprotein fractions, 125 mg. of each, were dissolved in 50 ml. of 0.04 *M* saline, dialyzed for 4 hr. at room temperature against 0.04 *M* saline, and then diluted to 250 ml. with the same solvent. The pH was adjusted to 7.5 ∓ 0.2 with 0.1 *N* NaOH. Twenty milliliters was measured out in 1-oz. bottles and specified volumes of benzalkonium chloride stock solutions (0.1, 1, and 10%) were added to the individual bottles to give the desired concentration of the surfactant. In studying the effect of urea, the lipoprotein solutions were subjected to the action of 3 *M* urea for 36 hr. before adding the surfactant.

The interaction of cephalin–cholesterol reagent and benzalkonium chloride was also studied. This reagent is employed as a liver-function test. The mechanism of the test was reviewed by Bauer (2).

RESULTS

Figure 1 shows the turbidity of the various lipoprotein–sodium lauryl sulfate systems as a function of the concentration of the surfactant. The curves show that α lipoprotein complexes are solubilized at a lower concentration than β lipoprotein complexes. Also they show the negligible effect of heating the lipoproteins solutions for 4 hr. at 60° and the significant effect produced by urea.

Figure 2 shows the turbidity of various lipoprotein–benzalkonium chloride systems and the system benzalkonium chloride–cephalin–cholesterol. The turbidity curves resemble in shape those obtained with sodium lauryl sulfate.

DISCUSSION AND SUMMARY

The plasma concentration of most lipids, including cholesterol, glycerides, and phospholipids, has been studied extensively in relation to many diseases and pathological conditions. But the fact remains that practically all these lipids are found in blood as lipoproteins. The physical-chemical properties of these colloids are very important in determining their behavior and interactions in the body regardless of the total concentration of a particular component, *e.g.*, cholesterol or phospholipids. α and β lipoproteins differ considerably in their colloidal properties. The main difference could be expressed in terms of their hydrophilic nature.

The results of this study show that negatively charged lipoproteins, both α and β, are precipitated and then resolubilized by cationic surfactants. Positively charged lipoproteins are, on the other hand, precipitated and resolubilized by anionic surfactants. The interaction does not require di- or polyvalent metal ions and the insoluble complexes are not dissociated at very high ionic strength (up to 20% NaCl). These results are in contrast to the interactions of polysaccharide sulfates with lipoproteins. The results show that the more hydrophilic α fraction is solubilized at a lower concentration than the β fraction. Heating the lipoproteins causes a slight modification of these interactions. Urea produces a major change in these interactions. The concentration at which no observable turbidity is attained is considerably lowered in the presence of urea. The effect is related to the action of urea on the lipoprotein structure, the water structure, and the critical micelle concentration of the surfactant. The pH profiles of the lipoprotein–surfactant systems as a function of the concentration of the surfactant throw a light on the mechanism of these interactions. This aspect will be the subject of a future communication.

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Chromatographic Study of the Alkaloids of *Aquilegia formosa*

By G. H. CONSTANTINE, JR., M. R. VITEK, K. SHETH, P. CATALFOMO, and L. A. SCIUCHETTI

Chromatographic investigation of alkaloid fraction from the roots of *Aquilegia formosa* Fisch. (*Ranunculaceae*) revealed the presence of five alkaloidal spots. Two of them were identified as magnoflorine and berberine. A thin-layer chromatographic procedure was developed which effectively separated berberine, palmatine, and jatrorrhizine.

A PHYTOCHEMICAL investigation of plants considered to be of medicinal value by certain Indian tribes of the Northwest, particularly those of the Warm Springs Reservation, Ore., revealed the presence of alkaloids in the roots of *Aquilegia formosa* Fisch.

An ethnobotanical survey conducted by French (1) noted that numerous species of *Aquilegia* have been widely used by the Indians of North America for a host of medicinal purposes. The reputed medicinal uses include diuretic and analgesic activity, treatment of diarrhea or smallpox, a stimulating tea, etc. Species of *Aquilegia* were employed not only as medicines but also as perfumes (2), and were used in certain magical rites. Hitchcock *et al.* (3) indicate that there are approximately 70 species of *Aquilegia* native to the north temperate zones. They are commonly referred to as "columbines," and many are available as horticultural varieties.

A comparative paper chromatographic screen of 10 horticultural varieties of *Aquilegia* was conducted by Winek *et al.* (4). Their screen did not include *A. formosa*. From the entire group, three alkaloids were established—magnoflorine, berberine, and aquileginine. Chromatography also revealed other alkaloids, but their identities were not established. The presence of palmatine remained equivocal because the solvent systems used did not separate this alkaloid from berberine. An investigation of *A. hybrida* by this same group (5) revealed eight alkaloids in this species. Three of the isolated alkaloids were identified as berberine, magnoflorine, and aquileginine. The others remain unknown.

Since preliminary studies indicated alkaloids in *A. formosa*, and readily available quantities could be collected in the wild, the present study was undertaken. The objectives of this investigation were to identify the alkaloids in this species and to develop a thin-layer chromatographic procedure to identify the alkaloids encountered.

EXPERIMENTAL

Collection and Extraction.—*A. formosa* was collected at the flowering stage during the month of May in Benton County, Ore. The roots were separated, cleaned, and dried in a forced-air dryer at 48° for 5 days. The dried roots were ground to a coarse powder in an Abbé mill. A 2.36-Kg. quantity of this material was continuously extracted with alcohol in a Soxhlet extractor until the extractive gave a negative test with Mayer's reagent. The extract was concentrated to a syrupy residue in a flash evaporator at 40° under reduced pressure. The residue (approximately 600 Gm.) was stored in a refrigerator while portions of it were investigated chromatographically.

Separation of Alkaloidal Fraction.—A 200-Gm. portion of the extract was dissolved in a solution containing 100 ml. of 2% hydrochloric acid and 500 ml. of distilled water. A 2% solution of ammonium reineckate was then added until precipitation ceased (approximately 400 ml.). The mixture was refrigerated for 6 hr., the liquid decanted, and the residue dried *in vacuo*. The dried residue was then taken up in warm acetone (250 ml.), and an equal volume of distilled water was added. To this mixture was added 430 ml. of 0.6% silver sulfate to precipitate silver reineckate (6). The mixture was filtered and the filtrate saved. The precipitate was washed with several portions of 50% acetone (total 500 ml.) and the washings added to the original filtrate. The filtrate was then concentrated to a thick syrup in a flash evaporator.

Chromatographic Procedures.—The initial separation and identification procedures utilized were modified techniques of Winek *et al.* (5). The concentrate described previously was adsorbed onto 28.0 Gm. of activated alumina[1] and pulverized to a fine powder with a mortar and pestle. Ten grams of this mixture was placed atop an activated alumina column (35 × 150 mm.). The column was then developed successively with benzene, chloroform, several varying chloroform–methanol mixtures, and finally methanol alone (Table I, AB). Each fraction was concentrated to approximately 10 ml. in a flash evaporator and subjected to analysis using paper chromatography. The extracts were spotted on Whatman No. 2 filter paper and developed with the upper phase of *n*-butanol–acetic acid–water (BAW 5:1:4) using the ascending technique, or *n*-propanol–ammonium hydroxide–water (PAW 2:1:1) employing the descending technique. The spots were revealed by examining the sheets under ultraviolet light and by spraying them with Dragendorff's reagent (7). The results obtained in this manner were not completely satisfactory, and subsequent analyses were conducted with thin-layer chromatography (TLC). TLC plates of Silica Gel G[2] were prepared according to standard techniques. Several recommended solvent systems for alkaloids (8) were tried. Effective separation of alkaloidal constituents was not realized. The combination of systems finally utilized were BAW (4:1:5) upper phase, BAW (4:1:1), and PAW (2:1:1). The spots were located as previously de-

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[1] Alumina activated, chromatographic grade, Matheson, Coleman and Bell, East Rutherford, N. J.

[2] According to Stahl, Brinkmann Instruments, Inc., Westbury, Long Island, N. Y.

TABLE IA.—PAPER CHROMATOGRAPHY OF COLUMN FRACTIONS AND REFERENCE COMPOUNDS[a]

Fraction	Solvent	Vol., ml.	Paper Chromatography: BAW (5:1:4) U.V.	BAW (5:1:4) *Rf*	PAW (2:1:1) U.V.	PAW (2:1:1) *Rf*
1	Benzene	500	..	...	..	...
2	Chloroform	500	..	...	..	...
3	$CHCl_3$–MeOH 97:3	500	..	...	..	...
4	$CHCl_3$–MeOH 94:6	1500		Traces		Traces
5	$CHCl_3$–MeOH 88:12	600	B	0.46	B	0.52
			B	0.56	B	0.59
			Y	0.75	..	0.87
			..	0.89	..	...
6	$CHCl_3$–MeOH 82:18	500	B	0.46	B	0.49
			B	0.57	B	0.57
			Y	0.73	..	0.87
			..	0.88	..	...
7	$CHCl_3$–MeOH 75:25	500	B	0.52	B	0.52
			B	0.62	B	0.61
			Y	0.71	..	0.87
			..	0.89	..	...
8	Methanol	500	..	Traces ...	..	Traces ...
	Reference Compd.					
	Magnoflorine		B	0.56	B	0.47
	Jatrorrhizine		Y	0.68	Y	0.56
	Palmatine		Y	0.68	Y	0.68
	Berberine		Y	0.68	Y	0.60

[a] BAW (5:1:4), *n*-butanol–acetic acid–water, 5:1:4 (upper phase); BAW (4:1:5), *n*-butanol–acetic acid–water, 4:1:5 (upper phase); BAW (4:1:1), *n*-butanol–acetic acid–water, 4:1:1; PAW (2:1:1), *n*-propanol–ammonium hydroxide–water, 2:1:1. Detection, U.V. fluorescence and Dragendorff's reagent: B, blue; Y, yellow.

TABLE IB.—THIN-LAYER CHROMATOGRAPHY OF COLUMN FRACTIONS AND REFERENCE COMPOUNDS[a]

Fraction	Solvent	Vol., ml.	Thin-Layer Chromatography: BAW (4:1:5) U.V.	BAW (4:1:5) *Rf*	PAW (2:1:1) U.V.	PAW (2:1:1) *Rf*	BAW (4:1:1) U.V.	BAW (4:1:1) *Rf*
1	Benzene	500	..	...	..	...	..	...
2	Chloroform	500	..	...	..	...	..	...
3	$CHCl_3$–MeOH 97:3	500	..	...	..	...	..	...
4	$CHCl_3$–MeOH 94:6	1500		Traces		Traces		Traces
5	$CHCl_3$–MeOH 88:12	600	Y	0.02	Y	0.60	Y	0.01
			B	0.07	B	0.63	..	0.08
			..	0.19	..	0.66	B	0.12
			B	0.24	..	...	..	...
			Y	0.35	Y	0.70	Y	0.32
6	$CHCl_3$–MeOH 82:18	500	Y	0.02	Y	0.60	Y	0.02
			B	0.05	B	0.64	..	0.07
			..	0.15	..	0.66	B	0.12
			B	0.21	Y	0.69	Y	0.32
			Y	0.31	..	...	..	...
7	$CHCl_3$–MeOH 75:25	500	Y	0.02	Y	0.62	Y	0.02
			B	0.07	B	0.64	..	0.08
			..	0.19	..	0.67	B	0.13
			B	0.24	..	...	..	...
			Y	0.35	Y	0.70	Y	0.33
8	Methanol	500		Traces		Traces		Traces
	Reference Compd.							
	Magnoflorine		B	0.24	B	0.64	B	0.12
	Jatrorrhizine		Y	0.42	Y	0.66	Y	0.33
	Palmatine		Y	0.34	Y	0.68	Y	0.26
	Berberine		Y	0.36	Y	0.68	Y	0.32

[a] See *Footnote a*, Table IA.

scribed. The results are summarized in Table I, AB. The U.V. fluorescence was noted only for those compounds which gave orange spots with Dragendorff's reagent.

Presence of Magnoflorine and Berberine.—Winek *et al.* (4, 5) reported the presence of magnoflorine and berberine in several *Aquilegia* species. Based on preliminary chromatography, it appeared that these alkaloids were also present in *A. formosa*. A portion of column fraction 5 (Table I, AB) was spotted heavily in three different channels on Silica Gel G TLC plates. Authentic magnoflorine[3] was spotted

[3] The authors are grateful to Professor J. L. Beal, School of Pharmacy, Ohio State University, Columbus, for authentic samples of magnoflorine, berberine, palmatine, and jatrorrhizine.

TABLE II.—RESULTS OF CO-SPOTTING EXPERIMENTS

Solvent	R_f Values					
	Eluate	Eluate + Magnoflorine Chloride	Magnoflorine Chloride	Eluate	Eluate + Berberine Chloride	Berberine Chloride
BAW (4:1:5)	0.22	0.22	0.22	0.36	0.35	0.36
PAW (2:1:1)	0.64	0.63	0.64	0.70	0.72	0.72

in a fourth channel. The plates were developed in BAW (4:1:5). The three zones possessing blue fluorescence and corresponding to magnoflorine (R_f 0.23) were removed from the plate by means of a zone extractor and eluted with 10 ml. of hot methanol. The solution was reduced to a small volume (approximately 0.1 ml.) and divided into two portions. A chromatoplate was prepared containing the one portion of eluate and magnoflorine spotted individually and one spot containing the other portion of the eluate plus magnoflorine. The plates were developed in PAW (2:1:1). Examination of the plate under U.V. light or spraying with Dragendorff's reagent (blue fluorescence under U.V., orange spot with Dragendorff's reagent) revealed that the eluate and the co-spot of eluate plus magnoflorine gave only one zone which had an identical R_f value to that of reference magnoflorine. Similarly, fraction 5 was chromatographed in PAW (2:1:1). The blue fluorescent zones corresponding to known magnoflorine (R_f 0.65) were extracted and eluted with hot methanol and the co-spotting experiment was run using BAW (4:1:5). Comparable results were obtained.

The presence of berberine was established in an identical manner. In this instance, column fraction 7 was used since visual inspection of preliminary chromatograms indicated higher concentration of the suspected berberine in this fraction. Yellow fluorescent zones corresponding to reference berberine [R_f 0.39 and 0.75 for chromatoplates developed in BAW (4:1:5) and PAW (2:1:1), respectively] were extracted and eluted with methanol. Concentrated eluates were co-chromatographed with berberine in a manner similar to that for magnoflorine. The results of these experiments are summarized in Table II.

TLC plates of fractions 5 and 7 were prepared and developed in BAW (4:1:1). Solutions of reference magnoflorine and berberine chlorides were spotted singly and in combination with extracts 5 and 7, and these were compared to plates of extracts without additions. Detection of spots was carried out as before. The procedure revealed the presence of both alkaloids.

Other Alkaloids.—Preliminary paper chromatography revealed the presence of other alkaloids in fractions 5 and 7. When these fractions were chromatographed in the TLC system, additional spots were noted (Table I). These spots were not identified, but co-chromatography experiments using the three solvent systems described earlier indicated that they were not palmatine or jatrorrhizine.

DISCUSSION

Winek *et al.* (4, 5) reported the presence of alkaloids in 10 species of *Aquilegia* which, however, did not include *A. formosa*. On the basis of chromatography, particularly TLC, the presence of at least five well-defined alkaloids has been demonstrated. Consistent with the results of Winek *et al.* (4, 5), *A. formosa* also contained magnoflorine as the principal alkaloid and berberine as one of the minor alkaloids. Since the majority of the conventional solvent systems (8) failed to provide effective separation of these alkaloids (perhaps because of their quaternary nature), two systems—namely, BAW (4:1:5, acidic) and PAW (2:1:1, alkaline)—were used during co-spotting experiments. In all instances, the alkaloidal mixture along with an authentic sample of either magnoflorine or berberine was run in one system and the subsequent co-chromatography was run in the other. Thus, the eluted spot and the authentic sample were subjected to an entirely new environment. In every case there was no separation of the co-spot.

Berberine and palmatine could not be distinguished by TLC employing BAW (4:1:5). However, BAW (4:1:1) separated these two closely related alkaloids cleanly. Co-chromatography using the combination of three TLC systems (Table I, AB) eliminated the presence of palmatine in *A. formosa*. In a similar manner, the presence of jatrorrhizine in the alkaloid mixture was also eliminated. It will be necessary to isolate adequate quantities of the other alkaloidal substances in order to establish their identity.

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Determination of *In Vitro* Release Rates of Sustained-Action Preparations by Paper Chromatography and Electrophoresis

By H. SCHRIFTMAN

Determination of *in vitro* release rates of experimental formulations requires multiple analyses by conventional methods. A general procedure is presented for determining *in vitro* release patterns using paper chromatography and electrophoresis techniques which eliminates the tedious manipulations involved with the evaluation of such formulations by conventional methods. Application of these chromatographic and electrophoretic procedures to a preparation containing phenylpropanolamine HCl, dextromethorphan HBr, and chlorpheniramine maleate is described. The procedures compare favorably in accuracy and speed with the conventional techniques. A scheme for the rapid evaluation of several experimental sustained-action formulations using the above techniques is suggested.

DRUGS that have been in general use for a long time are now becoming available in sustained-action formulations containing other active compounds. In handling the assay of these preparations, the customary methods, *i.e.*, ultraviolet spectroscopy and colorimetry, become laborious and unwieldy because additional filtering and extraction steps are generally necessary for their successful application. In addition, the difficulties are magnified by the need to assay many samples in order to determine the *in vitro* release patterns. The assays and release studies of these multicomponent preparations are considerably simplified by paper chromatographic and electrophoretic procedures. In general, separations of the various compounds are accomplished through ascending and descending paper partition chromatography by selection of appropriate solvent systems or by paper electrophoresis with selected pH buffer systems. The papergrams are sprayed with an appropriate color developing reagent and assayed quantitatively by photoelectric densitometry. These techniques have been described previously for conventional multicomponent formulations (1–3).

Application of these procedures to a sustained-action preparation containing phenylpropanolamine HCl, dextromethorphan HBr, and chlorpheniramine maleate is described. In addition, a scheme for the rapid evaluation of experimental sustained-action formulations using the above techniques is suggested.

EXPERIMENTAL

Reagents.—*n*-Butanol A.C.S., 1 *N* hydrochloric acid, absolute alcohol U.S.P., methanol A.C.S., modified Dragendorff's reagent (iodobismuthate) as described before (2, 3), ninhydrin (Fisher reagent), cadmium acetate·$2H_2O$ (Baker reagent), glacial acetic acid A.C.S., pH 4 acetate buffer (0.1 *M*), 1 *N* sodium hydroxide, petroleum ether (Fisher reagent), simulated gastric juice, and intestinal juice were employed.

Equipment.—Whatman No. 1 and 3 mm. chromatographic paper, micropipets,[1] 0.005 and 0.010 ml., photovolt densitometer, model 530 with recording and integrator attachments, pressure plate paper electrophoresis equipment, model EC-455-18 in. with power supply No. EC453 and ice water circulator No. EC322 (E. C. Apparatus Co.), rotating bottle apparatus (Smith Kline & French) as described before (4), and appropriate chromatographic containers were used.

Procedures.—Totals and release rates of a sustained-action capsule containing chlorpheniramine maleate, 4 mg., phenylpropanolamine hydrochloride, 50 mg., and dextromethorphan hydrochloride, 30 mg., are assayed as follows.

Paper Chromatography.—Powdered contents of two capsules are used directly for total assay while the residues obtained from the usual rotating bottle technique for release studies are dried and ground to a fine powder. These powders are transferred to a 25-ml. volumetric flask to which 15 ml. of absolute alcohol is added. After heating the flask on a steam bath and shaking mechanically for 15 min., the flask is cooled and diluted to mark with absolute alcohol. This mixture is well shaken and undissolved material is allowed to settle to the bottom before spotting the sample. A standard solution containing known amounts of "actives" are likewise prepared in absolute alcohol. These solutions are spotted with micropipets (0.04 ml. in 0.01-ml. increments) on Whatman No. 1 paper which was prewetted with a 1:1 mixture of butanol (saturated with 1 *N* HCl) and methanol. This moist, blotted paper is used for separating and quantitating the dextromethorphan HBr and the chlorpheniramine maleate, while another similarly prepared paper is spotted with 0.005 ml. for the assay of the phenylpropanolamine hydrochloride. A descending chromatographic technique is used with butanol saturated with 1 *N* HCl as the solvent system. An overnight migration of the solvent is sufficient for separating the chlorpheniramine maleate and dextromethorphan HBr. The air-dried chromatograms are sprayed with modified Dragendorff's reagent and the resultant orange-red spots are quantitatively evaluated and compared with a photovolt densitometer containing recording and integrating equipment (1). The other papers containing phenylpropanolamine spots are also analyzed by photoelectric densitometry after spraying with a special ninhydrin reagent (0.2% ninhydrin plus 0.5% cadmium acetate in 95% alcohol containing 2% glacial acetic acid) and heating for 10 min. at 100°. Figure 1 shows the relative migration of the active ingredients.

Paper Electrophoresis.—Again the powdered con-

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[1] Microcaps. Drummond Scientific Co., Philadelphia, Pa.

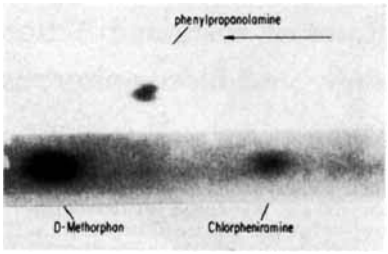

Fig. 1.—Paper chromatograms of phenylpropanolamine, dextromethorphan, and chlorpheniramine.

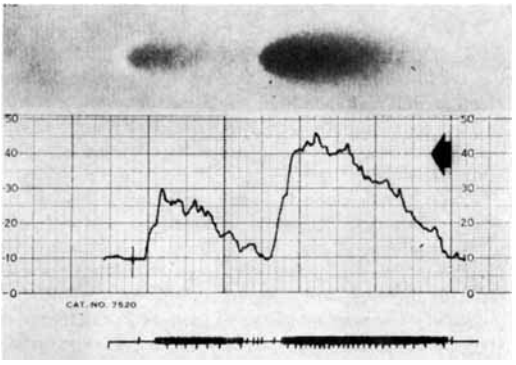

Fig. 2.—Electrophoretic separation of chlorpheniramine and dextromethorphan and their respective recorded and integrated density curves. Chlorpheniramine is the faster moving spot.

tents of two capsules and residues are dissolved and prepared as described for the paper chromatographic procedure. Whatman No. 3 mm. paper strips are prepared for electrophoresis with pH 4 acetate buffer (0.1 *M*) as described previously (1). Two strips are spotted with 0.04 ml. (in 0.01-ml. increments) of standard and sample solutions, while two other strips are spotted with 0.005 ml. of each solution. The papers are placed in an EC apparatus horizontal-type electrophoresis equipment (18 in.) for 16 hr. at 250 v. at 4° (in a walk-in refrigerator). Alternately, the migration can proceed at 600 v. for 6 hr. using salted ice water circulated by a peristaltic pump through the plates. One set of completed and dried electrophoretograms containing the 0.04-ml. spots are sprayed for the detection of the chlorpheniramine maleate and dextromethorphan HBr while the other strips are sprayed with ninhydrin reagent for phenylpropanolamine HCl as described above. Again, as described under *Paper Chromatography*, the resultant spots are analyzed and compared by photoelectric densitometry. Figures 2 and 3 show relative migration of spots as well as their typical recorded and integrated curves.

Application of Paper Chromatography and Paper Electrophoresis to Aqueous Portions of Release Studies.—It may be necessary at times (usually on initial runs) to assay the filtered aqueous portions as well as the residues from a sustained-release study in order to check out the assay and verify the release pattern. The filtered aqueous portions are placed into a 250-ml. separator and 5 ml. of 1 *N* sodium hydroxide is added. The alkaline solution is extracted with 2 × 75 ml. of petroleum ether, which is evaporated to small volume on a steam bath. Evaporation is continued to dryness at room temperature with the aid of a stream of nitrogen. The

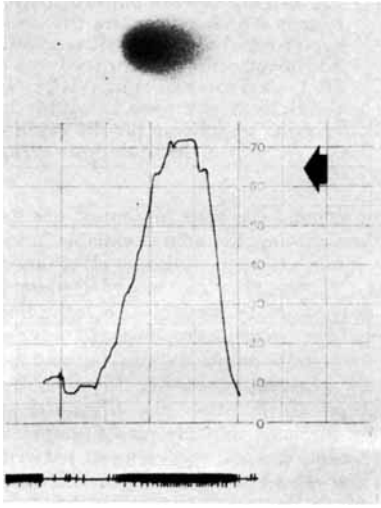

Fig. 3.—Ninhydrin sprayed pink spot of phenylpropanolamine after electrophoresis on 3 mm. paper, combined with its recorded and integrated density curve.

Table I.—Typical Data for Sustained-Action Cold Capsules[a]

	% Released		
	Paper Chrom.	Paper Electr.	Conventional
	Total		
P	96	95	93
D	100	99	100
C	100	99	98
	1 hr.		
P	35	38	37
D	30	30	34
C	27	26	25
	2 hr.		
P	55	57	57
D	54	47	49
C	51	50	50
	4 hr.		
P	70	72	72
D	58	50	60
C	58	60	61
	5 hr.		
P	72	74	73
D	...	54	60
C	...	62	61
	7 hr.		
P	80	78	79
D	67	71	64
C	65	68	66

[a] Theory: each capsule = 4 mg. of chlorpheniramine maleate (C), 50 mg. of phenylpropanolamine hydrochloride (P), and 30 mg. of dextromethorphan hydrobromide (D).

TABLE II.—COMPARISON OF ASSAYS OF RESIDUES AND AQUEOUS PORTIONS

	—% Released (Aqueous)— Chrom.	Electr.	—% Left in Residue— Chrom.	Electr.
		1 hr.		
P	35	33	63	64
D	31	30	68	69
C	24	25	76	73
		2 hr.		
P	57	55	41	43
D	53	52	46	46
C	50	49	48	51
		4 hr.		
P	69	70	30	28
D	58	55	40	44
C	60	61	38	39
		5 hr.		
P	73	74	24	24
D	62	60	35	38
C	63	65	34	33
		7 hr.		
P	80	81	20	17
D	64	66	34	31
C	67	66	31	33

residue is dissolved in absolute alcohol and transferred to a 25-ml. volumetric flask and brought up to mark with alcohol. The analysis of this solution is then continued *via* paper chromatography and electrophoresis as described.

RESULTS

The results listed in Table I indicate that the procedures presented here compare favorably with a conventional method involving a periodate oxidation of phenylpropanolamine hydrochloride as reported by Chafetz (5) followed by bicomponent spectral analysis of the other two ingredients, which must include corrections for interference from base materials. Thus, in addition to eliminating the tedious manipulations involved in the assay of these preparations by the conventional methods, paper chromatography and electrophoresis have dispensed with the need for correction factors which have to be determined every time a new formulation is devised. Reliance of the proposed methods are further substantiated by data contained in Table II, which compare the assays of the residues with their corresponding aqueous portions obtained from the release studies. In general, a scan of the results obtained from the respective and combined aqueous and residue contents of "actives" approaches within 2 to 3% of the theoretical 100% combined values.

DISCUSSION

The comparative ease and success of applying these procedures to a sustained-action formulation lends credence to the following proposed scheme for the rapid evaluation of these preparations. The pharmaceutical developer could semiquantitatively compare his various formulations using the above techniques, as follows.

Large sheets of Whatman No. 1 chromatographic paper or several strips of Whatman No. 3 mm. paper are prepared by spotting known quantities of the "actives" in the release pattern that is required, *i.e.*, 50% release of active No. 1, 20% release of active No. 2, and 25% of active No. 3 for 1 hr., followed by the requirements for 2, 3, 4, 5 hr., etc. The various formulations after the various hours of the release study are then subjected to procedures as described here, and their resultant spots are compared visually with the respective reference spots. This rapid surveillance could indicate which formulation (if any) approaches the desired release pattern. In this manner, many formulations could be evaluated and compared at the same time, thus saving the experimental formulator valuable time. In addition, the analyst obtains fewer samples for final and accurate quantitation; and using the methods presented here, he saves further time which frees him for the performance of other assays.

SUMMARY

Paper chromatographic and electrophoretic procedures have been successfully applied for studying the release pattern of a multicomponent sustained-action preparation.

The procedures compare favorably in accuracy and speed with the conventional techniques.

Simplicity and ease of application of these methods have led to the suggestion that several sustained-action formulations could be rapidly evaluated by the product developer.

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Some Factors Affecting Inhaler Dosing

By KENNETH R. HEIMLICH and MARY C. GINKIEWICZ

Some factors affecting the dose from an inhalant dosage form have been studied. It was found that rate of dosing, but not pressure, influenced the amine dose from propylhexedrine inhalant N.F.

SOME ASPECTS of inhaler technology were discussed in this journal in 1962 by Kennon and Gulesich (1). From their work they concluded that the vapor pressure of the drug and additives in an inhalant dosage form was not the dose-determining factor. They contended that inhaler dosing is a process controlled by factors affecting the rates of volatilization, whereas, vapor pressure values represent an equilibrium situation. Recently, in answer to an inquiry from the National Aeronautics and Space Administration, the authors have substantiated the above findings and further studied the factors affecting the dose from an inhalant dosage form. The inquiry from NASA concerned the possible inclusion of propylhexedrine inhalant N.F. in the medical kit for the Apollo Space Program. Since the pressure inside the spacecraft is maintained at 5 lb./sq. in. absolute (p.s.i.a.), it was necessary to know the amount of propylhexedrine per inhalation which would emanate from the inhaler at this reduced pressure.

EXPERIMENTAL

A dosing chamber (Fig. 1) which could be operated at reduced pressures and varying flow rates was constructed. An oxygen source was attached to the inlet valve of the system and the outlet was attached to an aspirator. The chamber then was adjusted to maintain the desired pressure at a specific flow rate of oxygen. At the time of dosing, a valve which maintained an open system between the chamber and the dosing tube was closed. This caused the oxygen flow to pass through the inhaler unit carrying vapors down into the dosing tube which contained a standardized solution of sulfuric acid. The assay of the amine was done by making the solution alkaline and extracting with chloroform. The chloroform extract was titrated potentiometrically in an isopropanol–ethylene glycol solution with hydrochloric acid. Three inhalers were dosed and assayed at each condition.

RESULTS AND DISCUSSION

The results of the inhaler dosings are given in Table I.

The data indicate that reducing the pressure above the inhaler to 5 p.s.i.a. does not affect the dose delivered by the inhaler. However, the rate at which the oxygen is passed through the inhaler significantly affects the dose.

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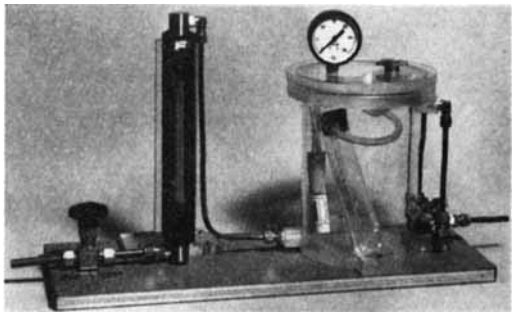

Fig. 1.—Dosing chamber for inhaler units. Chamber can be operated at varying pressures and flow rates.

TABLE I.—MILLIGRAMS OF PROPYLHEXEDRINE FROM INHALERS DOSED AT VARYING PRESSURES AND FLOW RATES

Flow Rate through Inhaler	Pressure	Propylhexedrine/L. of Oxygen passed through Inhaler, mg.
6 L./min.	5 p.s.i.a.[a]	0.39
6 L./min.	Atmospheric	0.45
1 L./min.	5 p.s.i.a.	0.71
1 L./min.	Atmospheric	0.75

[a] Pounds per square inch absolute.

The dosing of an inhaler may be considered analogous to a distillation process in that the active driving force of the system is the tendency for liquid and vapor to approach equilibrium. In the inhaler system this equilibrium is never attained. The fact that the vapor dose varied at different flow rates is direct evidence of this. If sufficient time were allowed for the passage of a constant volume of oxygen through the inhaler, the amount of drug evaporated would be independent of time since equilibration would occur. However, at a certain dosing rate of oxygen per unit time, the evaporation rate becomes dose determining. Thus, at a constant evaporation rate more drug is obtained per unit volume from a slow dosing rate than from a faster rate.

Since the change of pressure did not affect the dose, it may be assumed that the gases are behaving ideally at the pressures present in the system. Had the system been operated at higher pressures, collision with higher concentrations of molecules would have retarded the amount of amine vaporized and conceivably the dose would become pressure dependent.

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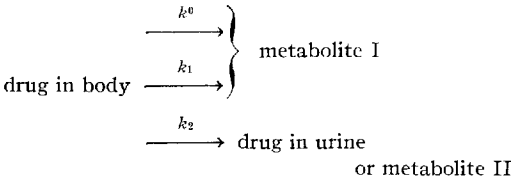
Possibility of Simultaneous Apparent Zero- and First-Order Kinetics in the *In Vivo* Formation of a Single Drug Metabolite

Sir:

The biotransformation of many drugs is describable by first-order kinetics and most of the theoretical models in pharmacokinetics have been developed on that basis (1, 2). It is now known that several important biotransformation processes have a limited capacity (3, 4) and proceed by apparent zero-order kinetics in the therapeutic dose range (4). The purpose of this communication is to propose the possibility that a single biotransformation product may be formed by a combination of simultaneously occurring apparent zero- and first-order processes.

Many biotransformation processes are known to occur in more than one tissue. For example, significant enzymic transformation of phenol to phenol sulfate has been demonstrated in human liver, adrenals, kidneys, and small intestine (5). These tissues differ in intrinsic (maximum rate per unit weight of tissue) and absolute capacity for this process. Therefore, it is likely that, at a sufficiently high drug concentration in body fluids, biotransformation in one tissue proceeds at a maximum rate while the same process in another tissue follows apparent first-order kinetics.

Such mixed kinetics in the formation of a single metabolite (metabolite I) might involve, for example, one apparent zero-order process[1] in parallel with one or more apparent first-order processes which do not reach "saturation" at any reasonable drug concentration. If the drug is also eliminated partly by renal excretion or by apparent first-order formation of another metabolite (metabolite II), the following model will apply:



where k^0 and k_1 are apparent zero- and first-order rate constants, respectively, for the formation of metabolite I, and k_2 is the apparent first-order rate constant for renal excretion of unchanged drug or for formation of metabolite II. Under these conditions, the fraction of the dose which is excreted as metabolite I will decrease with increasing dose. However, unlike the case where one of the metabolites is formed *solely* by apparent zero-order kinetics, the fraction of the dose excreted as metabolite I will approach a relatively constant value at high doses. This is so because the contribution of the apparent zero-order process to the total elimination process will become negligible at sufficiently high doses. At these high doses, the fraction of the total excreted drug which is metabolite I will approach the value $k_1/(k_1 + k_2)$ as in apparent first-order kinetics (6). The dose range where this occurs will depend on the values of k^0 and k_1.

[1] It is assumed as a matter of convenience that the capacity of this process is so low that a maximum rate is reached with very low doses.

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Stereochemistry of Methadol Diastereomers

Sir:

Previous investigations (1, 2) have shown that catalytic or lithium aluminum hydride reduction of methadone (I) proceeds stereospecifically to afford α-methadol (II*a*), while sodium in propanol reduction gives rise to a mixture of diastereomers in which the β-isomer (II*b*) predominates. The stereochemistry of these isomers is of great interest because of the remarkable changes in the configurational selectivity of analgesic receptors (Table I) which takes place in the α-series. Such changes recently have been rationalized in terms of differing modes of analgesic–receptor interactions (3).

In this paper evidence is presented which leads to the complete stereochemical assignment and conformational preference of these diastereomers.

Quaternization of racemic α- and β-methadol with methyl iodide afforded the corresponding methiodides, IV*a* (4) and IV*b*, m.p. 189–191°. Pyrolysis of IV*a* and IV*b* proceeded stereospecifically to give rise to isomeric tetrahydrofuran derivatives V*a*, m.p. 90–91° (4), and V*b*, m.p. 61–63°, respectively. Catalytic hydrogenation of VI which was obtained by the method of Easton (5, 6), afforded a mixture of V*a* and V*b* in 60% yield, whose composition was estimated by NMR analysis to be in a ratio of 2 to 1. Molecular models (Stuart-Briegleb) indicate that the top face of VI is more accessible to the hydrogenation catalyst than is the side which is *cis* to the C-5 methyl group. This strongly suggests that V*a* corresponds to the *cis*-isomer and V*b* represents the *trans*-diastereomer.

In view of the stereospecificity of the ring closure to V and the reported (7) formation of optically active VI from enantiomers of methadone methiodide, it is evident that the cyclization of IV*a* and IV*b* involves inversion at the C-6 asymmetric center. The relative stereochemistry of II*a* and II*b* therefore can be designated as (3S:6S) or (3R:6R) and (3R:6S) or (3S:6R), respectively. This assignment was corroborated by determining the dissociation constants of α- and β-methadol in methanol. The pKa of II*a* is 8.15 while that of II*b* is 7.85. According to the current concepts (8) concerning the base strength of diastereomeric amino alcohols, the pKa of these diastereomers is a reflection of the difference in the ability of the protonated species of II*a* and II*b* to form intramolecular hydrogen bonds of the type illustrated by formula VII. The diastereomer which is hydrogen bonded more strongly is more highly dissociated. The fact that α-methadol (II*a*) is a stronger base therefore indicates that there is less steric hindrance to intramolecular hydrogen bond formation. Molecular models show that this is consistent with the stereochemistry proposed above for II*a* and II*b*, since it can be seen that the cyclic hydrogen bonded conjugate acid of β-methadol (VIII*b*) has both the methyl and ethyl groups on the same side of the *quasi* ring, whereas the less hindered α-isomer (VIII*a*) has these groups on opposite sides.

High resolution infrared studies of II*a* and II*b* in carbon tetrachloride show that the bases are intramolecularly hydrogen bonded in as high a concentration as 0.25 *M*, thus indicating strong association. This was substantiated by NMR analysis of the bases in deuterochloroform. The hydroxylic protons of II*a* and II*b* resonate at unusually low field (8.6 and 7.9 p.p.m., respectively) and have peak half-widths of 30 and 23 c.p.s., respectively. The fact that the hydroxy-

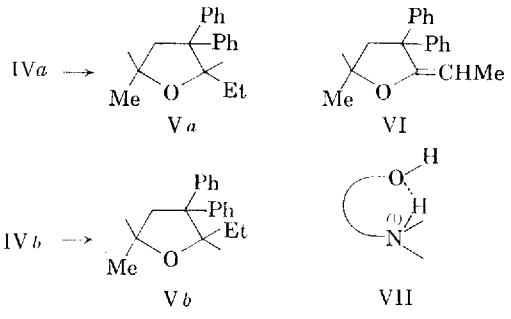
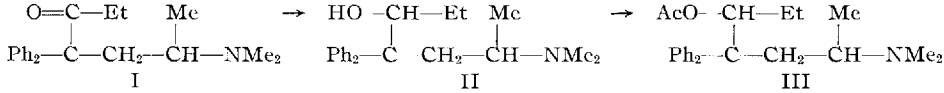
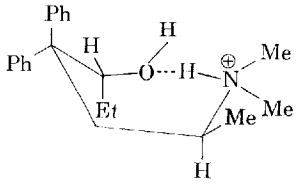


TABLE I.—ANALGESIC ACTIVITY OF METHADOLS AND ACETYLMETHADOLS (2)

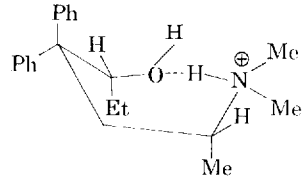


Configuration	ED_{50}[a]	Isomer	ED_{50}[a]	Isomer	ED_{50}[a]
S-(+)	25.7	*a*, (−)-α	3.5	(−)-α	1.8
		b, (+)-β	63.7	(+)-β	4.1
R-(−)	0.8	*a*, (+)-α	24.7	(+)-α	0.3
		b, (−)-β	7.6	(−)-β	0.4

[a] mg./Kg. injected subcutaneously in mice.



VIII *a*

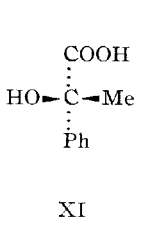


VIII *b*

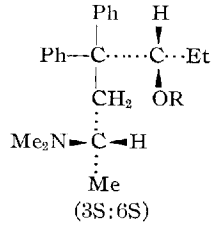
ylic proton resonance for II*a* is broader than that of II*b* indicates that the former free base forms stronger intramolecular hydrogen bonds than does the latter. The conformation of the bases, therefore, are very similar to the hydrogen bonded conjugate acids.

Further evidence for the stereochemistry of the methadols was obtained by application of Prelog's rule (9). Treatment of (−)-II*a* with benzoylformyl chloride produced in 95% yield the benzoylformate ester hydrochloride (IX), m.p. 195°, $[\alpha]_D^{25}$ −74.9°, which was converted to its methiodide (X), m.p. 208°, $[\alpha]_D^{25}$ −42.8° (70% yield). Reacting either IX or X with methylmagnesium iodide followed by saponification, afforded (80% over-all yield from X) S-(+)-atrolactic acid (XI), $[\alpha]_D^{25}$ +4.3° (1% in 1 *N* NaOH), corresponding to 8% optical purity (10). In accordance with Prelog's rule (9), the stereochemistry at C-3 therefore is assigned to the S-series.

Since the absolute configuration of (+)- and (−)-methadone, which are precursors of optically active α- and β-methadol (see Table I) has been determined (11), the complete absolute stereochemistry is as shown below. The (+)-α- and (−)-β-series are the mirror image forms corresponding to the projection formulas.

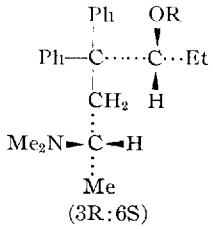


XI



(3S:6S)

II*a*, R = H
III*a*, R = Ac
} (−)-α



(3R:6S)

II*b*, R = H
III*b*, R = Ac
} (+)-β

Possible reasons for the changes in analgesic receptor stereoselectivity will be discussed fully in a definitive publication.

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* National Institute of Health Predoctoral Fello

Carbonate Prodrugs in Formulation and Therapeutics

Sir:

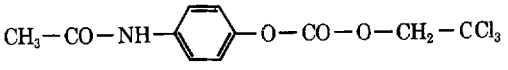
The authors have noted that numerous carbonate diesters can be made of drugs containing the hydroxy group. Often such carbonates are prodrugs, *i.e.*, new compounds having physical–chemical properties different from the parent drugs but retaining qualitatively identical pharmacologic effects and reverting to the parent drug in the body. That they are prodrugs which are hydrolyzed to the parent drugs in the body is attested to by *in vitro* cleavage studies where blood serum has been shown to be a good source of enzymes for catalyzing the hydrolysis reactions.

The principle of prodrug formation has special utility where the parent drug possesses undesirable pharmaceutical features. For example, trichloroethanol possesses interesting sedative properties; but it is a volatile liquid with an unpleasant odor and taste. As such, it is not conveniently suited for therapeutic use: but by reaction with phosgene, it can be converted into bis-trichloroethyl carbonate having a melting point of 86–87°. This previously unreported crystalline compound is virtually tasteless, has sedative properties, and can be encapsulated or tableted.

$CCl_3—CH_2—O—CO—O—CH_2—CCl_3$

bis-Trichloroethyl Carbonate

Similarly, for example, a trichloroethyl carbonate diester of acetaminophen has been made by reacting it with trichloroethyl chloroformate. It is a crystalline compound, m.p. 151–153.5°, possessing the analgetic and sedative properties of the parent drugs from which it is derived, and is also virtually free of taste.



2,2,2-Trichloroethyl-4-acetamido-phenylcarbonate

These compounds, and some related ones, are interesting new compositions of matter. With but few exceptions (quinine ethyl carbonate, alkyl erythromycin carbonates) carbonate diesters have not been utilized as therapeutic agents, and to our knowledge it has not been demonstrated, heretofore, that diester carbonates are prodrugs. Studies on their physical-chemical properties, cleavage rates, biochemistry, and pharmacology along with studies on their pharmaceutic and therapeutic utilities will be subjects of more detailed publications.

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REVIEWS

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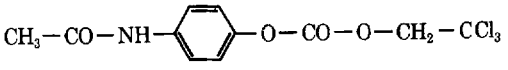

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Encyclopedia of Industrial Chemical Analysis. Vol. I, General Techniques A-E. Edited by F. D. SNELL and C. L. HILTON. Interscience Publishers, a div. of John Wiley & Sons., Inc., 605 Third Ave., New York, N. Y. 10016, 1966. xv + 763 pp. 18.5 × 26 cm. Price $35.00 per copy with subscription. $45.00 per single copy.

The structure of the chemical industry, the nature of the chemical products, and the methods and techniques of analysis have undergone radical changes in recent years. As a result, a comprehensive view of industrial analytical chemistry was felt to be a desirable undertaking. This is the first in a series of volumes to fulfill that need. Methods and techniques used throughout the world for the analysis of raw materials, intermediate, and finished products, as well as evaluation of the finished product for its intended use, will be included. The Encyclopedia will be presented in

but each chapter begins with an excellent table of contents. The binding of the book is fairly good; the print is readable; and the quality of the paper is good.

The book is addressed to research workers and graduate students, and it will be highly useful as a reference to organic chemists, biochemists, physical organic chemists, analytical chemists, and (to a lesser extent) to physical pharmacists. It is recommended for the research library, with the hope that the entire set of "Chemistry of Functional Groups" will be of the same quality.

Reviewed by Joseph G. Cannon
University of Iowa
Iowa City

Nonexistent Compounds: Compounds of Low Stability. By W. E. DASENT. Marcel Dekker, Inc., 95 Madison Ave., New York, N. Y. 10016, 1965. ix + 182 pp. 16 × 23.5 cm. Price $8.50.

To anyone concerned with preparing or using stable compounds, the unstable ones are of interest, if only for the purpose of avoidance. And, of course, there are many shades of gray between the possible and impossible.

This little book has taken as its theme those inorganic compounds that are on the verge of stability, usually arranged in such classes that some of the members are stable and some are not. The compounds are discussed in terms of the reasons for their variations in stability.

The book opens with a chapter on various general approaches to stability, *e.g.*, valence and structure, thermodynamics, tendencies in chain and multiple bond formation. A chapter illustrating each of several types of stability then follows. The semistable compounds of nitrogen, sulfur, phosphorus, and silicon have the most representatives, but there are illustrations from almost all the elements except the electropositive metals and the two rare earth series. There is a ten page discussion of compounds of the noble gases. It is difficult to give a typical example of the types of compounds included, so varied are they. The purely ionic compounds are specifically excluded.

Explanations and discussions are well grounded theoretically and are well documented. There are extensive tabulations of energetic data. The author leans heavily on Pauling's "Nature of the Chemical Bond" as concerns valence and bond energies, and writes generally in the same spirit as Pauling.

The book is not a mere curiosity, as suggested by the facetious title. It will find its best use as a rich source of illustrative material for the principles involved in the stability of inorganic and metal-organic chemistry. Some readers will be disappointed that it contains little of direct value for the organic chemist. A book in that subject would have a larger audience, and its point of view would be much different. Of course, it would have a different author.

Reviewed by L. Dallas Tuck
School of Pharmacy
The University of California
San Francisco

Remington's Pharmaceutical Sciences. XIII ed. Editor-in-Chief ERIC W. MARTIN. Mack Publishing Co., Easton, Pa., 1965. xii + 1954 pp. 21 × 29 cm. Price $23.50

In keeping with the increased emphasis on the scientific basis of pharmacy, this classic has changed its title—from "Remington's Practice of Pharmacy" to "Remington's Pharmaceutical Sciences." The basic outline of earlier editions has been followed; however, many chapters have been revised and several new chapters have been added to reflect the latest developments in analytical and manufacturing procedures. The greatest amount of new material has been in the field of physical pharmacy—four areas are covered in new individual chapters—biopharmaceutics, quantum theory, reaction kinetics, and thermodynamics. A chapter on plastics has been added to the manufacturing section and includes the more recent developments in the nature, uses, and testing of plastics used in medicine.

Drugs are discussed individually as in previous editions. The use statements might more properly be called a therapeutic summary; uses are given, of course, but also included in the discussion are action, special advantages and/or disadvantages, side effects, and cautions.

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Review Article

Techniques Utilized in the Evaluation of Psychotropic Drugs on Animal Activity

By WILLIAM J. KINNARD, JR., and NATHAN WATZMAN

CONTENTS

INTRODUCTION

THE EVALUATION of chemical compounds for potential psychotropic activity involves their sequential evaluation in a battery of pharmacologic tests. This screening protocol serves to eliminate toxic or inactive compounds and at the same time allows for the stepwise formation of a profile of pharmacodynamic and toxicological information. The sensitivity of this entire program is heavily dependent upon the initial screening tests involving measurements of animal activity. It is ironic that, of all of the procedures that comprise a screening program, these are the most critical, yet the least standardized, most highly individualized, and most vulnerable to environmental factors. It is therefore, important that careful thought and organization of test criteria precede actual laboratory work. The investigator should determine the optimum set of experimental conditions for his laboratory, since external influences on drug responses will vary from one laboratory to another. It is the purpose of this paper to acquaint the reader with the problems of evaluating animal activity, the advantages and limitations of present-day test apparatus and procedures, the parametric variables which may alter drug response, and the design and statistical techniques which may increase the efficiency of this type of experimentation.

OBSERVATIONAL TECHNIQUES

General Considerations.—The subjective observation of drug-induced changes in the activity of laboratory animals has long been a preliminary procedure in drug testing. This initial procedure can provide information concerning general pharmacological and behavioral drug actions as well as preliminary toxicological data. These total data comprise one facet of the preclinical experimental protocol needed for any attempt on the part of the investigator to predict the clinical activity

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of the drug. Two approaches can be utilized in the evaluation of potential psychotropic agents through the use of gross observational techniques (1). The first of these is the *criterion behavior* approach in which a form of abnormal human behavior is selected and its basic characteristics are determined. The next step is the induction in test animals of a form of behavior which is as nearly as possible similar to this human behavior. The search is then made for new drugs which will modify this behavior at low doses. This might be exemplified by the production of "experimental neuroses" in laboratory animals and the examination of test drugs on this behavior. The second approach is the use of *criterion drugs*. The effects of clinically established drugs are determined in animals using a wide variety of tests, and then the new compounds are studied on the most sensitive of these tests. Most laboratories actually use a composite of these procedures.

The generation of an adequate rating scale for observational procedures requires that the behavior patterns of the experimental animals be carefully examined. As the spontaneous behavior of animals is studied, the events occurring within the animal's behavior can be organized into patterns that are dependent upon the variability of these events (2). Careful examination of these behavior patterns can be made and documented to provide a basis for rating programs (3). Since these observable patterns are under the influence of innumerable internal and external factors, the emphasis on the development of a rating program must be placed upon the use of carefully controlled conditions. There are many variables that must be carefully regulated before the observational test procedure can be used efficiently. The all important variable is the test animal itself. Certainly, all of the attributes of the proper experimental design used in any pharmacologic procedure should be followed in the selection of the test animal. The health of the animals should be a primary concern, and the operations of the animal colony should ensure that a minimal stressing of the animals occurs. If female animals are to be used, the effect of the estrus cycle must be taken into consideration. The development of estrus in the cat periodically alters baseline activity of the animal, and total ovariectomy will eliminate this problem, yet allow for general behavior patterns to remain virtually unchanged (4).

Environment will play a major role in the animal's total behavioral response. As noted by Brady (5), the effects of drugs upon behavior depend upon the environmental conditions affecting the animal when the pharmacologic agent is being evaluated. The initial standardization of the observation area in regard to temperature, humidity, range area of the animal, etc., should not be altered throughout the experiment in order to prevent minute behavioral changes from occurring. Consideration must also be made of the "arousal-provoking" quality of the experimental situation (6). Irwin (7) used a soundproofed or sound attenuated room for the examination of cat behavior, whereas other investigators have used a standard laboratory room that permitted external auditory stimuli to reach the test animals. The question arises here as to whether unexpected external stimuli can be used as an indication of animal awareness. It would appear that they could contribute to a more adequate assessment of the animal's attentiveness.

The observer is an extremely important variable in the test procedure. This person must be a highly trained, observant, and patient individual. He must be fully cognizant of the animal's normal behavior and totally aware of the possible changes in behavior that a drug can produce. He must be able to recognize and record not only those events that are listed in the test program, but also any others that may occur. The training of an individual is based on experience and is very critical; it cannot be achieved by the presentation of written or oral material describing the procedure. Each of the behavioral changes must be shown to the observer through the use of control drugs, and many experimental trials must be performed to ensure adequate reproducibility of the observer's rating.

The mechanics of the rating procedure should be considered in the design of the rating sheet. Most investigators use a single sheet to note any drug effects over the experimental time period. In order to negate subjective bias of the observer, a booklet form can be used for rating (4). Each of the observational time intervals is placed on a separate page so that the observer does not see the previous rating entries. Also, to help ensure that the observer is examining each item on the list, a place can be allowed at each rating point for "no observable change." The design of the sheet or booklet may also be governed by the type of information retrieval program used by the laboratory. If the material is to be placed on punch cards for later printing, the rating sheets should also be designed so that the key punch operator may accurately transfer the information from the rating sheet to the punch card. The choice of items to be placed on any rating scale must be dependent

upon the skill of the supervisor and the observer in the determination of the animal's ongoing behavior patterns in response to the test situation. A rating scale should originally include as many items for rating as possible, based upon the general behavior of the animal and the discrete items that occur within each behavior pattern, as well as upon known changes elicited by the prototype compounds. Following use of the test scale, certain items can be eliminated from the program if the frequency of these events is found to occur at a very low level. As noted by Irwin (8), it is better to have an excess of rating points rather than a deficiency for the test scale.

There are several weaknesses present in multidimensional observation procedures. First, observers vary in their ability; this is only overcome by standardizing the rating scale and quantifying the observed behavior on the basis of an all-or-none rating of events. Second, a tendency toward subjective bias exists as well as a tendency to have an insufficient number of steps in the rating scale. This latter point would possibly cause a loss of information from the behavioral observation. Subjective bias may be overcome by the use of blind procedures for drug administration and observation, and the loss of information can be overcome by adding more rating items. This excessive subdivision does not appear to distort the data (8). Two further problems must be considered: reproducibility of procedures and data between laboratories, and the difficulty of summarizing the information developed from the test procedure (9). The difficulty in transferring the experimental method from one laboratory to another is well known. These procedures are highly individualized and dependent upon the scientist generating the scale. Extremely well-defined profiles are required to overcome this limitation. Their definition must not only be in writing but in some pictorial form as well.

Observational Procedures with Rodents.—Observational test procedures involving rodents vary greatly depending upon the specific need of the particular laboratory. Some investigators (10, 11) use little or no detail in their rodent tests, but these programs are only a minor part of a larger over-all program of drug evaluation. Janssen (12) used a protocol which involved a large number of tests in rodents and dogs; among these were an open field measure, hot and cold plate tests, a toe pinching procedure, a rotarod test, etc. The question which arises from this type of program is whether these multiple tests are necessary for the evaluation of drug effects at the early level of pharmacologic screening. Perhaps early screens should not be overly burdened, but rather allow for fewer, more definitive tests to show potential drug action. A fairly complete rating scale for mice or rats has been clearly defined by Irwin (13). His procedure for the evaluation of the general activity and acute toxicity produced by the test drug is written in a manner that allows for accurate interlaboratory replication. The paper includes a rating chart used in his laboratory, and shows drawings of the animal postures rated for the righting reflex and passivity items on the scale. Norton (2) has developed a rating scale that is more behaviorally oriented than those produced by others. Her profile for the hamster is well defined and includes five main behavior patterns: sociability, contentment, excitement, defensive hostility, and aggressive hostility. Under each of these main categories there are five subheadings which are specified items of behavior. As an example, the items of squawk, pulling, chasing, biting, and rearing are subheadings under the category of aggressive hostility. She compared the results with the hamster to those obtained from monkey and cat studies, and noted that the test animals responded differently to the individual test drugs. For example, chlorpromazine caused an increase in sociability in all animals, but caused an increase in contentment in the monkey and hamster with a decrease in contentment in the cat. It induced an increase in excitement in the cat and monkey, but a decrease in the hamster.

The depressant activity of drugs has been identified rather well for many years, and the utilization of some of these procedures may contribute to the efficiency of rating programs in psychopharmacology. Lim *et al.* (14) used a procedure to evaluate sedation in rats. Animals were housed individually in small cages and pictures taken of their posture and degree of eye closure, with the activity of the animals being measured by a photocell system. The animals were startled periodically with a mild air blast, and the changes in posture and degree of eye closure rated. The illustration of depressed postures of rats and dogs in this paper can aid in the replication of its data. Cohen and Nelson (15) rated depressive activity of rats, noting the effect of chlorpromazine and pentobarbital on the loss of spontaneous motion, response to stimuli, and the degree of ataxia. Each of these were subdivided into four or five rating items. A simple measure of motor activity that has been used to test depressants is the open field test, a procedure that can easily be incorporated into a rating

program. Furthermore, Brimblecombe (16) considered the open field test with the rat to be a valid measure of emotionality. He noted, however, that by itself it may not be accurate, but in a battery of tests it does give some indication of the drug action on the emotional behavior of the animal. In the classical test, the animals are placed at the center of a circular (17) or rectangular (18) open area which has been suitably marked off, and the number of times that an animal moves across the zone lines is recorded. In addition other rating points such as the degree of grooming and defecation can be checked simultaneously (16, 19, 20). This test can utilize individual or paired rats (19), and has been used as a measure of motor deficit, emotionality, and exploration. These behavior patterns can also be analyzed in different ways. Randrup and Munkvad (21) rated the behavior of individual rats by counting the different types of grooming that occurred as well as recording their locomotion within an area through the use of multiexposure photography. They noted that amphetamine produced a stereotyped activity which they could alter through the use of perphenazine. Bindra and Baran (22) evaluated drug-induced changes on sniffing, lying, grooming, and general activity of male hooded rats, which were individually housed in foot square boxes. Many other behavioral signs can be added and scored in this procedure. Cole and Dearnaley (23) utilized a simple rating program in the evaluation of the effects of reserpine and morphine in rats and mice. They illustrated Straub tail reactions, and also noted the effects of the test drugs on such items as the grasp reflex, piloerection, tremor, and posture. Catalepsy and palpebral ptosis have also been used as observational test items (24). Toman and Everett (25) called particular attention to unusual aspects of behavior; for example, hunching and squinting which are characteristic responses to reserpine. Lim (26) included items of morphine-like fixation of posture and pseudohypnosis in his profile for rating drug effects on rats.

One hesitates to talk about emotionality in rodents, but there are certain aspects of behavior that investigators use for its evaluation. Raitt *et al.* (27) and King (28) used a six-item program with each component rated on a 5- or 6-point scale. Rats were evaluated on their reaction to the presentation of a probe to their snout, response to a light rap on the back with a probe, resistance to capturing, resistance to handling, vocalization during stages of capture and handling, and urination and defecation during these stages. This procedure certainly allows for the assessment of drug effects on the stress induced by the observer's interaction with the test animal, but one wonders if this type of emotional rating can be correlated with clinical effects.

Sulser *et al.* (29) have utilized a different test situation in their rating of drug effects on behavior in the rat. The animals were placed on top of a box about 1 ft. high. Control animals would move about and explore their surroundings for a few minutes, then cluster together, and groom themselves, but would not leave the top of the box. Test drugs altered this response; for example, rats given a benzoquinolizine compound (RO-41284) remained motionless and isolated from each other for about 3 hr. Rats treated with amphetamine would dash about helter-skelter, while rats given RO-41284 plus desmethylimipramine would move around the edge of the box and fall off or leave the box. Votava *et al.* (30) examined the effect of experimental drugs on the central nervous system with the aid of a test involving orientation activity in the rat. The animal was placed in a small chamber and after a short interval was allowed to move into a second and larger chamber when a barrier was removed. The animal was then rated on various motor responses, such as the number of times it moved through the door, the amount of grooming, etc. Welker (31) has examined sniffing as an aspect of exploratory behavior in the albino rat. In a rather detailed paper, he noted the various aspects of this behavior and how it was altered by various stimulants and depressants.

Most rodent rating scales utilize individual animals, but the social interaction of these animals should not be overlooked. If social interaction is to be included in a rating scale, some excellent discussions (32, 33) of this behavior in rats and mice can be consulted. Silverman (34) has recently discussed the use of ethology as a means of observing animal behavior with increasing precision. He has developed a well-defined and integrated rating profile for social interaction, which includes the categories of exploration, investigating, mating, aggression, and other signs. Rats were isolated for several days and then introduced into the observation cages in pairs; only one of the test animals was treated with placebo or chlorpromazine, and a counterbalanced order of presentation was introduced into the experimental design. Chance and Silverman (35) evaluated chlorpromazine, amphetamine, and amobarbital on the latter test. It is possible that within groups of rats, different degrees of social interaction will occur. Irwin (36) reported that there was a significant negative

correlation between individual treadwheel counts and the time devoted to interactional behavior of rats; *i.e.*, animals which showed a high degree of spontaneous activity in the treadwheel did not score high in social interaction.

Certain aspects of social behavior lend themselves well to the testing of psychotropic drugs. The aggressive tendency of rodents in certain situations has been used in an attempt to duplicate clinical situations. When mice are isolated for a period of time and then placed together, an aggressive behavior, which can be rated (37–39), is produced between the two mice. An instinctive behavior that is seen in some rats is the mouse-killing reaction. Certain rats will almost immediately kill a mouse when it is introduced into the rat's cage. While many psychotropic drugs are not specific antagonists of this response, it has been noted that antidepressants will block it (40).

If animals are to be re-used in these test procedures, prior experience must be taken into consideration. Marriott and Spencer (41) examined the exploratory behavior of rats under the influence of various psychotropic drugs. Their studies, based on that of Steinberg *et al.* (42), used a Y-shaped box and noted the number of complete entries that the rat made with all four feet into one of the arms of the box. They found that chlorpromazine reduced exploratory behavior, but that meprobamate and chlordiazepoxide increased it. However, the effect of chlordiazepoxide on exploration was completely inhibited by a single previous exposure to the Y box. This fact should be taken into consideration if open field tests or other forms of exploratory behavior measurements are to be used.

The addition of many of the aforementioned behavioral signs or procedures to the standard pharmacological screening test used in observational studies may aid in the identification of potential psychotropic agents. These rating scales in their final form should be as quantitative as possible. It is relatively easy to quantify certain physiological signs within the test structure; these include such items as pupil size, cardiac and respiratory rates, and body temperature. One caution that must be noted is that the desire for exacting quantification may yield a rating scale that, through the time and effort required for its usage, drastically reduces the efficiency of the total rating program.

Observational Procedures with Cats.—A requisite for gross observational studies is that the test animal must possess a fairly stable personality for the prolonged periods of time required for laboratory study; in addition, the animals must show a sufficient range of spontaneous behavioral patterns to allow accurate studies to be made. Cats seem to fit these qualifications (43). Irwin also favors the cat as a test animal for its value in predicting drug effects in man. He noted that its sensitivity to various behavioral drug effects appears to be more like that of man than any other laboratory animal, with the ratio between doses producing behavioral and side effects in the cat being closely approximate to that of man. A disadvantage in the use of the cat is that it shows atypical effects to certain drugs; for example, it is stimulated by certain narcotic analgesics and antihistamines (44); also chlordiazepoxide produces an effect in the cat that lasts for several days. The cat, along with the dog and monkey, allows a wider range of behavior and drug personality interactions to be observed than one would see with the rat or the mouse (45).

The cat observational technique is not truly a preliminary test in that the rodent screen should be initially utilized to eliminate a large number of inactive and toxic compounds from the test series. This is necessary since the procedures involving cats require the use of colonies that become invaluable because of the time involved in establishing behavioral baselines. Drugs that are to be tested on these animals must also have some toxicological data available for the estimation of initial drug dosage. The drugs should be given orally to allow a better clinical correlation of data, although other routes have been considered (8). Drugs can be administered in capsule form to the cats who are restrained either in a box or in the arms of an assistant. The capsules are placed on the back of the tongue using a long curved forceps or hemostat, and the animal's mouth is then held shut. However, the oral route is not without its disadvantages; for example, chlorpromazine often produces vomiting in the cat (46), and thus, the animal will not receive the full dose of the drug.

As with the other species, the environment and method of observation can influence the behavior of the cat. Individual animals can be observed within (43, 47, 48) or when removed from their home cage (49). Multiple animals have been tested either as pairs of unrestrained, free-roaming cats (4) or as four cats restrained by leashes to allow only minimal overlap of animal test areas (6). The rating of individual animals allows for the notation of behavioral and physiological signs influenced by the interaction with the observer or with other aspects of the test environment, but does not allow for the evaluation of social interaction between test animals. This disadvantage

can readily be overcome by using multiple animals in the test sequence. As noted before, Irwin *et al.* (6) chose to rate multiple animals within a sound attenuated room to minimize external stimuli, while others (4) rated the animals in a regular laboratory room which allowed for the animals to react to random sounds from the external environment.

The rating scale should be developed with an intimate knowledge of normal behavior of the cat in the surroundings in which the test is to be conducted. It would appear that the test procedure and environment should generate as much behavior as possible in the test animals. The behavior patterns to be observed should be reliable and consistent within the test sequence. As an example, the authors, during the preliminary development of a cat rating program, presented to the cats a mouse within a plastic chamber. All of the cats did not respond with the typical mouse-kill pattern of behavior, even when the lid was removed from the plastic cage. This type of test then could not be used as a part of the behavioral scheme unless the cats were screened for this specific reaction. This type of selection, involving the elimination of various cats because of the lack of reactivity to certain test sequences, reduces the heterogeneity of the test sample. It would appear to be a better course to randomly select a series of healthy cats and build the test around these animals rather than to find the animals to suit the test.

Various types of rating scales have been developed for cats. Norton and deBeer (43) examined behavior patterns of individual cats and selected four main rating categories (sociability, contentment, excitement, and hostility). Sociability and hostility were categories selected to represent opposite reactions of the animals directed toward the observer, while contentment and excitement were selected to represent opposite patterns reflecting the emotional attitude of the cat in his accustomed surroundings. Each of these main headings had five subheadings of behavioral signs, and the scoring system was based on the frequency of occurrence of these signs. Using this procedure, drugs were administered orally to cats; and it was noted that chlorpromazine, among the compounds tested, reduced sociability to the least degree, but produced the greatest reduction in hostility. Sharma *et al.* (47), using the same type of rating profile, also showed a decrease in sociability with chlorpromazine and reserpine. Irwin (6, 13) used a much more comprehensive scoring system for the evaluation of drug effects. Cats were restrained on leashes so that they had approximately 1 ft. of interanimal overlap to allow some play or aggressive activity. The observer conducted the experiment for 5 hr. following drug administration. During this time the animals were rated on major changes of behavior, changes in interaction with the observer, and changes in interaction between the animals. Items rated included: time to sleep, lying down, alertness, curiosity, reactivity, locomotion, restlessness, stereotypy, grooming, vocalization, effect (playful, placid, fearful, aggressive), staggering gait, pupil size, heart rate, respiratory rate, limb weakness, relaxation of the nictitating membrane, and deep sleep. Chlorpromazine produced a diminution of play, grooming activity, fearfulness, or aggressiveness, especially in low doses. Hostile behavior was almost always suppressed, but some animals did show an increased fear or aggression after drug administration (45). Kinnard *et al.* (4) used a 32-item rating scale which included measures of social interaction, interaction with the observer, and general behavioral or physiological changes of the individual animal. Activity of the pairs of free-roaming cats was stimulated through the use of play objects and periodic presentations of catnip to the animals. Using this method, chlorpromazine or perphenazine, imipramine, pentobarbital, and *d*-amphetamine could be readily differentiated from each other. Data output from this and other multi-item rating programs tends to become voluminous, and a need for data reduction is apparent. Through the use of a computer program, adapted from one used for the clinical rating of patient symptoms, the 32 variables in the latter test were reduced to nine factors of behavior. In the past, investigators have preselected behavioral patterns and then subdivided these patterns through the use of specific animal actions, such as the subdivisions of yowling, hissing, and piloerection for aggression. Through the use of the computer program, the results of a test can be grouped into major factors based upon the intercorrelation of the responses of the animals to the experimental drugs. This type of analysis of the data may lead to a more efficient definition of the drug activity.

Rice and McColl (50) rated cat behavior using a small profile that included autonomic signs, somatomotor effects, and behavioral effects. The behavioral signs rated were howling, habit change, and hostility. Cole and Glees (51) rated the ability of cats to obtain food from a horizontal glass tube, their ability to walk along a ladder to obtain this food, and their performance in a placing reflex test involving the hind legs.

The procedures previously described have used

the normal behavior of laboratory cats as their baseline. However, it is quite conceivable that the effect of the drug on this type of behavior might not correlate with the drug's clinical activity. Investigators have attempted to induce abnormal behavior in cats and then test drugs upon this altered activity. Masserman (52) trained cats to open a box to receive a pellet of food following a light signal; after learning this event, the cat was trained to turn a switch to initiate the program. The animals were then subjected to an air blast or a mild shock which induced a motivational conflict behavior between conditioned hunger and fear. Under these conditions the animals developed startle and phobic reactions to sound and light stimuli as well as other neurotic behavioral patterns (53). Jacobsen and Skaarup (54, 55) have used this technique to study psychotropic drugs. They noted that chlorpromazine, in total doses of 0.1 to 2.0 mg. s.c., did not alter the neurotic reaction in cats, although benactyzine did. This type of response might be compared to the conditioned emotional responses (CER) used in rat behavioral techniques. Mixed results have been obtained from this procedure, and it was reported that chlorpromazine and reserpine did not alter the emotional response of the rat in this test (56). It is possible that this specific type of conflict behavior in the cat may not be a definitive test for all types of psychotropic drugs. Sacra *et al.* (57) produced a conflict behavior in cats by administering shock through the tail of a mouse whenever the cat attacked the mouse. The cat thus received a shock when it went to pick up the mouse. Following several presentations of this response, a conflict behavior pattern developed. Chlorpromazine and meprobamate were found to be effective in protecting against this type of response.

The environment and observer also play a major role in the final drug effects observed in the conflict studies. Masserman (58) noted that sedative and tranquilizing drug effects were greater when the test animals (cats or dogs in conflict behavior) were in the accustomed security of their home cages than when in a state of alert anticipation during transportation to the laboratory; the new environment, on the other hand, enhanced the stimulant effects of the test drugs. He also noted that the drug effects were dependent upon the difference in handling of each animal by different experimenters no matter how constant the research protocol was. It has been pointed out that observer reliability and consistency is extremely important and that this should be rated within the test program (59). The age of the animal used is also important. Pechtel *et al.* (60) showed that kittens, compared to older cats, adapted less well to laboratory routine and learned tasks of lever pressing and audiovisual discrimination. The young animals readily developed neurotic patterns under the stress of adaptive conflict.

Observational procedures have been used in experiments that involved different routes of drug administration or types of pretreatment. Feldberg and Sherwood (61) have analyzed the behavior of cats following the injection of test drugs into the lateral ventricle of an unanesthetized animal. Behavioral changes were not rated, but were noted in a general manner. Haley and Dasgupta (62) observed the changes caused by an intracerebral injection of LSD in conscious dogs and cats, but again no rating scale was used. Elder and Dille (63) administered LSD to singly caged cats and rated autonomic responses, spontaneous behavior, and response to types of stimuli (auditory, visual, and tactile). Various pharmacologic agents were then used in an attempt to antidote the LSD response. Sturvesant and Drill (64) analyzed the effect of mescaline on the behavior of cats. Rowe *et al.* (65) noted the behavior of reserpinized cats following monoamine oxidase inhibitors, and included photographs of the cat's behavior. Burdock *et al.* (66) analyzed the behavior of laboratory animals before and after the production of hypothalamic and midbrain lesions in the animals. Behavior of the animals can also be observed and rated in conjunction with neurophysiological recordings of brain activity (67).

Observational Procedures with Other Species.—The dog has been used by many investigators as a test animal for observational techniques, but apparently it is secondary to the cat in these procedures. Lang and Gershon (68) have used the dog in a procedure involving the intravenous administration of yohimbine to the animal. The induced behavior before and after the use of potential antagonists was then rated. The rating program presented was extremely well defined with 17 main divisions of behavior, each with a specified 3-point scale.

The monkey would appear to be the ideal test animal to use in observational techniques, especially if one considers its place on the phylogenetic scale. However, the use of the monkey, possibly because of costs and other reasons, is not so widespread as it should be in this type of pharmacologic program. Many investigators (69–75) have tested potential psychotropic drugs on monkey behavior. The viciousness of monkeys, such as the rhesus and the cynomolgus,

can be used as a tool for the evaluation of potential psychotropic agents. The animals, as in the rodent or cat studies, can be tested singly or in a procedure which involves chained pairs of monkeys to allow interaction (74). Knapp *et al.* (70) tested chlorpromazine and piperacetazine in dogs and squirrel monkeys, and noted that the drug effects may have been more discernible in the dog than in the monkey. In spite of this, the monkey should still be considered a prime test animal for observational studies since its behavioral patterns, in comparison to other laboratory animals, more closely resemble those of the human.

INSTRUMENTAL TECHNIQUES

Spontaneous Locomotor Activity.—Methods for measuring small animal activity have been used since the turn of the century. The devices were, for the most part, mechanically operated and used by psychologists to study the normal behavioral patterns of laboratory animals (76, 77). However, the search for psychoactive agents gave impetus to the need for instruments which would accurately assess the ability of these compounds to alter the normal spontaneous locomotor activity of small animals.

Activity recording units can be classified into four main groups involving four different types of activity cages: those which are immobile and record activity independently of cage movement (photocell activity cage); those which rotate about a central axis as the animal runs (treadwheel); those cages which are vertically or horizontally displaced as a result of animal movement (jiggle cage); and those which tilt on a fulcrum (tilt cage). The first type (fixed) is considered a direct recording apparatus because animal movement itself is recorded, whereas the latter three instruments are the indirect type because they basically record cage movement rather than animal movement. Since Riley and Spinks (78) reviewed the early prototypes of these instruments in 1958, this phase of the paper will emphasize the types of activity recording units developed since then.

An ideal device for measuring spontaneous activity would be one that is (*a*) sensitive to all types of motor movements, (*b*) sensitive to minor changes in animal activity, (*c*) free of positive feedback of stimuli, (*d*) free of carry-over momentum, (*e*) independent of animal weight variation, (*f*) capable of delineating between the activity of animals receiving placebo and animals treated with small doses of psychotropic compounds, (*g*) capable of recording a stable baseline of activity over short trials, (*h*) capable of simple operation and adjustment, (*i*) capable of being used with a simple direct-reading digital recorder. Needless to say, an instrument possessing all of these characteristics has yet to be developed.

The photocell activity cage has been used extensively as a drug screening device to determine the effects of psychotropic compounds on the spontaneous activity of small animals. It operates on the photocell system, in which light beam interruptions due to animals in motion are converted into electrical impulses which are transmitted to digital counters. Activity of the animal is thus reflected as a summation of light beam interruptions due to lateral movements. Many modifications of the photocell activity cage have been described in the literature and reflect the attempts of investigators to maximize its sensitivity. Dews (79) utilized a rectangular single beam unit to study psychomotor stimulants; Winter and Flataker (10) also used a single beam but reflected it twice off the sides of the cage; Kinnard and Carr (80) used a circular single beam unit to determine the activity patterns of central nervous system depressants, *e.g.*, secobarbital and meprobamate. A circular two-beam unit was utilized to study the ataractic properties of chlorpromazine and sodium pentobarbital (81). Most investigators employ horizontal beams; however, an apparatus with a single vertical light source has been used satisfactorily (82). The beam is directed from the ceiling of the unit downward onto several photocells placed below a Lucite floor. The use of a single light source precludes the possibility of variations in light intensity among the beams as in units with multiple light sources. The disadvantage is that fecal boluses may block off the operation of the vertically directed beams. Woodard (83) designed an apparatus with six photoelectric cells equally spaced around the perimeter of a circular raceway. A single light source was housed in a circular compartment in the center of the cage. This design takes advantage of the natural instinct of the rodent to confine its activity to the periphery of any confined environment; however, it places limitations on the spontaneous activity which satisfies the animal's strong instinct for exploratory behavior.

Tedeschi *et al.* (84) used another approach to the evaluation of psychoactive compounds on the motor activity of rats. They devised a photocell unit which was only large enough for a rat to rear up but precluded him from moving laterally. Rats placed in such a test situation at first elicited exaggerated behavior which progressed to complete inactivity toward the end of their

confinement. The authors suggested that drugs which reduce motor activity can be tested during the initial period of increased activity and, conversely, drugs which increase motor activity may be tested during the latter part of confinement when activity is markedly reduced. The effects of established psychoactive agents were measured in both the confined motor activity (CMA) test and the conventional photocell activity cage. Caffeine, in an oral dose of 5 mg./Kg., produced a 200% increase in activity in the CMA test; whereas, 5 to 350 mg./Kg., orally, did not increase the activity level in the conventional photocell apparatus. Similarly, tranylcypromine (5 mg./Kg. orally), a clinical antidepressant, produced a 200% increase in activity in the confined motor activity test; whereas, the chronic administration of tranylcypromine (5 mg./Kg., orally, twice a day) failed to produce any consistent or significant effects on motor activity in the conventional apparatus (85). In the CMA test, the effect of *d*-amphetamine was greatly magnified in that 0.24 mg./Kg., orally, increased activity 200%; whereas, in the conventional motor activity test, an oral dose of 5 mg./Kg. produced only a 40% increase in activity. A significant limitation of the conventional photocell activity cage was noted (86) when an oral dose of 16 mg./Kg. of *d*-amphetamine was administered to mice. This dose produced a decrease in lateral movement and induced tremors of the head and limbs analogous to the well-known clinical side effects of restlessness, irritability, and emotional disturbance. This type of activity resulted in a decrease in beam interruptions in the conventional unit, but under the CMA tests, may have been accurately registered as an increase in activity. Thus, by monitoring only one component (rearing) of spontaneous activity, Tedeschi has been able to correlate the preclinical and clinical effects of well-established stimulants and antidepressants. The CMA test was also effective for quantifying the effects of drug which decreased spontaneous activity, but the sensitivity was not significantly greater than that of the conventional photocell unit. The data suggest that it may not be necessary to measure total activity but only selected components of activity known to be affected by certain classifications of compounds. The problem is that psychotropic agents have a diffuse action on the nervous system and psychopharmacological techniques in existence today cannot, with certain exceptions, delineate the component effects of drug action.

Another novel approach to the recording of animal activity was the use of electroconductive slats placed across the floor of the test cage (87). Each slat was made of varnished Masonite and painted with silver-based conductive paint. As each animal made contact with the slats, an electronic relay activated an externally powered counter. This appears to be a satisfactory method of recording locomotor activity of small animals. It is less bulky than the photobeam units and can readily be installed to activate timers and measure areas of the test environment explored. Mitchell (88) devised another type of unit in which the lateral motion of an animal on a galvanized iron plate caused it to move slightly, inducing an electric current in a coil with a bar magnet core. The currents thus produced were then amplified electronically and recorded as activity.

More sophisticated methods of monitoring small animal activity have been developed, such as the transmission of vibrations produced by animal motion, using a piezo-electric head (89), a 30-gauge galvanized steel diaphragm (90), or a crystal phonograph cartridge (91). In most of these procedures, activity is recorded by means of a stylus recorder, resulting in records which are difficult to quantify. Alvarez-O'Bourke (91) solved the problem of quantifying such graphs by eluting each record with acetone and quantitatively evaluating it with a spectrophotometer on the principle that the amount of ink laid down by the pen is proportional to the frequency and intensity of the movements of the animal. Otis (92) designed a chamber which operated on a floor displacement principle. Normal floor vibrations which occurred during locomotion were transduced into modulated electrical signals that operated from 1 to 3 electromagnetic counters depending on the intensity of floor movements. Only downward motion was recorded, and the counter circuits were preset so that one counter will fire at the slightest movement of the animal, a second counter only if the animal's activity is moderate, and a third, only if violent activity occurs. The value of this apparatus is that it detects both the occurrence and intensity of movement, which the photocell cage is not capable of. Otis attached this device to a treadwheel so that the experimental animals would have access to either unit. Therefore, he was able to compare the effects of psychoactive compounds on activity with both devices separately and combined. The data of his studies indicated that the treadwheel failed to discriminate between intraperitoneal injections of placebo and 2.5 mg./Kg. of amphetamine, 30 mg./Kg. of imipramine, and moderate doses of other psychostimulating agents. In contrast, the floor displacement apparatus did delineate between

placebo treatment and equivalent doses of the same stimulating drugs. The over-all conclusion of this study was that the combined chamber–treadwheel combination was the most sensitive method for detecting drug effects. Knoll (93) developed an electronic device in which an animal moved over four aluminum plates, and counters registered every crossing from one plate to another. The advantage of this method is that as many as 24 mice can be tested at one time. Shillito (94) measured mouse locomotor movements by a capacitor system including six brass probes. When an animal moved past a probe, its capacitance was altered, resulting in an imbalance in the electrical circuit, which caused a dot to be recorded on a moving kymograph. The data were analyzed by merely counting the dots, and as many as four mice were tested simultaneously without causing an overlapping of marks. This device is similar to the "antenna cage" (95) in which a rat moves around a radio antenna placed in the middle of the cage; his movements change the capacity between the cage and the antenna. The latest electronic activity device[1] generates a soundwave of a frequency and level that cannot be detected by most animals. Any motion within an experimental cage into which this sound wave is directed produces disturbances in the received portion of the wave, causing the receiver to produce electrical impulses which can activate many types of recording and counting devices.

Melander (96, 97) used a photographic technique similar to Rothlin and Cerletti (98). The experimental mice were painted with a dye which emits visible light when activated by ultraviolet light. The animals were then exposed for 5 min. to panchromatic film. The resulting films afforded the investigator information on the types of movement, but they were not quantifiable.

Several modifications of the tilt-type activity cage have been described in the literature. Basically, the cage pivots on a central fulcrum, tilting in response to the weight of the animal as he moves from one part of the cage to the other. Sensing contacts (microswitches), below the platform, register the gross movement of the animal on digital counters. The apparatus is sensitive only to movement in line with the sensing contact on the platform below. Minor movements such as rearing, grooming, etc., which are not large enough to tilt the cage are not monitored. Tilt-type units have been described by Campbell (99) and Kissel (100), and the latter reported a lack of habituation to the unit probably due to the positive feedback typical of all moving-type devices. The limitation of these units is that only running activity is recorded to the exclusion of important minor movements (tremors, etc.) which may be drug induced. This limitation was overcome by Caviezel *et al.* (101), who added a closed air-Marey tambour system which recorded activity by means of a work adder. Thus, while the microswitches below the tilt platform recorded running movements, the downward displacement of the ball-bearing fulcrum set in motion the tambour membrane, and this recorded minor activity. Bastian (102) and Bourgault *et al.* (103) utilized a rectangular cage which tilted on a wide metal fulcrum, perpendicular to its axis; thus, an animal must run along the long axis of the cage in order to register a count; movement perpendicular to the long axis of the cage was not recorded. Also, minor movements were not monitored, and various types of activity were not differentiated as is true of other instruments of this class.

The jiggle cage has been used extensively to evaluate the effects of drugs on small animal activity. Earlier designs were usually of the spring-suspended type in which animal movement vertically displaced the freely swinging cage (104). Several interesting modifications of the jiggle cage have been described. Cho (105) used a 500-Gm. Toledo scale for the rat and a 500-Gm. dietetic scale for the mouse; movement of the animal vertically displaced the platform of the scale causing upward and downward deflections of a pen writing on a moving kymograph. Schallek (106) and Sandberg (107) used a unit (Williamson Development Co., West Concord, Mass.) in which the cage was suspended on a resilient cantilever beam which permits slight sideways motion of the cage in response to the animal's activity. The sideways motion is proportional to the acceleration imparted by the animal. Such motion caused a switch to close momentarily whenever the integrated accelerations reached a predetermined level. A similar apparatus was described by Chappel (108); the cage was suspended by a ball and socket joint from a spring steel or spring bronze cantilever beam. Movements of the animals were recorded by electrical counters activated by contact of the cantilever with a stationary screw. The sensitivity of the apparatus can be adjusted for body weight differences by turning the screw regulating the gap between the stationary contact and the cantilever beam. The advantage of this unit is that small movements such as

[1] Ultra-Sonic Motion Detector, Alton Electronics Co., Gainesville, Fla.

grooming, biting, gross respiration, head bobbing and swaying can be recorded, although the movement of the cage, itself, is relatively slight. A jiggle platform (Lehigh Valley Electronics, Fogelsville, Pa.) has been developed which oscillates horizontally on a set of ball bearings. Sudden movements, *e.g.*, turning, running, jumping, etc., cause the platform to move slightly, making and breaking an electrical contact. A wide range of sensitivity is accomplished by the raising or lowering of a conical pendulum into or out of a metal ring. However, this apparatus is only suitable for measuring the activity of rats or hamsters and cannot monitor mouse activity. In general, the suspended jiggle cage has several limitations. First, most of the units lack a dampening effect so that carry-over momentum generally exaggerates the amount of activity; second, it is a moving or mobile unit which may generate a small amount of positive feedback stimuli; third, the animals satiate rapidly so that accurate estimation of peak drug effect is required or else the effect will be missed; finally, the units are difficult to calibrate; attempts to maximize the sensitivity of such cages in order to pick up minor movements result in excessive residual excursions after animal activity has stopped; on the other hand, decreasing the sensitivity precludes the monitoring of minor movements. An advantage is that various types of behavioral patterns can be monitored on a pen recorder; however, such records are difficult to quantitate and are only useful for *visual* comparison of the changes in behavior elicited by various drugs.

The revolving treadwheel turns as the animals run or walk, and revolutions in either direction are recorded on counters. For a review of the early units (prior to 1958) the reader is referred to Riley and Spinks (78). Skinner (109) also reviewed the parameters of the exercise wheel in 1933. Because of the large positive feedback generated by animal movement, habituation to this unit does not occur so that it is possibly the only unit in which cross-over studies can be carried out (110). This apparatus is capable of detecting the stimulating effects of monoamine oxidase inhibitors (110) which cannot be monitored with immobile units. Irwin (110) indicated that the treadwheel can also detect doses of drugs which disorganize behavior. Because of the mobility of the apparatus, animals generally sustain their behavior over a long time interval and, therefore, it appears that the unit is of more value in the study of depressants where it is desirable to obtain stable, sustained levels of activity as a baseline. In order to study stimulants in this type of unit, Wiedemeijer *et al.* (111) limited the mice to a section of the turning wheel by fixing a partition within it causing the animals to produce a smaller baseline of spontaneous activity. Since the apparatus only measures running activity in revolutions, Royer *et al.* (112) increased the information output by attaching a tachometer–generator and recorded the output on an Esterline–Angus pen recorder.

Investigators have used both graphic and numerical recording techniques to illustrate drug-induced changes in spontaneous activity. Graphic recording illustrates types of activity (rearing, jumping, grooming, etc.), and, therefore, can indicate specific qualitative alterations on behavior; digital counters, on the other hand, reflect only quantitative changes. The tendency today is to record only quantitative changes, because it is relatively simpler and less expensive to attach some type of digital counter to the activity unit. However, this must be done with guarded caution, for each method of recording gives important information about drug effects. For instance, Tonini (113) has indicated that sedative, subhypnotic doses of barbiturates did not decrease numerical values in his actograph unit, but did alter the activity graphs (short, intense, and more frequent bursts of activity with long periods of rest between); whereas, tranquilizers, in therapeutic doses, decreased numerical values but did not alter the graphic record. Thus, both procedures should be used in the preliminary evaluation of psychoactive compounds.

Forced Motor Activity.—The neurotoxic effects of psychoactive compounds have been evaluated by "fall-time" tests which measure motor coordination of experimental animals. There are two types of "fall-time" methods: those using inclined planes, and those using rotating rods and cylinders. A discussion of the fixed incline plane procedure can be found in the paper by Riley and Spinks (78). The data are usually expressed as the average time that a group of animals can stay on or as the percentage of animals falling off within an arbitrary period of time. The first of the horizontal rod-type of instrument was the hollow screen cylinder devised by Young and Lewis (114) for testing insulin. Mice which could not hold on to the rotating cylinder fell into metal trays. It was later used in the assay of curare (115) and the measurement of sedation (116). The horizontal rod method appears to be the more popular device today because of the simplicity of construction and objectivity of measurement. It was used by Dunham and Miya (117) for detecting neurological deficits of psychotropic agents in rats and

mice; Herr (118) compared the effects of antidepressants and tranquilizers on the rotarod performance of mice; and Plotnikoff *et al.* (119), studying the effect of stimulants, was able to distinguish between amphetamine which enhanced rotarod performance and caffeine which was inactive; Kinnard and Carr (80) studied several types of depressants and suggested the combined use of the rotarod and the photocell activity cage to characterize and differentiate between various types of depressants. The objectivity of the apparatus was increased recently by an electronic circuit which automatically stops a timer when the animal falls to the compartmentalized platform beneath the rod (120).

Otis (121) assessed motor coordination by forcing animals to perform on a drum 18 in. in diameter. When an animal could not maintain his performance, he slipped to the back wall plate of his compartment, tripping a microswitch which transmitted an impulse to a digital counter. Both the number of times and the total time the wall plate was depressed were recorded.

An important factor which has been neglected in forced motor studies is the "free ride" animals will take periodically throughout the trial. A "free ride" is defined as one revolution in which the animal holds on without walking. This could result in the failure to detect minor depressive actions of drugs and neurotoxicity, because a drug might decrease motor coordination without impairing the ability of the animals to wrap themselves around the rod and ride. In one study (86) the average per cent rides for the training trials ranged from 0.2–11.1%, and the range among the placebo-treated animals was 0.0–10.5%. While 0.2% riding may be insignificant, especially at the higher speeds and longer time trials, the higher value of 11.1% may mask drug effects. The results may be affected to a greater degree when large doses of a depressant drug are administered, because the animals may have enough strength to hold on, even though the drug has affected their coordination. Per cent rides as high as 57.6% were recorded with 16 mg./Kg. of chlorpromazine, administered orally to mice. It is suggested that investigators observe animal performance carefully and take into consideration the possibility that erroneous conclusions concerning drug effects may be drawn from such data.

Parametric Influences on Drug Response.—Although the literature is filled with many papers dealing with the effect of psychotropic drugs on motor activity, very few investigators have been concerned with the various parameters which might significantly influence drug response as measured in the various devices described above. Such parametric effects may differ not only among the various classifications of compounds but from drug to drug within a single classification. Thus, by experimental manipulation of environmental factors, a drug may be made to have a stimulant effect, a depressant effect, or no effect on motor activity (122). It is, therefore, of extreme importance that investigators take into consideration the parameters which have been shown to influence drug response and utilize the combination of variables which will produce the optimum results. For instance, test and housing aggregation size, sex, illumination of the test environment, availability of food prior to the test, and route of administration can be powerful determinants of the magnitude of drug effect. Wright *et al.* (83) reported that promazine produced an immediate equal decrease in the activity of individual mice and groups of mice; whereas, pentobarbital in low doses produced a greater increase in the activity of animals tested singly than those tested in groups. Brown (123) found no differential effect of chlorpromazine on the activity of single and grouped mice, but Watzman *et al.* (124) reported that chlorpromazine had a greater depressant effect on activity of mice tested in aggregations of five than on those tested singly. This drug–aggregation interaction may indicate that chlorpromazine potentiates the tendency for grouped animals to clump together and thus register relatively low activity. When dose-response curves for test aggregations of one, five, and nine animals per test unit were compared (125), the slopes indicated that chlorpromazine produced a greater depressant effect on the larger test aggregations than on animals tested singly; furthermore, the slopes for both multiple animal groups were approximately the same so that there appeared to be no advantage in using as many as nine (126) animals per test unit. It has also been reported (125) that chlorpromazine produced a significantly greater effect on animals housed and tested under the same aggregation conditions than those housed and tested under different conditions. Since amphetamine has been shown to stimulate aggregated animals more intensely than isolated animals (127), the group situation gives evidence of accentuating both depressant and stimulating drug effects.

An experimental condition producing a powerful effect on drug response is the illumination of the test environment (125). It is well documented that the spontaneous activities of rodents (128) and rabbits (129, 130) are affected by illumination conditions. Chlorpromazine had a greater effect

on spontaneous motor activity of mice tested in the dark than in the light during the latter half, but not the first half, of a 2-hr. test (125). Under the placebo condition the decrement in activity of mice in the last hour of the trial was much steeper and decreased to a much lower level in the animals tested in the illuminated condition. Testing in the dark produced no increase in activity in the initial exploratory phase but did greatly decrease adaption during the latter part of the session. Apparently, the high curiosity and exploratory behavior in the initial part of the test was powerful enough to overcome the effects of illumination.

It has also been reported that the activity of females was consistently higher than males in the photocell activity cage, and that chlorpromazine had a significantly greater depressant effect on the spontaneous activity of females than males, during the first 0.5 hr. of the trial (125). It was thus concluded that conditions which elevate normal activity (aggregation, darkness, female sex, same housing, and test groupings) enhance the depressant effect of chlorpromazine on spontaneous activity. On the other hand, when the normal activity level is low, such as at the end of a test session in an illuminated environment, the effect of chlorpromazine is slight. The influence of these factors appears to be class-specific. For instance, while the activity response to perphenazine and chlorpromazine (phenothiazines) is influenced by the same environmental factors and in the same direction, the response to pentobarbital is not (83, 126). The response to pentobarbital was not differentially affected by test illumination, as was the response to phenothiazines; however, it did produce a consistently greater effect on fasted than satiated animals, while responses to the phenothiazine compounds were not differentially affected by the feeding condition of the animals. The authors recommended (125) that the most sensitive measure of chlorpromazine effect in the photocell activity cage could be obtained by testing an aggregation of five female mice for a period of 0.5 hr. beginning 30 min. after i.p. administration of the drug (86).

Another parameter, often neglected, which may alter response to a drug is the difference in sensitivity among the measuring units. If more than one instrument is being used, then the investigator must demonstrate equality of sensitivity (131). Failure to do so may lead to inaccurate interpretations of drug effects. Since there is a difference in sensitivity among photocell units, even from the same manufacturer (125), it is imperative that the various levels of all experimental variables be exposed to each unit in a factorial design or else all animals under all conditions should be tested in a single unit.

The location of the photocell beams is also an important determinant of drug effect on motor activity. The use of two right-angle (crisscross) beams yielded a better delineation of low doses of chlorpromazine than three parallel beams (132). However, there was a greater drug effect with the two peripheral beams than middle beam in the parallel arrangement, with no significant drug–beam interaction in the crisscross arrangement. The data suggested that the most sensitive measure of chlorpromazine effect would be with an arrangement of two pairs of peripheral beams at right angles to each other.

The importance of evaluating drugs at certain test intervals is borne out by Dews (79) in measuring the influence of certain drugs on the locomotor activity of mice with the photocell activity cage. He found that the initial part of the trial (first 15 min.) produced more reliable data than any other test interval. This is the period of exploratory hypermotility which occurs when animals are first placed into a new environment. The high reproducibility of the data during this period is probably due to the fact that the animals search to the same degree to satisfy their curiosity and, therefore, the variability of movement among the animals is small. Borsy *et al.* (133) studied several classical tranquilizers by their ability to inhibit this orientational hypermotility. This appears to be a sound approach to the evaluation of depressants because the test compounds were made to challenge a natural, unlearned reaction similar to the clinical situation for which these compounds are used. Bonta (134) reinforced this exploratory behavior by moving mice from a large rectangular cage in a dark room to a small round test cage in an illuminated room and measured spontaneous activity for the first hour only. Reserpine, azacyclonol, benactyzine, and chlorpromazine abolished this hypermotility, but meprobamate was successful only in ataxic doses. These tests are in contrast to the studies in which drug-induced hyperactivity is used as the baseline of performance (135, 136).

Otis (92) illustrated the importance of evaluating drug effects over long, as well as short, periods. He reported that deanol, phenelzine, and imipramine produced quantitative differences in spontaneous activity relative to placebo controls, depending on whether they were tested for 3 or 16 hr. The data suggested that different drugs may require different testing periods in order to achieve significance.

Irwin (137) has reported on the influence of

internal and external factors on spontaneous activity in the treadwheel; female rats were more responsive to drug effects than male animals; hyperactive animals were found to be significantly more responsive to both stimulant and depressant drugs than hypoactive animals. In the female rat, peak running activity occurred every fourth day in correlation with the estrus cycle (138). Older rats performed more intensely but with shorter spurts of activity and maximum treadwheel activity was observed in animals between 87 and 120 days (139), 175 days (140), and 300 days old (141). Jones *et al.* (142) reported that running activity in the wheel varies inversely with age and directly with experience although these relationships are not linear. Desroches (143) also found that activity in the treadwheel declined with age but reported that prior experience in the unit did not influence this decrease. The discrepancy in the literature is probably due to differences in environmental conditions, such as temperature, illumination, and sound levels of the experimental room. Ström has described extensive studies on the suitability of the treadwheel for the evaluation of substances having potential central depressant action (144).

The literature is sparse with regard to the influence of experimental conditions on rotarod performance. However, there is some evidence that manipulation of speed and rod diameter alters the magnitude of drug response. Plotnikoff *et al.* (119) studied the effect of amphetamine and other stimulants at three different speeds (11.44, 18.30, and 29.28 r.p.m.) and reported that the effect of amphetamine was smallest at the lowest speed. Watzman *et al.* (86) found that chlorpromazine produced a greater depressant effect on mice when they performed on a 2-in. diameter rod rather than on a 1 or 1.5-in. rod.

It is apparent from the foregoing discussion that environmental and experimental factors must be carefully controlled; for, as mentioned before, the effects of drug on behavior are largely a function of the situation in which they are studied.

Repeated Tests of the Same Animals.—The advisability of re-using animals in experimental situations has been controversial and was briefly discussed earlier. It is highly desirable, if possible, to give repeated tests to the same animals when evaluating the effects of psychotropic agents on behavior. Not only is it an economic advantage, but a more sensitive test is obtained because the consistency of individual performances usually found in repeated tests means a smaller variation in scores against which the effects of drug can be measured. On the other hand, the disadvantage of repeated tests on the same animals is that both drug and behavioral carry-over effects may occur from one test to the next, especially when there is a short time interval between sessions. Adler (145), utilizing the photocell activity cage, found a greater depressant effect of tetrabenazine on the motor activity of rats in the second of two tests with a 1-week intertrial period. Rushton *et al.* (146) have shown that even a single brief experience in a Y-shaped maze, lasting only 3 min., markedly modified subsequent reactions to an amphetamine–amobarbital mixture. Ross and Schnitzer (147) reported an elevation of activity level of mice 2 weeks after the animals had been tested for locomotion while under the influence of a single dose of *d*-amphetamine sulfate. The same authors later showed in a separate experiment, that the drug was not directly involved in the later elevation of activity and theorized that the animals learned to be more active due to their prior treatment of the stimulating drug. Watzman *et al.* (124) tested mice twice in the photocell activity cage, 1, 3, 7, and 14 days apart. The scores in the second test were generally lower than those of the first test, and this behavioral carry-over effect was greater for groups given a second test at intervals of 1 or 3, rather than 7 or 14 days after the first test. Even at the 7 and 14-day intervals the recovery of the original activity level was not complete. A more important finding in this experiment was that chlorpromazine produced a greater depressant effect on activity in the first 0.5 hr. of the second session than of the first session. This effect was clearly not due to accumulation of the drug from the first session because the greater drug effect was found in the second session after the 14-day interval as well as after the 1-day interval. The authors concluded that investigators must be careful when using the same animals more than once in tests of drug effects. They recommended that the sequence be counterbalanced giving placebo first to half of the animals, and the drug first to the other half. The high over-all correlation in activity between the first and second sessions showed that the use of repeated tests on the same animals can increase the sensitivity of the test of drug even though there is a difference in performance and a different magnitude of drug effect between the two sessions. Thus, the effects of the drug can be tested adequately with a greatly reduced number of animals by the use of a repeated test design.

Statistical Treatment of Activity Data.—Parametric tests of statistical significance, such as the analysis of variance and *t* tests, assume

that the frequency distribution of scores is nonskewed and that the variance among the experimental groups is approximately equal. Usually, because of the extreme variations in activity performance among small animals, it is necessary to transform the data and thus normalize the frequency distribution. If this is not done, the non-normality is likely to be accompanied by a loss of power in the F and t tests and a corresponding loss of efficiency in estimation of treatment effects (148). Furthermore, extremely high raw scores are likely to lead to a misinterpretation of relative magnitudes if they are farther from the mean than the lowest scores. Logarithmic or square root transformation of data may normalize the distribution of scores by reducing the magnitude of the high scores more than the low ones. In a recent study of the photocell activity cage (124), a comparison of the raw, square root, and logarithmic forms of experimental data indicated that the raw scores were skewed in a positive direction with extreme high scores being much farther from the mean than were the extreme low scores. The logarithmic scores were skewed in the opposite, negative direction, whereas the square root scores were skewed to the least degree (positive direction). Also, spontaneous activity appeared to be most stable when the scores were in the square root form. Irwin (110) has used square root transformation data on locomotor activity in the treadwheel, and Kissel (100) found it necessary to transform tilt cage data into logarithmic form. The skewness of a frequency distribution can be roughly estimated by comparing the relative distance of the highest score and the lowest score from the mean or median value. A more refined procedure (149) requires the estimation of the mean (M), median (Mdn) or mode, and standard deviation (S.D.). Either of three equivalent equations may be used as follows:

$$\frac{\text{M} - \text{mode}}{\text{S.D.}} = \frac{\text{M} - [\text{M} - 3(\text{M} - \text{Mdn})]}{\text{S.D.}} = \frac{3(\text{M} - \text{Mdn})}{\text{S.D.}}$$

The value of a normal frequency distribution is 0, and therefore data which are distributed in a manner approaching normality would have a value close to 0.

Because reliability and reproducibility of data are of extreme importance to scientific investigators, it is desirable to use the most sensitive measure of reliability. One statistical procedure which is extremely useful is the split-half method (150), in which stability of performance for each animal within the same session can be readily computed. For example, if the spontaneous motor activity of an animal is measured over a 2-hr. period in 15-min. segments, the performance of the even time intervals (2nd, 4th, 6th, and 8th) is correlated with the scores recorded in the odd time segments (1st, 3rd, 5th, and 7th). The resulting correlation coefficients can then be tested for statistical significance of differences by the method described by Edwards (151).

Rotarod performance of rodents treated with central nervous system depressants has been recorded as the per cent decrease in performance times (81) or the per cent of animals (118, 152, 153), falling off at a predetermined time (all-or-none method). A more consistent dose response effect was obtained for chlorpromazine when the "performance time" method of recording was used (87). The all-or-none method, in principle, does not appear to be satisfactory. If an animal scores 179 sec. under a low dose and 20 sec. under a higher dose in a 3-min. trial, a satisfactory dose response curve would be achieved under the time-response procedure; whereas, under the all-or-none method, 100% depression would be reported for both doses. There is even some question as to the suitability of the "performance time" method of recording the data. Because all trials are terminated at some point of time, the data are usually skewed with the majority of trained animals scoring at the "ceiling" or maximum time limit of the experiments with some animals scattered throughout the middle and lower part of the frequency distribution. Therefore, parametric tests of significance, such as the analysis of variance and t tests, cannot be properly applied for the reasons given above.

Since the drug effect may vary with environmental factors, it is desirable in activity experiments to study the effect of a drug over a range of conditions. This can be done efficiently by the use of the factorial design which permits broader generalizations to be drawn than a group of individual studies which are limited to a single set of conditions (154). The statistical treatment of activity data of this type can be efficiently analyzed by the analysis of variance test. It is a flexible statistical procedure which is capable of treating several levels of experimental variables simultaneously. By providing estimates of interactions between doses and the other variables of the experiment, it gives important information on parameters which govern or influence the appearance of a particular drug effect.

SUMMARY

It would appear that a total pharmacological evaluation of psychotropic drugs must include the use of at least two different animal species in an

observational program. If time and funds are available, a third species can be added. The rodent (mouse or rat) and the cat would seem to be the favored animals for this procedure with the monkey being the third species. The rodent screen should provide not only behavioral or pharmacodynamic data, but also toxicological information for the protection of animals used in subsequent studies. The rodent observational scale should include the signs characteristic of autonomic and central nervous system changes, and certain behavioral measurements obtained through the use of open field tests and the rating of signs, such as grooming, etc. More important, an additional sequence should involve the measurement of social interaction of rodents. This latter point is too often omitted from the typical observational scale. The cat can be used as a test animal once sufficient toxicological data has been obtained. It provides the stable baseline of behavior that may give a better correlation of drug effects than the data obtained from the rodent studies. The cat procedure should allow the generation of a high degree of behavior, and the rating scale should include a sufficient number of rating points so that information is not overlooked. The use of paired, free-roaming cats may be advantageous over other techniques in that their activity may be increased over caged or restrained animals. Data reduction is extremely important in these tests, and the use of certain computer programs may serve to fulfill this need. The clinical predictiveness of observational data is undoubtedly more reliable when the number of species involved in the tests is increased. The addition of the monkey to the rating procedure increases this reliability and provides some natural behavior patterns (aggression) that are not seen routinely in other test animals. Even with the additional species, preclinical predictiveness is at best difficult. To overcome this and other inadequacies, better models of behavior must be developed for the test animals. It would appear that the "experimental neuroses" developed in test animals should be re-evaluated and a new set of baselines formed. The present methods of developing conflict behavior by using adverse stimuli may not be the suitable method for attempting the differentiation of drug effects, but this would appear to be a major area for future work in observational research.

The fixed type of activity unit such as the photocell cage appears to be the preferable apparatus for monitoring small animal activity. It measures lateral motion directly and does not introduce any movement artifacts typical of the indirect type of activity cage. The indirect measuring units, such as the jiggle cage, are difficult to calibrate and the data, too often, represent frequency of cage movements rather than animal movements. The sensitivity of the direct type of activity apparatus has been vastly increased by investigators with the use of intricate electronic circuitry. Further efforts to increase sensitivity with still more complex and sophisticated instrumental design does not appear to be necessary. Present concepts of drug evaluation are based on the assumption that *total* movement must be measured in order to monitor drug effects sensitively. However, investigators (84) have accurately simulated the clinical effects of stimulants by recording only one component of behavior, while the recording of total locomotor activity failed to do so. This is not surprising in view of the fact that the primary effects of amphetamines in humans are nonlocomotor in nature (restlessness, irritability, anxiety). Similarly, depressant compounds may alter selectively only a few component behavioral patterns of total activity. Therefore, research should turn its attention toward delineating, if possible, the specific behavioral patterns affected by particular compounds or classification of compounds. If successful, the use of complex, expensive apparatus capable of recording every type and all degrees of movement would not be justified. Papers have been reviewed which indicate that the qualitative and quantitative effects of a drug are a function of the environment in which it is tested. Because such parametric influences vary from laboratory to laboratory, it is incumbent upon each investigator to determine the optimum set of experimental conditions for his laboratory.

Because of the great variability in activity between animals, proper statistical treatment of data is extremely important to the behavioral scientist. He depends to a great extent on the power of tests of statistical differences to give meaning to his data. Since parametric tests (analysis of variance, t tests) assume that frequency distributions are normal, it is imperative that investigators consider the transformation (log, square root) of raw data before applying these statistical tests.

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Research Articles

Synthesis of ^{14}C-Labeled Isomers of Dichlorodiphenyldichloroethanes (DDD)

By R. E. COUNSELL and ROBERT E. WILLETTE

1,1-Dichloro-2-(*o*-chlorophenyl)-2-(*p*-chlorophenyl-^{14}C)ethane (*o,p'*-DDD-^{14}C), 1,1-dichloro-2-(*m*-chlorophenyl)-2-(*p*-chlorophenyl-^{14}C)ethane (*m,p'*-DDD-^{14}C), and 1,1-dichloro-2,2-bis-(*p*-chlorophenyl-^{14}C)ethane (*p, p'*-DDD-^{14}C) were synthesized by acid catalyzed condensation of chlorobenzene-^{14}C with excess 2,2-dichloro-1-(*o*-, *m*-, and *p*-chlorophenyl)ethanols. The carbinols were prepared in good yields by reverse addition of the chlorophenyl Grignard reagent to dichloroacetaldehyde. Purity was determined by thin-layer and gas chromatography. The I.R., U.V., and NMR spectra of these compounds are discussed.

INTEREST in the development of radiopharmaceuticals suitable for adrenal photoscanning prompted the present study. For this purpose, an agent that selectively concentrated in the adrenal and could be labeled with a γ-emitting radionuclide was necessary. This paper describes the synthesis of ^{14}C labeled isomers of 1,1-dichloro-2,2-bis-(chlorophenyl)ethane (DDD) to be utilized in tissue distribution studies. The synthesis of other DDD isomers and ^{125}I and ^{131}I isomers will be reported elsewhere.

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Research Articles

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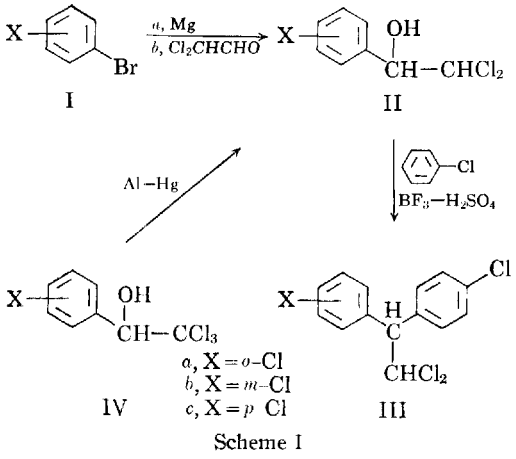
TABLE I.—2,2-DICHLORO-1-(CHLOROPHENYL)ETHANOLS

Isomer	moles (n)	Yield, %	B.p., °C./mm.
ortho	0.2	35	105–120/0.4[a]
meta	0.13	53	81–84/0.04[b]
para	0.4	63	118–123/1.2[c]

[a] Lit. b.p. 126–129°/3 mm. (12) and 115–123°/0.5 mm. (13). [b] Lit. b.p. 115–118°/3 mm. (14). [c] Lit. b.p. 140–145°/2 mm. (12).



Scheme I

Isomers of DDD were chosen for this study because they have been shown to have a predilection for adrenal tissue. During an investigation of the toxicological properties of various insecticides, Nelson and Woodard (1) observed that *p,p'*-DDD (III*c*) caused extensive necrosis and atrophy of the adrenal cortex in dogs. This observation led to unsuccessful attempts to utilize this substance clinically to produce a chemical adrenalectomy (2). Cueto and Brown (3) demonstrated, however, that the adrenocorticolytic activity ascribed to *p,p'*-DDD was actually due to the *o,p'*-isomer (III*a*) which was present as a contaminant. They also showed that *o,p'*-DDD concentrated to a greater extent than *p,p'*-DDD in the adrenals of dogs.

Several reports concerning the actions of *o,p'*-DDD have appeared recently. Studies have shown it to produce tumor regression in cases of metastatic adrenal cortical carcinoma (4) and to cause remissions of symptoms in patients with Cushing's syndrome (5, 6). It was found ineffective, however, for the treatment of mammary carcinoma (7). *o,p'*-DDD has also been found to have an effect on steroid metabolism and this aspect has received considerable attention. These studies have shown it to stimulate cortisol metabolism (8), to alter the extra-adrenal metabolism of cortisol (9), and to inhibit specific enzymes (10).

Although *o,p'*-DDD has been studied to a considerable extent, Nichols *et al.* (11) indicated that the *m,p'*-isomer (III*b*) was more effective in causing regression of adrenal carcinoma with fewer side effects. Despite the interest in *o,p'*-DDD and its isomers, tissue distribution studies with labeled compounds have not been reported. The first synthesis of these labeled DDD isomers is reported here.

EXPERIMENTAL[1]

Synthesis of Isomeric 2,2-Dichloro-1-(chlorophenyl)ethanols (II).—The Grignard reagent, prepared from the appropriate bromochlorobenzene (n mole), magnesium (n mole), and ether in a three-necked flask provided with a stopcock-fitted outlet on the bottom, was added over 1 hr. to a well-stirred solution of dichloroacetaldehyde[2] (n mole) in ether (total $n \times 200$ ml.) with ice cooling under a nitrogen atmosphere. After stirring an additional 30 min. at room temperature, 6 *N* sulfuric acid or concentrated ammonium chloride solution was added and the mixture separated and extracted with ether. The combined organic layers were dried ($MgSO_4$) and the ether removed at atmospheric pressure. The residue was distilled under reduced pressure. (See Table I.)

1,1 - Dichloro - 2 - (o - chlorophenyl) - 2 - (p-chlorophenyl-^{14}C)ethane.—A mixture of 2.26 Gm. (0.01 mole) of 2,2-dichloro-1-(*o*-chlorophenyl)ethanol, 29.7 mg of chlorobenzene-^{14}C (uniformly labeled, 0.50 mc.),[3] and 530.3 mg. (total 0.005 mole) of chlorobenzene was stirred at 40° and 6 ml. of boron trifluoride-saturated sulfuric acid added dropwise over 5 min. The mixture was heated at 40° with vigorous stirring for 3 hr. and then extracted with petroleum ether (b.p. 30–40°, 3 × 25 ml.). The combined extract was washed with water (2 × 25 ml.) and dried ($MgSO_4$ and charcoal). The filtered extract was concentrated under vacuum and the residue chromatographed on a column of 35 Gm. of silicic acid, prepared and developed with benzene–hexane (1:1). The eluate was monitored with a Geiger survey meter and the radioactive fraction collected and evaporated. The oily residue was taken up in methanol and refrigerated to give,

[1] Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Elemental analyses were performed by Spang Microanalytical Laboratories, Ann Arbor, Mich. Infrared spectra were taken in KBr disks on a Perkin-Elmer 337 spectrophotometer. Ultraviolet spectra were recorded on a Beckman DK2A spectrophotometer in 95% ethanol. The NMR spectra were obtained with a Varian A-60 spectrometer in $CDCl_3$ at a concentration of 10%, with tetramethylsilane as internal reference. Thin-layer chromatograms (TLC) were run with 1 in. wide Eastman Chromagrams, type K301R with fluorescence indicator, developed with benzene, and spots detected with U.V. light and iodine vapor. Chromagrams of ^{14}C-labeled compounds were scanned with an Atomic Associates RCS-363 Radiochromatogram Scanner. Gas-liquid chromatography (GLC) was carried out with a F&M, model 400, gas chromatograph equipped with an electron-capture detector; column, 122 cm. × 3 mm. i.d. glass tube; packing, 3.8% by wt. silicone rubber SE-30 on 80–100 mesh Diatoport S, prepared according to Horning *et al.* (21); column temperature, 185°; detector temperature, 210°; flash heater, 230°; inlet pressure, 40 p.s.i.; flow rate, about 70 ml./min. Radioactivity measurements were made with an Atomic Associates FC-72A gas-flow planchet counter and Baird-Atomic 146 scaler. Silicic acid used in the column chromatography was Baker and Adamson reagent grade, dried at 105° for 1 hr. just prior to its use.

[2] Columbia Organic Chemicals Co., Columbia, S. C.

[3] Purchased from New England Nuclear Corp., Boston, Mass.

in two crops, 656 mg. (41%) of colorless needles, m.p. 75–76° [lit. m.p. 76–78° (12) and 75.5–76.5° (13)]; specific activity 99.5 μc./mmole. It showed one spot (R_f 0.67) on a TLC strip, which scanned as a homogeneous radioactive area. $\nu_{max.}$ 3070, 3050 (Ar—H); 2980, 2930 (CH); 1480, 1465 (C=C); 1100, 1051, 1029, 770 (1,2-disubstituted Ar—H); 1083, 1040, 1010, 810 (1,4-disubstituted Ar—H); 757 (CCl_2); 608; and 509 cm.$^{-1}$. $\lambda_{max.}$ 229.5 (ε 16,970) and 267.5 mμ (ε 640). The NMR spectrum showed doublets at 5.21 δ (benzylic proton) and 6.38 δ (—$CHCl_2$) with J = 8.5 c.p.s., and a single aromatic-proton peak at 7.35 δ.

1,1 - Dichloro - 2 - (m - chlorophenyl) - 2 - (p-chlorophenyl-^{14}C)ethane.—A mixture of 2.26 Gm. (0.01 mole) of 2,2-dichloro-1-(*m*-chlorophenyl)ethanol, 29.7 mg. of chlorobenzene-^{14}C (uniformly labeled, 0.50 mc.)[3] and 530.3 mg. (total 0.005 mole) of chlorobenzene was stirred at 40° and 6 ml. of boron trifluoride-saturated sulfuric acid added dropwise over 1 hr. After heating at 40° with vigorous stirring for 1 hr., 2 ml. of cyclohexane was added. After heating 2 hr. longer, the mixture was worked up as above and chromatographed with benzene–hexane (1:4). The radioactive fraction was dissolved in an equal volume of 95% ethanol and after allowing to stand in the refrigerator 3 weeks gave 490 mg. (31%), m.p. 51–52°, which was recrystallized from ethanol to give 388 mg., m.p. 53–54° [lit. m.p. 54° (14)], specific activity 89.3 μc./mmole. TLC showed one spot (R_f 0.67), which was the only radioactive area on scanning. $\nu_{max.}$ 3050 (Ar—H); 2995, 2910 (CH); 1580, 1565, 1480, 1470 (C=C); 1090, 1072, 836, 785 (1,3-disubstituted Ar—H); 1085, 1040, 1009, 808 (1,4-disubstituted Ar—H); 759 (CCl_2); 615; and 502 cm.$^{-1}$. $\lambda_{max.}$ 219 (ε 19,770), sh. 226.5 (ε 16,700), and 268.5 mμ (ε 800). The NMR spectrum showed doublets at 4.57 δ (benzylic proton) and 6.31 δ (—$CHCl_2$) with J = 8.0 c.p.s., and an aromatic peak split at 7.26 and 7.29 δ.

1,1 - Dichloro - 2,2 - bis - (p - chlorophenyl - ^{14}C)-ethane.—A mixture of 2.26 Gm. (0.01 mole) of 2,2-dichloro-1-(*p*-chlorophenyl)ethanol, 107 mg. of chlorobenzene-^{14}C (uniformly labeled, 0.50 mc.),[4] and 456 mg. (total 0.005 mole) of chlorobenzene was stirred at 40° and 6 ml. of boron trifluoride-saturated sulfuric acid added dropwise over 30 min. The reaction was conducted and worked up as described for the *meta* isomer above.

The radioactive fraction afforded from methanol 694 mg., m.p. 107–110°, which was recrystallized from methanol to give 602 mg. (43%), m.p. 109–110° [lit. m.p. 109.5–110° (12)], specific activity 106.9 μc./mmole. TLC showed one spot which corresponded to the only radioactive area on scanning (R_f 0.72). $\nu_{max.}$ 3050, 3020 (Ar—H); 2970, 2910 (C—H); 1580, 1490, sh. 1475 (C=C); 1087, 1040, 1010, 803 (1,4-disubstituted Ar—H); 763 (CCl_2); 532; and 499 cm.$^{-1}$ $\lambda_{max.}$ sh. at 211.5 (ε 17,060), 217.5 (ε 15,910), 231 (ε 19,710), and 268 mμ (ε 713). The NMR spectrum showed doublets at 4.58 δ (benzylic proton) and 6.33 δ (—$CHCl_2$) with J = 8.0 c.p.s., and a single aromatic peak at 7.33 δ.

DISCUSSION

The syntheses of these three DDD isomers have been reported previously. After initial isolation of *o,p'*- (III*a*) and *p,p'*-DDD (III*c*) from technical "DDT,"[5] Haller *et al.* (12) synthesized them in 39 and 63% yields, respectively, by the Friedel-Crafts alkylation of chlorobenzene with the appropriate carbinol (II) in the presence of 100% sulfuric acid. They obtained the *ortho* carbinol (II*a*) in only 6% yield by the addition of dichloroacetaldehyde to the Grignard reagent prepared from *o*-bromochlorobenzene (I*a*). The *para* carbinol (II*c*) was prepared in good yields by this method [67% (12)] or by reduction of 2,2,4'-trichloroacetophenone [96% (15)]. (See Scheme I.)

In an approach to the synthesis of the more biologically interesting *o,p'*-DDD, Inoi, Gericke, and Horton (13) obtained the *ortho* carbinol (II*a*) by reduction of the more readily available 2,2,2-trichloro-1-(*o*-chlorophenyl)ethanol (IV*a*) with aluminum amalgam in 90% ethanol. They synthesized several diphenyldichloroethanes by this method, including *o,p'*-, *m,p'*-, and *p,p'*-DDD (III*a*, *b*, and *c*), all in good yield. They also improved on the condensation of carbinol (II*a*) with chlorobenzene by using boron trifluoride-saturated sulfuric acid (47% yield).

Although the biological properties of *m,p'*-DDD (III*b*) have been mentioned several times in the literature, reports of the synthesis and physical properties have been limited. It was prepared by the reduction outlined above, being reported as an oil, b.p. 178–180°/0.5 mm. (13). Synthesis of the *meta* carbinol (II*b*) by the Grignard procedure was reported in 32% yield and *m,p'*-DDD (III*b*), using sulfuric acid in the Friedel-Crafts reaction and a laborious work-up, in 19% yield (14).

With the specific intention of preparing the ^{14}C labeled compounds, interest centered on the Friedel-Crafts route since this enabled incorporation of the labeled precursor at the last step. In preliminary investigations on the synthesis of the carbinol intermediates, the authors were unable to repeat the aluminum amalgam reduction of the trichloroethanol (IV*a*) [prepared according to Haller *et al.* (12) and Bergmann *et al.* (16)]. Attention turned to the Grignard synthesis. It was anticipated that slow addition of the Grignard reagent prepared from the bromobenzenes (I) to a cold ethereal solution of the unstable dichloroacetaldehyde would give an improvement in the yields. In this manner, yields of 35, 53, and 63% were obtained for the *o*-, *m*-, and *p*-carbinols (II), respectively.

The condensation reaction was studied extensively in an effort to maximize the radioactive yield. It is usual in Friedel-Crafts alkylations of benzene and its readily available derivatives to have them in excess relative to the alkylating agent, and to serve as solvent. As the chlorobenzene to be used was the labeled precursor and it seemed likely that the carbinols (II) would exhibit some instability with possible decomposition, trial reactions were run with varying proportions of chlorobenzene and carbinols and a twofold excess of the latter was found to give maximum yields. These varied from 56 to 75%, depending somewhat on the isolation procedure used. It was found most convenient to chromato-

[4] Purchased from Tracer Lab, Waltham, Mass.

[5] DDT, of various grades, is the product obtained from the condensation of chloral with chlorobenzene in the presence of sulfuric acid, and usually contains about 70% 1,1,1-trichloro-2,2,-bis-(*p*-chlorophenyl)ethane.

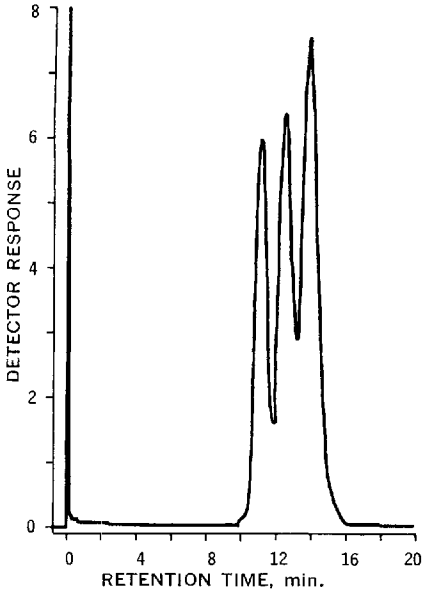


Fig. 1.—GLC tracing of 2.5 ng. each of *o,p'*-, *m,p'*-, and *p,p'*-DDD-^{14}C, injected in 6 μl. of benzene.

graph the crude product on activated silicic acid with benzene–hexane (1:4). In all three cases, an aromatic by-product was eluted before the DDD isomers. Since heterogeneous reactions tend to give variable results, tetrachloroethane and cyclohexane were tried as solvents. Yields were lower with the former and unaffected by the latter, although it did facilitate stirring. The labeled compounds were obtained in about 40% yields following recrystallization.

It has been pointed out frequently (17) that special purity analyses are required for radiochemicals intended for biological distribution studies. In this investigation the labeled isomers were checked for purity by thin-layer (TLC) and gas-liquid chromatography (GLC). With TLC, chemical purity was established by spot detection with ultraviolet light and iodine vapor, and radioactive purity by means of a radiochromatogram scanner. GLC was found to be the most effective analytical tool for assessment of purity. The type of column and conditions employed have been used extensively for detection of pesticide residues (18) and gave satisfactory separation of the three isomers (Fig. 1). The electron-capture detector used was capable of detecting trace impurities. This made it possible to show that each was isomerically pure. This was necessary as the condensation of *o*-, *m*-, and *p*-carbinols (II) with chlorobenzene can occur at both the *ortho* and *para* positions. Of most concern was the complete elimination of *o,p'*-DDD from *p,p'*-DDD, as the former is known to localize to a much greater extent in various tissues. This was readily seen as the gas chromatogram of *p,p'*-DDD-^{14}C showed a single peak even when 25 ng. (10 times that used in obtaining Fig. 1) was injected. It is curious to note that their order of retention does not correspond to their melting points, the lower melting *m,p'*-DDD following the sterically hindered *o,p'*-DDD. Additional isomers are being prepared to study this and other properties.

We examined the I.R., U.V., and NMR spectra of the three DDD isomers for possible use in assessing purity. Reports on spectral data for these compounds have been limited. McDonald and Watson (19) employed I.R. spectroscopy for differential analysis of *o,p'*-, and *p,p'*-DDD and used the distinguishing bands at 685 and 765 cm.$^{-1}$, respectively. In addition, *o,p'*-DDD has been observed to exhibit a strong characteristic band at 608 cm.$^{-1}$.

Inoi *et al.* (13) reported on the U.V. spectra of *o,p'*-DDD and some related compounds but made no comparisons. In the studies reported here, comparison of the *o,p'*-, *m,p'*-, and *p,p'*-isomers showed them to have similar but distinguishable U.V. absorption spectra.

The NMR spectra showed the greatest distinctions. In contrast with *m,p'*- and *p,p'*-DDD, the benzylic proton of *o,p'*-DDD showed a shift to lower field of 38 c.p.s. due to the deshielding effect of the *o*-chloro substituent. A similar downfield shift has been reported for the benzylic proton of benzhydryl derivatives possessing *o*-methyl groups (20). In addition, whereas the *o,p'*- and *p,p'*-isomers showed a single strong peak at approximately 7.34 δ for the aromatic protons, the *m,p'*-isomer displayed two major peaks at 7.26 and 7.29 δ.

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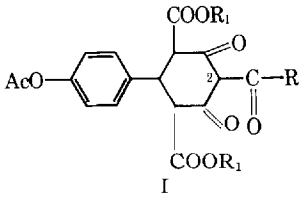
Potential Anticancer Compounds

Synthesis of 2-Carbamoyl and 2-Phenyl Carbamoyl Derivatives of 1,3-Cyclohexanedione-Type Compounds

By PHILIPPOS E. PAPADAKIS and GUY HAVEN

2-Carbamoyl and 2-phenylcarbamoyl derivatives of 1,3-cyclic diketones (I–VI) were synthesized as potential antibacterial and anticancer reagents.

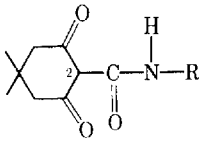
IN PREVIOUS publications (1, 2) the synthesis of 2-acyl and 2-aroyl derivatives of 1,3-cyclohexanedione-type compounds were reported. Compound I is an example where R = alkyl or aroyl and R_1 = ethyl.



I

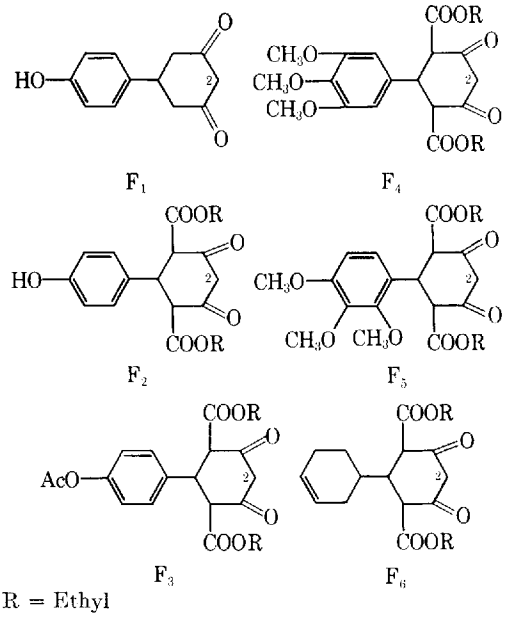
Carbon 2 in formula I has a tricarbonyl methane structure.

A Japanese team of scientists (3) found that the acylated or aroylated derivatives, at position 2 of the cyclic diketones they used, inhibited the multiplication of the Gram-positive bacteria while the carbamoyl and phenyl carbamoylated derivatives showed potency against both Gram-positive and Gram-negative bacteria, as well as a wide spectrum of antitumor activity. An example of such derivatives of the 5,5-dimethyl-1,3-cyclohexanedione (dimedone) is given by the formula (II) where R = H or C_6H_5.



II

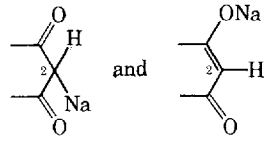
Papadakis *et al.* have synthesized derivatives of 1,3-cyclohexanedione (4–6) which have been used as intermediates for the preparation of physiologically important compounds (2, 7–10). Following are the formulas of such intermediates which have structural relationship to dimedone.



F_1 F_4 F_2 F_5 F_3 F_6

R = Ethyl

In view of the findings of Ukita *et al.* (3), it was thought advisable to prepare derivatives of each of the compounds I–VI having the —$CONH_2$ or —$CONHC_6H_5$ attached to 2-C of the cyclohexanedione ring. There are several methods by which such derivatives may be obtained but each has its own difficulties and side reactions. The phosgene (ClCOCl) reaction with the sodio derivative of each of the compounds I–VI would seem to afford a direct method for the preparation of the products desired. The method has the disadvantage that when each of the compounds F_1–F_6 is treated with NaOR, two structures result.



When the sodio derivatives are treated with phosgene and subsequently with ammonia, the following products may be obtained:

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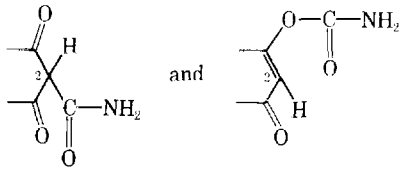
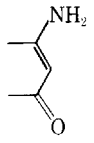
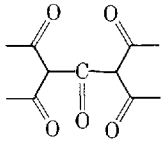


III IV

Product IV predominates. Ammonolysis of IV may render as by-products urea and

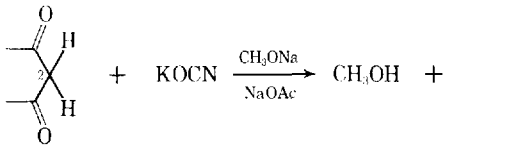


Another possibility is the formation of bis derivatives type V. When the cyclic diketone,



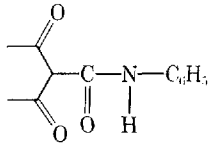
V

F_1, reacts with phosgene in the molar proportions of 2:3 and the product is treated with ammonia or aniline, compound VI is formed.



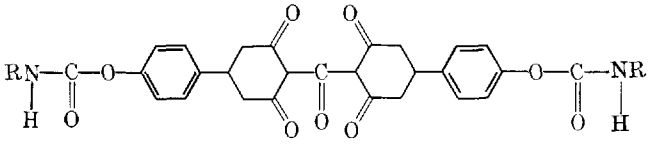
Scheme I

The 2-phenyl carbamoyl derivatives



of any of the formulas F_1–F_6 can be prepared by one of the reactions shown in Scheme II.

Similar side reactions can occur here as in the case of the preparation of the —$CONH_2$ derivative. In reaction *B* where aniline was used, there



VI

R = H or C_6H_5, respectively.

Such carbamoate or phenylcarbamoate derivatives have been isolated and analyzed (11).

There is also the possibility of polymerization between the sodioderivatives of F_1 and phosgene.

In another method each of the structures (F_1–F_6) was treated with phosgene in the presence of pyridine. Here also there is the possibility of formation of bis compounds. When any of the compounds (structures F_1–F_6) suspended in dioxane is mixed with the pyridine and the mixture, from a dropping funnel, is allowed to fall dropwise into the reaction mixture flask containing excess of a solution of 12% phosgene in benzene, the formation of the bis compound decreases.

Another method involves the reaction of any of the compounds (F_1–F_6) with potassium cyanate in the presence of $NaOCH_3$ and sodium acetate using dioxane and refluxing temperature. (Scheme I.)

is the possibility of carbanilide formation, C_6H_5-$NHCONHC_6H_5$. The method of mixing the reagents as indicated previously can adjust the relative concentrations, thereby decreasing the amount of the carbanilide formation.

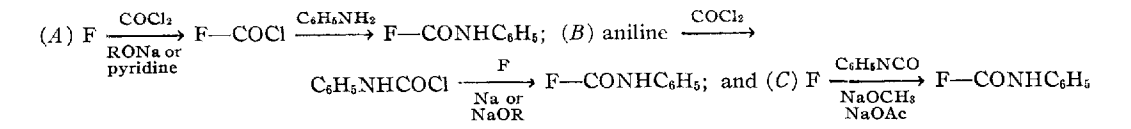
EXPERIMENTAL

In experiments *1*, *9*, and *12*, the phosgene method was used. The apparatus set up, safety precautions, and the general procedure are described in experiment *1*.

Instead of writing the long names of the compounds F_1, F_2, F_3 . . . etc., the respective letters will be used to represent the compounds. The names of the products will be given in the beginning of each experiment.

In experiments *10*, *11*, and *13*, the procedure was similar to that of experiment *1*, except that aniline was used instead of ammonia, followed by refluxing, concentrating, and processing.

Experiments *3*, *6*, *7*, and *14* were done using the phenyl isocyanate method as described.



Scheme II

1.—5 - (p - Hydroxyphenyl) - 2 - carbamoyl - 1,3-cyclohexanedione.—A mixture of 10.5 Gm. of F_1 (0.0515 mole), dioxane (30 ml.), and pyridine (8 ml.) was added slowly through a dropping funnel into a three-neck flask which contained 130 ml. of a solution of 12% phosgene in benzene. The reaction mixture flask was equipped with a dropping funnel, a condenser, and a magnetic stirrer. The top of the reflux condenser was connected with a flask containing NaOH to trap any $COCl_2$ vapor. All the apparatus was set up under the hood.

The phosgene–benzene solution was kept cold during the addition of the diketone–pyridine–dioxane mixture. The reaction mixture was allowed to stand at room temperature under the hood for 2 days, then it was refluxed for 2 hr. The flask containing the NaOH was connected to the water aspirator and controlled reduced pressure was applied to remove any unreacted phosgene. Arrangements were made for distillation. The heating was continued till the temperature reached 98°. After cooling, 30 ml. of dioxane was added to the mixture, and ammonia gas was bubbled through it for 2 hr. The mixture was allowed to stand overnight. The precipitate formed was filtered. The filtrate was concentrated to a red-orange syrup which was stirred with ether 3 successive times. The ether was decanted each time. The light tan residue was dried on porous tile. The combined solids (9 Gm.) were boiled with a limited amount of absolute alcohol and filtered hot. On cooling, the filtrate rendered crystals which were washed with distilled water and dried on porous tile, m.p. 167°–176°.

Anal.—Calcd. for $C_{13}H_{13}NO_4 \cdot H_2O$: C, 58.87; H, 5.64; N, 5.28. Found: C, 59.21; H, 5.30; N, 5.40.

The material above was dried at 100° and 1.5 mm., m.p. 183°.

Anal.—Calcd. for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.26; N, 5.66. Found: C, 63.27; H, 5.65; N, 5.25.

The material which was not readily soluble in the hot alcohol, m.p. 185°, corresponds to the formula $C_{14}H_{14}N_2O_5$.

2.—5-(p-O-Carbamoate phenyl)-2-carbamoyl-1,3-cyclohexanedione.—*Anal.*—Calcd. for $C_{14}H_{14}N_2O_5$: C, 57.93; H, 4.82. Found: C, 58.02; H, 4.70.

3.—2 - Phenylcarbamoyl - 5 - (p - O - phenylcarbamoate phenyl) - 1,3 - cyclohexanedione.—F_1, 7.28 Gm. (0.035 mole), 0.805 Gm. of sodium dissolved in methanol, and 30 ml. of dioxane were mixed in a three-neck flask equipped with a reflux condenser and magnetic stirrer. After refluxing for 20 min., the apparatus was arranged for distillation of the methanol. The distillation was continued until the temperature reached 90°. Twenty milliliters of dioxane was added and 4.2 ml. of phenyl isocyanate. After 5 hr. of refluxing, the dioxane was distilled off under reduced pressure using a water bath. The residue was cooled and then washed with ether. The ether was decanted and the residue was stirred with water. The solution was acidified and the precipitate which formed was filtered and recrystallized from ethanol. It sinters at 193°. It turns to syrupy droplets at 198° and to a red-black melt at 223°–229°. Recrystallized from acetone, m.p. 209°–213°.

Anal.—Calcd. for $C_{26}H_{22}N_2O_5$: C, 70.57; H, 4.97; N, 6.33. Found: C, 70.16, 70.31, 70.53; H, 4.73, 5.22, 5.10; N, 6.32.

4.—5 - (p - Hydroxyphenyl) - 2 - carbamoyl - 4,6-dicarbobutoxy - 1,3 - cyclohexanedione.—F_2 (10.4 Gm.), sodium (0.92 Gm.) dissolved in butanol, urea (3.6 Gm.), and butanol (50 ml.) were refluxed 4 hr. Some urea sublimed and was deposited in the inner wall of the reflux condenser. Some ammonia was also forming, as shown when a piece of litmus paper held on the top of the condenser turned blue. Some methyl alcohol was added to lower the boiling temperature to 95° and prevent the subliming of urea. The materials dissolved in the beginning. Later a fine precipitate formed which changed from a greenish to a brown color.

After 4 hr. of refluxing, most of the butanol was distilled under reduced pressure. Water was added, and the mixture was acidified. The precipitate was stirred with cold alcohol and filtered. From the filtrate, after evaporation of the alcohol, the material obtained melted at 80°–85°, then resolidified, and remelted at 175°.

Anal.—Calcd. for $C_{13}H_{29}O_8N \cdot {}^1/_2H_2O$: C, 60.52; H, 6.57. Found: C, 60.08; H, 6.57.

5.—5 - (p - Hydroxyphenyl) - 1 - amino - 2-carbamoyl - 4,6 - dicarbethoxy - 1 - cyclohexenone-3.—F_2, 10.4 Gm. (0.03 mole), urea, 3.6 Gm. (0.06 mole), and dimethylformamide were refluxed 8 hr. After the refluxing, most of the solvent was evaporated under reduced pressure. Water was added. The mixture was stirred and filtered. The residue had a melting point of 187°–190°. The product shows blue fluorescence under ultraviolet light.

Anal.—Calcd. for $2(C_{19}H_{22}O_7) \cdot H_2O$: C, 59.84; H, 5.51; N, 7.34. Found: C, 59.39; H, 5.33; N, 7.40.

6.—2 - Phenylcarbamoyl - 5 - (p - hydroxyphenyl) - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione.—F_2, 10.4 Gm. (0.03 mole), NaOAc, 2.46 Gm., C_6H_5NCO, 7.14 Gm. (0.06 mole), and $NaOCH_3$, 3.18 Gm. (0.03 mole) were refluxed in dioxane medium for 8 hr. After that, the mixture was acidified and the precipitate was filtered off, washed with distilled water and with alcohol, m.p. 226°. The filtrate was concentrated by evaporation under reduced pressure and the residue recrystallized from ethanol, m.p. 195°.

Anal.—Calcd. for $C_{25}H_{25}NO_8 \cdot H_2O$: C, 61.84; H, 5.60. Found: C, 61.54; H, 5.75.

7.—5 - (p - Acetoxyphenyl) - 2 - phenyl carbamoyl-4,6 - dicarbethoxy - 1,3 - cyclohexanedione.—F_3, 3.9 Gm. (0.01 mole), C_6H_5NCO (1.2 Gm.), NaOAc anhydrous (1 Gm.), and 30 ml. of dioxane were mixed. After 4 hr. of refluxing, most of the dioxane

was distilled under reduced pressure. The residue was stirred in water 3 hr. and then filtered. The crystals were dried and then washed with ether three times, 223° dec. Product was almost white (little pale yellow).

Anal.—Calcd. for $C_{27}H_{27}O_9 \cdot H_2O$: C, 61.48; H, 5.54. Found: C, 61.91; H, 5.05.

Anal.—Calcd. for $C_{25}H_{21}O_8 \cdot H_2O$: C, 62.37; H, 5.00. Found: C, 61.91; H, 5.05.

8.—5 - (p - Acetoxyphenyl) - 2 - carbamoyl - 4,6-dicarbethoxy - 1,3 - cyclohexanedione.—A mixture of F_3 (11.7 Gm.), KOCN (2.43 Gm.), $NaOCH_3$ (1.62 Gm.), NaOAc (2.46 Gm.), and 50 ml. of *N*-dimethylformamide was refluxed for 5 hr. The solution became greenish, then red-orange, and orange-brown. Chromatographic paper is stained orange. Under the influence of U.V. it shows strong greenish cream fluorescence. The calculated amount of hydrochloric acid was added to react with the KOCN, sodium methoxide, and sodium acetate. The solution was evaporated under reduced pressure and the residue stirred with ice water. The insoluble part was washed with ether and recrystallized from methanol, m.p. 193°.

Anal.—Calcd. for $C_{24}H_{28}N_2O_2$: C, 56.91; H, 5.57. Found: C, 56.50; H, 5.57.

The analytical results suggest that the amide of the carbamoyl of the title compound may have reacted with the dimethylformamide as follows:

$$F\text{—}CONH_2 + O{=}C(H)\text{—}N(CH_3)_2 \rightarrow RCON{=}CH\text{—}N(CH_3)_2$$

9.—5 - (p - Acetoxyphenyl) - 2 - carbamoyl - 1,4-carbolactone - 6 - carbethoxy - 1 - cyclohexenone-3.—An attempt was made to make the compound (experiment *8*) by the phosgene method used in experiment *1*, using as starting material F_3 instead of F_1. The product begins to gum at 210° and tars at 280°. It is insoluble in ether, dioxane, cold alcohol, and water.

Anal.—Calcd. for $[C_{21}H_{23}NO_9 \cdot H_2O] \cdot C_2H_5OH$: C, 56.29; H, 4.72. Found: C, 56.50; H, 5.11.

10.—5 - (3′,4′,5′ - Trimethoxyphenyl) - 2 - phenyl carbamoyl - 4,6 - dicarbethoxy - 1,3 - cyclohexanedione.—F_5, 4.22 Gm. (0.01 mole) (6), dioxane (30 ml.), and pyridine (1 ml.) were added slowly through a dropping funnel into a three-neck flask containing 15 ml. of a 12% solution of $COCl_2$ in benzene. The apparatus and procedure were similar to experiment *1* with the exception that 1 ml. of aniline instead of ammonia was used. After refluxing for several hours, the dioxane was evaporated under reduced pressure. The residue was stirred with distilled water and acidified, filtered, and dried, and then it was washed with ether, m.p. 193°–195°, as aniline hydrochloride melts at 198°, mixed melting points were taken, 150°–155°.

Anal.—Calcd. for $C_{28}H_{31}NO_{10}$: C, 62.10; H, 5.73. Found: C, 62.58; H, 5.48.

11.—5 - (3′,4′,5′ - Trimethoxyphenyl) - 1 - iminophenyl - 2 - phenylcarbamoyl - 4,6 - dicarbethoxy-1,3-cyclohexanedione.—Experiment *10* was repeated with the difference that excess aniline was used. A material was obtained which decomposed at 122°.

Anal.—Calcd. for $C_{34}H_{36}N_2O_9$: C, 66.88; H, 6.23. Found: C, 67.11; H, 5.82.

The analytical data correspond to the title compound.

12.—5 - (4′ - Cyclohexene) - 1 - amino - 2 - carbamoyl - 4,6 - dicarbethoxy - 1 - cyclohexenone - 3.—A mixture of F_6, 11.2 Gm. (0.033 mole), dioxane (50 ml.), and pyridine (3 ml.) was added slowly through a dropping funnel into a three-neck flask which contained 70 ml. of a solution of 12% phosgene in benzene. The apparatus set up, safety precautions, and the procedure were described in experiment *1*. After the ammonia treatment, the mixture was allowed to stand overnight at room temperature. The precipitate formed was filtered and then heated with alcohol. The part that did not dissolve was washed with ether and dried on porous tile. The material had a light tan color, m.p. 150°, clear at 152°.

Anal.—Calcd. for $C_{19}H_{26}N_2O_6 \cdot {}^1/_2\ H_2O$: C, 58.91; H, 6.99. Found: C, 58.70, 58.90; H, 7.29, 7.25.

13.—5 - (4′ - Cyclohexene) - 2 - phenylcarbamoyl-4, 1 - carbolactone - 6 - carbethoxy - 1 - cyclohexenone-3.—F_6, 3.35 Gm. (0.01 mole) in dioxane, was mixed with 1 ml. of pyridine. The mixture was added dropwise through a dropping funnel into a three-neck flask containing 15 ml. of a 12% solution of phosgene in benzene. The apparatus set-up, safety precautions, and procedure were similar to experiment *1*, except that after the distillation of any unreacted phosgene, aniline instead of ammonia was used. To the reaction mixture 1 ml. of aniline in 24 ml. of dioxane was added dropwise with stirring.

The mixture was refluxed for 1 hr., then concentrated. The precipitate formed was filtered then stirred with water to get rid of the pyridinium chloride. The residue was dried, washed with ether, and recrystallized from acetone, m.p. 230°.

14.—In another experiment equimolar quantities of F_6 and CH_3ONa in dioxane medium were refluxed for 0.5 hr. The apparatus was arranged for distillation. Part of the solvent was allowed to distil until the temperature reached 100°. To the remaining mixture, equimolar quantities of phenyl isocyanate and anhydrous sodium acetate were added, and the mixture was refluxed for 2 hr. The precipitate formed was washed with water, filtered, dried, and then washed with ether. The ether was decanted and the residue recrystallized from acetone, m.p. 228°–229°; m.p. with carbanilide 208°.

Anal.—Calcd. for $C_{23}H_{24}NO_6 \cdot 2H_2O$: C, 61.88; H, 6.27; N, 3.13. Found: C, 62.04; H, 5.86; N, 2.90.

The material dried at 100° and 1.5 mm. gave the following analysis.

Anal.—Calcd. for $C_{23}H_{24}NO_6$: C, 68.03; H, 6.57. Found: C, 68.25; H, 6.56.

15.—Diethyl - (3 - cyclohexenal) - malonate.—3-Cyclohexene-1-carboxaldehyde, 218 Gm. (2 moles), 320 Gm. of diethylmalonate (2 moles), and 15 ml. of piperidine were mixed and refluxed 24 hr. at 105°. The mixture became orange in color. Fractional distillation was applied under reduced pressure. The fraction boiling at 132°–138° and 0.7 mm. was taken as representing the title compound and was used for the next step.

16.—5 - (4′ - Cyclohexene) - 4,6 - dicarbethoxy 1,3 - cyclohexanedione.—Sodium (7.8 Gm.) dissolved in absolute ethanol was added with stirring to 50 ml. of ethyl acetoacetate. After 15 min. of refluxing it was poured into a 1000-ml. flask contain-

ing 100 Gm. of diethyl-(3-cyclohexenal)-malonate. The mixture was refluxed for 6 hr. The color of the solution turned red. Distillation of the solvents under reduced pressure followed using a flash evaporator. The residue was dissolved in cold distilled water. The solution was extracted twice with ether. The aqueous layer was adjusted to pH 7 and then it was extracted with ether once more. The water layer was acidified with 3 *N* hydrochloric acid. White crystals resulted which were filtered, washed with distilled water, filtered, and then washed with a 50:50 mixture of ether–petroleum ether, m.p. 111°–113°. Recrystallized 4 times from absolute alcohol, m.p. 133°–137°.

Anal.—Calcd. for $C_{18}H_{24}O_6$: C, 64.26; H, 7.19. Found: C, 64.45; H, 7.32; C, 64.45; H, 7.19.

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Comparative Study of the Alternating and Direct Current Polarography of Several Δ^4-3-Ketosteroids

By JAMES L. SPAHR and ADELBERT M. KNEVEL

A study was undertaken to compare alternating current polarography with direct current polarography as a method of analysis of testosterone, methyltestosterone, and progesterone. A solution consisting of 50 per cent ethanol, buffer (pH 1.3), and tetrabutylammonium iodide was used as the sample medium. Results showed that the lowest practical concentrations of detection for both a.c. and d.c. polarography was $3.3 \times 10^{-5}M$. However, a.c. polarography gave greater precision than did the d.c. method.

A STUDY OF the analysis of Δ^4-3-ketosteroids by direct current (d.c.) polarography has been reported by several groups of workers (1–3). In the study conducted by Kabasakalian and McGlotten (3), it was reported that the diffusion current of testosterone and other related Δ^4-3-ketosteroids was directly proportional to the concentration in the range of 2×10^{-4} to $1 \times 10^{-2}M$. At low concentrations, however, deviations from linearity were observed in some cases. These workers pointed out that the deviations may have been due to the method of measuring the diffusion current rather than a change in diffusion properties, because the diffusion current plateau at low concentration was too steep. This explanation seems reasonable since ill-defined polarographic waves are not uncommon with ketones. One factor contributing to this poor definition may be that ketone half-wave potentials occur very close to the discharge potentials of the buffer components. This effect often makes it difficult to separate ketone diffusion current from buffer discharge current. Alternating current (a.c.) polarography offers the advantage of producing polarograms in which the reduction waves of the ketone and buffer components are often sufficiently separated so that diffusion currents can be measured more accurately. Furthermore, this technique is often more sensitive to organic compounds than is d.c. polarography. The objective of this study was to compare a.c. polarography with d.c. polarography as a method of analysis for several different Δ^4-3-ketosteroids.

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EXPERIMENTAL

Apparatus.—The dropping mercury electrode capillary used in this study had a length of 9.3 cm. Under a pressure of 26.5 cm. of mercury and with an open circuit, the drop time was 4.86 sec. and *m* was 1.13 mg. $sec.^{-1}$. These characteristics were determined at 25° with the mercury dropping into 50% ethanol which was 0.1 *M* in tetrabutylammonium iodide.

The electrolysis cell was a tube 7 cm. in length with an inside diameter of 2.1 cm. The saturated calomel reference used throughout this work was contained in a Hildebrand half-cell. Junction be-

ing 100 Gm. of diethyl-(3-cyclohexenal)-malonate. The mixture was refluxed for 6 hr. The color of the solution turned red. Distillation of the solvents under reduced pressure followed using a flash evaporator. The residue was dissolved in cold distilled water. The solution was extracted twice with ether. The aqueous layer was adjusted to pH 7 and then it was extracted with ether once more. The water layer was acidified with 3 *N* hydrochloric acid. White crystals resulted which were filtered, washed with distilled water, filtered, and then washed with a 50:50 mixture of ether–petroleum ether, m.p. 111°–113°. Recrystallized 4 times from absolute alcohol, m.p. 133°–137°.

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The electrolysis cell was a tube 7 cm. in length with an inside diameter of 2.1 cm. The saturated calomel reference used throughout this work was contained in a Hildebrand half-cell. Junction be-

tween the sample solution and the reference was made with a bridge of 4% agar in saturated KCl.

The instrument used in this study was a Sargent polarograph model XXI (E. H. Sargent & Co., Chicago, Ill.) modified according to Miller (4) for a.c. polarography. During the course of this study it was found necessary to modify the Miller circuit by adding a downscale compensator. The reason for the modification was that the base current of a.c. polarography was somewhat larger than the corresponding residual current of d.c. polarography. Many times at high sensitivity settings the supporting electrolyte alone caused a pen deflection so large that the a.c. peak went off scale. The downscale compensator on the model XXI polarograph could not be used to bring the peak on scale, because it is inoperative during a.c. operation using the Miller modification. In this study it was found necessary to introduce a downscale compensator in the a.c. circuit since low concentrations of steroid required high sensitivity values. Although this accessory was introduced into the circuit specifically for this study, it should increase the value of the a.c. modification of the model XXI in cases where high sensitivity is required. The details of this circuit have been reported elsewhere (5).

Reagents.—The buffers used were those reported by Kabasakalian and McGlotten (3). The components were observed to be polarographically inert in the voltage range employed. The need for a medium of 50% ethanol was dictated by the limited solubility of the steroids. The ethanol was distilled from commercial absolute ethanol in the presence of sodium ethoxide and diethyl phthalate (6). This preparation was necessary because the commercial material contained some reducible impurities. Alkyl phenoxy polyethoxy ethanol[1] was used as the maximum suppressor. A polarographic grade of tetrabutylammonium iodide was used as the supporting electrolyte. All other reagents were of A.R. grade. The steroids studied were testosterone,[2] methyltestosterone,[3] and progesterone.[4]

Sample Preparation and Handling.—In most of this work 50% ethanol containing the buffer and supporting electrolyte was used as the sample medium. Although potassium chloride is the most commonly used supporting electrolyte in d.c. polarography, it was not satisfactory for this study. Preliminary a.c. polarograms using methyltestosterone in 50% ethanol with 0.5 *M* KCl revealed that ethanol produced a tensametric peak. This phenomenon has been described by Breyer and Hacobian (7). The peak appeared at a potential very close to the reduction potential of the steroid and completely obscured the steroid wave. Other common inorganic supporting electrolytes such as KNO_3 gave the same results. Organic quaternary ammonium compounds were then tested and found to eliminate the ethanol tensametric peak in the potential region of interest. The supporting electrolyte finally chosen for this study was tetrabutylammonium iodide.

Electrolysis solutions were prepared by pipeting the appropriate aliquot of a standard ethanolic steroid solution into a 10-ml. volumetric flask, adding 0.1 ml. of 0.2% alkyl phenoxy polyethoxy ethanol, sufficient ethanol to measure exactly 5 ml., a calculated amount of supporting electrolyte, and finally diluting to the mark with aqueous buffer. Since the volume of the aliquot varied with the final concentration of the steroid desired, it was necessary to add enough ethanol to measure exactly 5 ml. before diluting with buffer. Tetrabutylammonium iodide was added to the flask before diluting with the aqueous buffer because it is only slightly soluble in water. The concentrations of the aqueous buffers were twice the values desired so that after dilution by the ethanol the final buffer concentrations were those listed in Table I. Sample solutions were transferred to the electrolysis cell and deaerated with nitrogen for 15 min. During the deaeration period and during the polarographic run the sample container was partially immersed in a water bath held at 25° ± 0.1°. The d.c. run was made immediately after deaeration. The instrument was then switched to a.c. operation and the a.c. run was made. pH determinations of each solution were made after electrolysis. The pH of individual samples varied slightly (0.1–0.2 units) from the values listed in Table I.

TABLE I.—COMPOSITION OF BUFFERS IN 50% ETHANOL

pH		Buffer Components
1.3	0.100 *M* HCl	
2.9	0.100 *M* malonic acid	0.025 *M* KOH
3.8	0.100 *M* malonic acid	0.075 *M* KOH
5.3	0.075 *M* acetic acid	0.025 *M* NaOAc
5.6	0.050 *M* acetic acid	0.050 *M* NaOAc
6.1	0.025 *M* acetic acid	0.075 *M* NaOAc
6.9	0.100 *M* malonic acid	0.170 *M* KOH
8.7	0.100 *M* trimethylamine	0.075 *M* HCl
9.1	0.100 *M* trimethylamine	0.050 *M* HCl
9.4	0.100 *M* triethylamine	0.075 *M* HCl
9.9	0.100 *M* triethylamine	0.050 *M* HCl
10.3	0.100 *M* triethylamine	0.025 *M* HCl

pH Effect on Wave Form.—The object of this portion of the research was to determine the pH at which the polarographic wave had the form best suited for quantitative analysis. The judgment was made on the basis of peak height and form.

Alternating current and d.c. polarograms were recorded for all three steroids at all pH values. In order to assure that observed effects were due to pH it was necessary to keep the ionic strength constant. The amount of tetrabutylammonium iodide required to bring the ionic strength to 0.2 was calculated and added to the sample at the time of preparation.

The solutions for the pH study were prepared by pipeting 5 ml. of an alcoholic steroid solution containing 1 mg./ml. of steroid into a 10-ml. volumetric flask, adding maximum suppressor and supporting electrolyte, and diluting to the mark with the appropriate aqueous buffer.

The a.c. calibration point was determined as follows: using a 2800 ohm resistance[5] substituted for the cell; a sensitivity setting of 0.300; and an applied a.c. voltage of 70 mv., the slidewire calibration potentiometer was adjusted until the re-

[1] Marketed as Triton X-100 by Rohm & Haas Co., Philadelphia, Pa.
[2] Courtesy of Eli Lilly and Co., Indianapolis, Ind.
[3] Courtesy of Wyeth Laboratories, Inc., Philadelphia, Pa.
[4] Courtesy of The Upjohn Co., Kalamazoo, Mich.

[5] Leeds & Northrup AC–DC Decade Resistor.

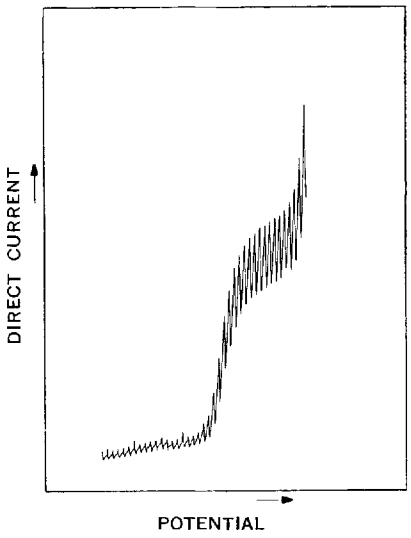

Fig. 1.—Direct current polarogram of methyltestosterone in 50% ethanol and tetrabutylammonium iodide at pH 1.3.

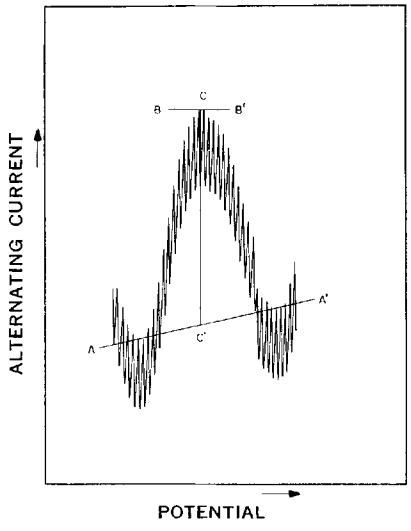

Fig. 2.—Alternating current polarogram of methyltestosterone in 50% ethanol and tetrabutylammonium iodide at pH 1.3. CC′ is the peak current.

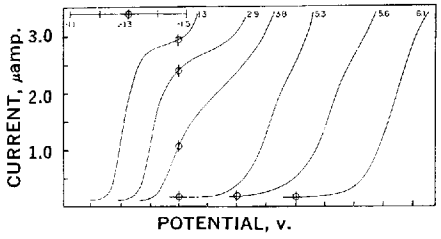

Fig. 3.—Effect of pH on the half-wave potentials and wave form of testosterone in 50% ethanol with tetrabutylammonium iodide as supporting electrolyte.

corder read 250 mm. The samples were then run at a sensitivity setting of 0.600.

Quantitative Studies.—All the quantitative work was carried out at pH 1.3 using methyltestosterone. For the steroid concentration range from $3.3 \times 10^{-4}M$ (100 mcg./ml.) to $6.6 \times 10^{-3}M$ (2 mg./ml.) a 50% ethanol solution was used. For the lower concentration range, from $3.3 \times 10^{-5}M$ (10 mcg./ml.) to $3.3 \times 10^{-4}M$, 25% ethanol was used. It was observed that the a.c. peaks were slightly higher in the lesser alcohol concentration. This was to be expected due to the series resistance effect.

For all the d.c. studies a direct potential scanning rate of 2.34 mv./sec. was used. For the a.c. studies a scanning rate of 2.34 mv./sec. was used in the high concentration range and 1.85 mv./sec. in the low concentration range.

The following a.c. calibration point was used for the high concentration range. The resistance substituted for the cell was 2800 ohms, the sensitivity setting was 0.300, the applied alternating potential was 70 mv., and the slidewire calibration potentiometer was adjusted until the recorder pen read 250 mm. The sensitivity was adjusted to the setting which produced a peak height of 50–125 mm.

For the low concentration range the instrument was standardized as follows. The resistance substituted for the cell was 2800 ohms, the sensitivity setting was 0.300, the applied alternating potential was 70 mv., the slidewire calibration potentiometer was adjusted until the pen read 250 mm., the downscale compensator was adjusted until the pen read 100 mm., and the slidewire potentiometer was readjusted until the pen again read 250 mm. The sensitivity was then set to 0.020 when the samples were polarographed.

In this low concentration range the downscale compensator was of the most value because it permitted the use of high sensitivity settings not possible without it. It enabled zero to be shifted downscale and thus the a.c. curve could be positioned on the chart paper. In other words, at high sensitivity settings the a.c. curve appeared high on the electrical scale due to high base current, and the downscale compensator enabled the electrical scale to be shifted with respect to the chart paper.

RESULTS

Results of the pH Study.—The current values of the d.c. polarographic waves which are shown are the average of the oscillations due to the dropping mercury. All the polarograms were recorded with the damping switch off. The amount of oscillation which resulted is shown by a typical polarogram of methyltestosterone in 50% ethanol at pH 1.3 in Fig. 1.

The current values of the a.c. curves are the values at the maximum age of the mercury drop, *i.e.*, the highest point in the recorder pen oscillation. All alternating current scales are arbitrary since the recorder scale was not calibrated in absolute alternating current. The values on this scale were obtained by multiplying the peak height in mm. by the polarograph sensitivity setting. Figure 2 is an a.c. polarogram of methyltestosterone which shows: (*a*) the oscillations due to the dropping mercury, (*b*) how the a.c. peak height was measured.

Figure 3 shows the d.c. waves for testosterone at

pH values 1.3 to 6.1. Figure 4 shows the corresponding a.c. waves. Methyltestosterone and progesterone gave essentially the same a.c. and d.c. results as those obtained for testosterone. Above the pH range shown, the waves were very similar to that obtained at pH 6.1.

In general, the polarographic waves of ketones are not ideal. They occur at very negative potentials and the diffusion current plateau and residual current have distinctly different slopes. In order to obtain reliable analytical results with the d.c. procedure, the method of evaluating the wave must be rigidly standardized. The a.c. wave tends to facilitate quantitative interpretation. The reduction of the steroid and buffer discharge are separated and the chances for error are lessened.

From Fig. 4 it was decided that pH 1.3 was optimum for quantitative work.

Results of Quantitative Studies.—The method used for determining the diffusion current from recorded d.c. polarographic waves was that described by Willard, Merritt, and Dean (8). Figure 2 illustrates how the a.c. waves were evaluated.

The d.c. results of the high concentration range are shown in Fig. 5 and the a.c. results are shown in Fig. 6. The relationship between a.c. current and concentration is not linear at high concentration and, therefore, is not as analytically useful as the d.c. curve.

The d.c. results of the low concentration range are shown in Fig. 7 and the a.c. results are shown in Fig. 8. Each concentration shown in Figs. 7 and 8 was run in triplicate. Where only two points appear, it was found that two results were identical. The concentration-current curves are calculated regression lines and the experimental points are plotted. R for the d.c. relationship is 0.994 and for the a.c. it is 0.997.

The value of the downscale compensator is illustrated by comparing Fig. 8 and Fig. 9. These are plotted on the same scale. The slope for the line in Fig. 8 is 0.034 and the slope in Fig. 9 is 0.019. Using the downscale compensator, the peak was about twice as high as without it.

DISCUSSION

While d.c. diffusion current depends on concentration gradients near the electrode surface, the a.c. current depends on the concentration of electroactive species on the drop surface. When adsorption of either the oxidized or reduced form occurs on the mercury drop, as is usually the case with organic compounds, the concentration-peak current curve is nonlinear. According to the Langmuir adsorption theory, molecules will be adsorbed on a surface until the surface is covered with a monolayer of adsorbed molecules (9). This means that no matter what the bulk concentration of a solution of electroactive species, the a.c. current reaches a limiting value when the surface of the mercury drop becomes saturated. The a.c. current is, therefore, not a linear function of bulk concentration, but approximates an adsorption isotherm. However, at low bulk concentration, when the mercury drop surface is nearly bare, the peak current-concentration relationship is essentially linear, and can be used for quantitative analysis.

In the quantitative study of methyltestosterone,

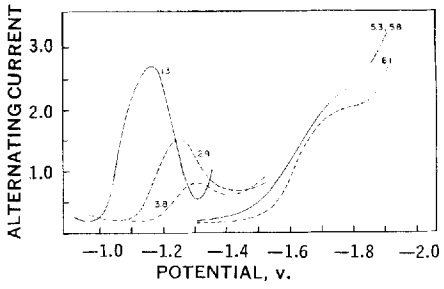

Fig. 4.—Effect of pH on the a.c. wave form of testosterone in 50% ethanol with tetrabutylammonium iodide as supporting electrolyte.

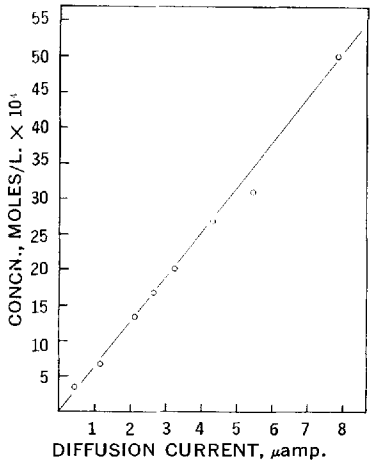

Fig. 5.—Diffusion current-concentration curve for methyltestosterone: concentration range, 6.6 × 10^{-3} *M* to 3.3 × 10^{-4} *M*; medium, 50% ethanol and tetrabutylammonium iodide at pH 1.3.

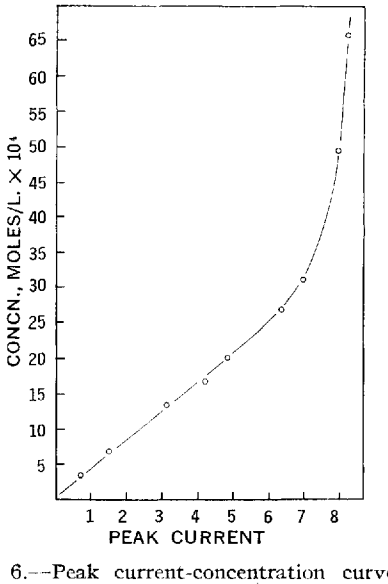

Fig. 6.—Peak current-concentration curve for methyltestosterone: concentration range, 6.6 × 10^{-3} *M* to 3.3 × 10^{-4} *M*; medium, 50% ethanol and tetrabutylammonium iodide at pH 1.3.

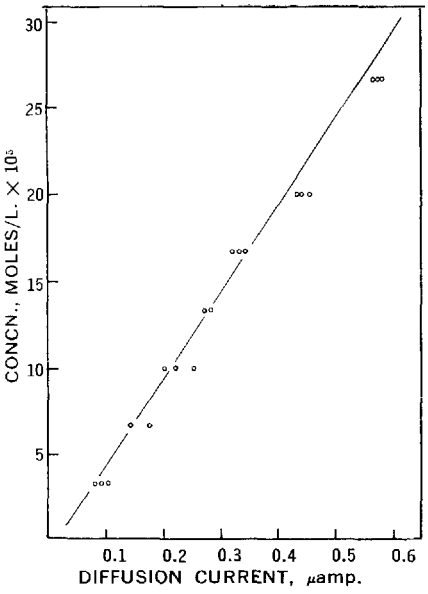

Fig. 7.—Diffusion current-concentration curve for methyltestosterone: concentration range, 3.3 × 10^{-4} *M* to 3.3 × 10^{-5} *M*; medium, 25% ethanol and tetrabutylammonium iodide at pH 1.3.

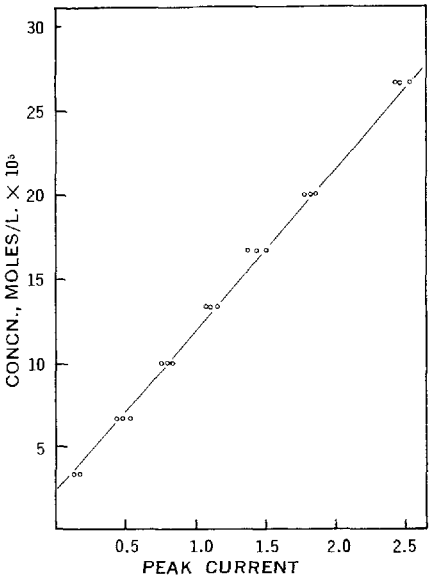

Fig. 8.—Peak current-concentration curve for methyltestosterone using the downscale compensator: concentration range, 3.3 × 10^{-4} *M* to 3.3 × 10^{-5} *M*; medium, 25% ethanol and tetrabutylammonium iodide at pH 1.3.

it was determined that the lowest practical limit of detection for both the a.c. and the d.c. methods was 3.3 × 10^{-5}*M*. Below this concentration the instrument did not give a reliable response with either method.

Although the lower limits of detection are essentially the same for a.c. and d.c., the a.c. procedure was found to be more precise. The presence of trace amounts of oxygen at low concentrations

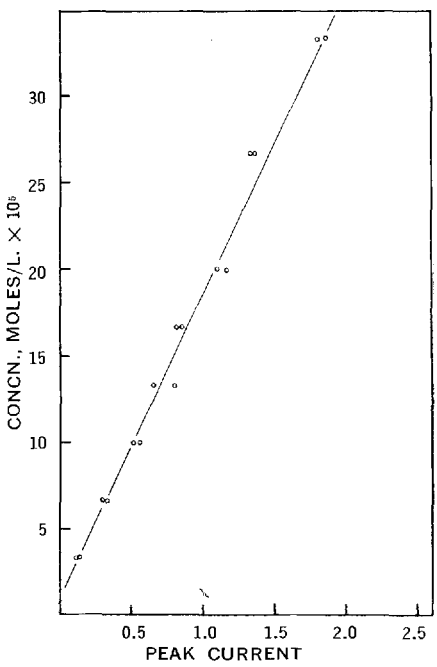

Fig. 9.—Peak current-concentration curve for methyltestosterone without using the downscale compensator: concentration range, 3.3 × 10^{-4} *M* to 3.3 × 10^{-5} *M*; medium, 25% ethanol and tetrabutylammonium iodide at pH 1.3.

of steroid can result in large errors in d.c. polarography, whereas the presence of oxygen does not affect a.c. results. It was observed during this study that occasionally 20 min. of deaeration did not completely remove all of the oxygen present. It was further observed that adequate grounding of the chassis of the a.c. accessory was absolutely essential. Without grounding a stable reference point could not be maintained. Stray a.c. voltages from the line cause undesirable electrical noise which can be minimized by shielding leads to the polarograph.

SUMMARY

An a.c. polarographic method has been developed for the analysis of several Δ^4-3-ketosteroids and compared to the d.c. method. The results show that lower limits of detection for both methods were essentially the same. However, the a.c. procedure was found to be more precise.

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Antagonism of Free Fatty Acid Release from Rat Epididymal Fat Tissue by Desmethylimipramine

By K. F. FINGER and J. G. PAGE

Desmethylimipramine (DMI) has been shown to be an antagonist of free fatty acid (FFA) mobilization from rat adipose tissue by both *in vivo* and *in vitro* techniques. Utilizing *in vitro* adipose tissue slices, homogenates, and cell-free preparations, it has been established that (*a*) the *in vitro* antagonism of FFA mobilization occurred whether the mobilization was induced by catecholamines or by other means and (*b*) the addition of DMI to an already activated lipase preparation caused a prompt cessation of lipolytic activity. These results indicate that DMI directly antagonized lipolytic enzymes and that effects on the adrenergic receptor are secondary to the primary inhibition phase. Desmethylimipramine antagonism of catecholamine-induced free fatty acid (FFA) mobilization was also observed to occur *in vivo*.

DURING THE past decade, considerable research effort has been placed on the investigation and development of antagonists of the catecholamine-induced mobilization of free fatty acids (FFA). In addition to providing agents of potential therapeutic usefulness, this type of research can and has served the purpose of elucidating the role of fat mobilization in certain disease states. Among the agents found to antagonize catecholamine-induced mobilization of FFA have been nicotinic acid (1), nethalide (2), the classical α and β adrenergic blocking agents (3), prostaglandins (4), and most recently, analogs of methoxamine (5, 6). The therapeutic value of antagonists of FFA mobilization, however, remains to be proved.

During the course of the authors' studies pertaining to the interaction of catecholamines and certain other phenethylamines with the adipose tissue adrenergic receptor system, desmethylimipramine (DMI) was investigated for its possible effects in modifying the action of catecholamines in this system. Previous work had shown that DMI was capable of enhancing adrenergic effects on certain receptor systems (7, 8), and it was with this in mind that DMI was investigated. Results indicated that DMI was an antagonist of catecholamine-induced mobilization of FFA, *in vitro* (9). This report is concerned with further investigations of the action of DMI on adipose tissue, and, in particular, its locus of action.

EXPERIMENTAL

Materials.—The chemicals used in this study and their sources are as follows: *l*-epinephrine bitartrate (Winthrop Laboratories); *l*-norepinephrine bitartrate (Nutritional Biochemical Corp.); and desmethylimipramine[1].

Methods.—The *in vitro* experiments described in this report utilized nonfasted male, white, Holtzman rats weighing between 200 and 250 Gm. All animals were maintained in their animal quarters at least 1 week prior to their use. The method of sacrifice, preparation, and handling of the epididymal fat tissue and the assay procedures employed have been described previously (9).

In those experiments employing homogenates of adipose tissue, the homogenates were prepared by adding a known weight of epididymal fat tissue to a volume of 0.25 *M* sucrose to make a 1:3 final homogenate, homogenization being accomplished by a motor driven, chilled Tenbroeck glass homogenizer.

Cell-free preparations of lipase were prepared by centrifuging the above-described homogenate at 12,000 × g for 10 min. at 4°, discarding the accumulated fat cake and aspirating off the supernatant phase. It was found that the supernatant phase contained the active lipase with little or no lipolytic activity being associated with either the fat cake or the 12,000 × g sediment in this system.

One milliliter of supernatant was then added to a media containing 2.5 ml. of 20% of 0.06 *M* phosphate buffer, pH 6.8, and 5.0 ml. of distilled water. At zero time, 0.5 ml. of substrate (12.5% emulsion of peanut oil prepared with acacia as the emulsifier) was added, and samples were taken at 0, 20, 40, and 60 min. for assay of FFA by the procedures previously described (9). Assays of the homogenate system were conducted in an identical manner except no exogenous substrate was employed, the endogenous triglycerides of the adipose tissue serving as the substrate in these experiments. It should be pointed out that acacia was found to be the emulsifier of choice primarily because other emulsifiers tried, such as the polysorbates, interacted with the DMI to an extent sufficient to prevent DMI from exerting any effect on the system.

For the *in vivo* experiments described herein, male Holtzman rats weighing between 400 and 600 Gm. were anesthetized with sodium pentobarbital, 35 mg./Kg., i.p., their femoral and carotid arteries cannulated to provide for i.v. administration of drug and the taking of blood samples for FFA analysis,

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[1] The authors thank the Geigy Pharmaceutical Co. for the supplies of desmethylimipramine used in these studies.

respectively. The DMI was administered *via* the femoral vein over a 3-min. period, while norepinephrine (20 mcg./Kg.) was administered rapidly *via* the femoral vein. Blood samples were collected in heparinized tubes and stored over ice until centrifuged to obtain plasma samples. The FFA assays were conducted on 0.5 ml. of plasma obtained in this manner by the modified method of Dole (10, 11).

RESULTS AND DISCUSSION

Effect of DMI on Catecholamine-Induced FFA Release from Adipose Tissue Slices.—In an earlier study, it was reported that DMI antagonized catecholamine-induced release of FFA from rat epididymal fat tissue (9). Those studies were conducted utilizing a preincubation period in which the DMI was allowed to interact with the tissue for approximately 15 min. prior to the addition of the catecholamine. In the present experiment, it was decided to first allow the catecholamine (epinephrine) to maximally stimulate lipolytic activity in the adipose tissue slices and to add the DMI at a time when FFA release was occurring at a maximal rate. The results of this study are shown in Fig. 1. In these experiments, the adipose tissue slice preparation was divided into two portions. Epinephrine (final concentration 6.5×10^{-6} *M*) was added at zero time to each vessel and samples taken at 15 and 30 min. Immediately after the 30-min. sample, DMI (final concentration 1×10^{-3} *M*) was added to one incubation vessel and distilled water added to the other. Samples were then taken at 45 and 60 min. from both vessels. FFA analyses indicated that DMI caused the rapid cessation of FFA release as is indicated in Fig. 1. The control preparation continued releasing FFA at a constant rate. These results indicated that DMI was capable of antagonizing an already activated lipolytic system suggesting that the site of inhibition may be beyond the activation step, although this does not rule out the possibility that the inhibition is at the site of activation. Recent studies (12, 13) have indicated that the activation of lipolytic activity by the catecholamines involves the catecholamine stimulated conversion of ATP to 3′,5′-cyclic AMP which, in turn, activates the lipolytic enzymes. Thus, in such a sequence of events, several possible sites of inhibition are possible. Attempts were made to stimulate FFA release by the addition of 3′,5′-cyclic AMP to the adipose tissue slices. However, these efforts failed presumably because the cyclic AMP did not enter the adipose tissue slices or, as has been suggested (12), the 3′,5′-cyclic AMP is not sufficiently stable in this system to stimulate effectively lipolytic activity. Further attempts will be made, in this regard, utilizing more stable and lipid soluble analogs of cyclic AMP.

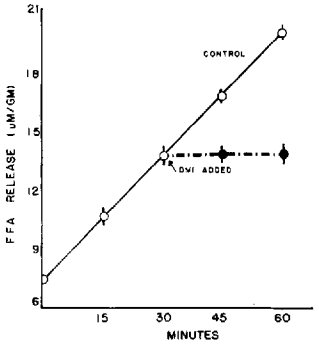

Fig. 1.—Effect of DMI on catecholamine-induced FFA release from adipose tissue slices. Vertical lines indicate standard error of the mean. Each point represents the mean of six determinations.

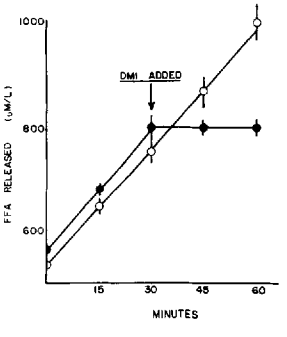

Fig. 2.—Effect of DMI on lipolytic activity of adipose tissue homogenates. Key: ○, control; ●, system to which DMI was added at 30 min. Vertical lines indicate standard error of the mean. Each point represents the mean of six determinations.

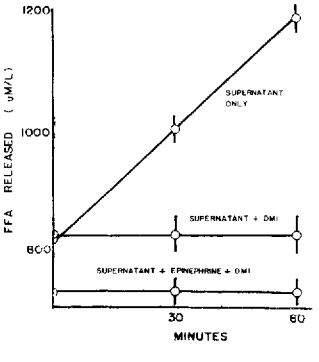

Fig. 3.—Effect of DMI on cell-free lipolytic activity. Vertical lines indicate standard error of the mean. Each point represents the mean of six determinations.

Effect of DMI on Lipolytic Activity of Adipose Tissue Homogenates.—It is characteristic of this type of preparation that the process of homogenization causes the maximal activation of lipolytic activity in the absence of any added catecholamine or other stimulant. Indeed, the addition of epinephrine to this system caused no further release of FFA above that observed in the absence of added catecholamine. Thus, the use of this system allowed the authors to investigate the effects of DMI on lipolytic activity in the absence of any added catecholamine.

In these experiments, a homogenate of adipose tissue was divided into two identical portions. Each incubation mixture was assayed for FFA at 0, 15, and 30 min. After the 30-min. sample was taken, DMI (final concentration 1×10^{-3} *M*) was added to one mixture and distilled water was added to the other. Samples were then taken at 45 and 60 min. from each preparation. The results of these experiments are shown in Fig. 2. It is again apparent that the addition of DMI caused the rapid cessation of FFA release while there was no diminution in the control FFA release. Although inhibition of the activation step is not entirely disproved, these results again suggest that the antagonism of FFA release exhibited by DMI occurred at a site remote from the activation step.

Effect of DMI on Cell-Free Lipolytic Activity.—Figure 3 illustrates the results obtained in studies conducted with the cell-free preparations. In

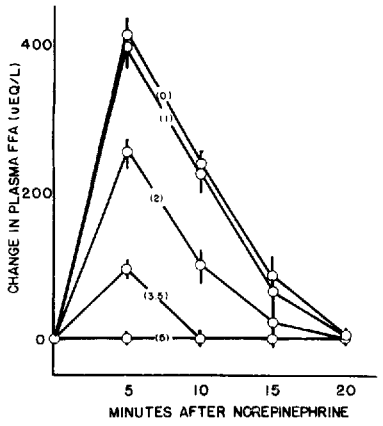

Fig. 4.—Inhibition of catecholamine-induced FFA mobilization, *in vivo*. Figures in parentheses indicate i.v. dose of DMI. Vertical lines indicate standard error of the mean. Each point represents the mean of six determinations.

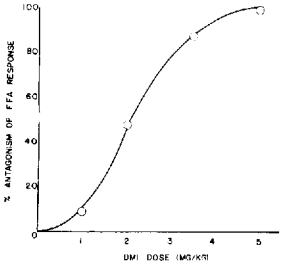

Fig. 5.—Dose-response relationship for inhibition of catecholamine - induced FFA mobilization, *in vivo*, by DMI. Each point represents the mean of six determinations.

these studies, employing a peanut oil emulsion as the substrate, maximal FFA release occurred without the addition of exogenous stimulants. The addition of DMI to yield a final concentration of 1×10^{-3} *M* completely abolished the FFA releasing activity of the preparations. The addition of epinephrine (1×10^{-6} *M*) did not reverse the antagonism as can be seen by the zero slope shown in Fig. 3. These results show that DMI is capable of antagonizing lipolytic activity with or without the addition of catecholamine.

In preliminary experiments, not reported here, the inhibition exhibited by DMI was overcome by the addition of additional enzyme (replacing the water in the media with an equal volume of supernatant enzyme preparation) but not with the addition of large quantities of catecholamine. It would thus appear that the DMI inhibition of lipolytic activity is dependent upon its interaction with enzymic material rather than at an adrenergic receptor site.

In Vivo Effects of DMI.—To ascertain whether DMI possessed the ability to inhibit FFA release *in vivo*, the drug was administered to animals, intravenously, at doses of 1.0, 2.0, 3.5, and 5.0 mg./Kg. Thirty minutes later, following the removal of a control blood sample, norepinephrine (20 mcg./Kg.) was administered *via* rapid i.v. injection. Norepinephrine was chosen for these experiments to minimize any variations in FFA mobilization due to the hyperglycemic responses so characteristic of other catecholamines. Blood samples were then taken at 5-min. intervals for a 20-min. period. The results of these experiments are shown in Fig. 4.

It can be seen in this graph that DMI possessed appreciable *in vivo* activity in antagonizing the norepinephrine-induced release of FFA, significant reductions in FFA mobilization being apparent at DMI doses of 2.0, 3.5, and 5.0 mg./Kg. The dose-response relationship for this inhibition is shown in Fig. 5.

It has been reported by Santi and Fassina (14) that the administration of DMI caused an elevation of plasma FFA, the maximal elevation being observed 150 min. after the administration of 25 mg./Kg. of DMI. These findings were confirmed in this laboratory. The apparent discrepancy in regard to the effects of DMI on plasma FFA can be explained in terms of the doses employed in the two studies and in the time course of events. Utilizing the interperitoneal route of administration, significant elevations of plasma FFA were obtained 150 min. after drug administration at a DMI dose of 25 mg./Kg., but not at a dose of 10 mg./Kg. or lower. Intravenous administration of DMI at doses up to 5.0 mg./Kg. caused no elevation of FFA. Furthermore, the elevations of FFA observed at the higher dose of DMI were rather slow in onset, becoming apparent only after approximately 100 min. following drug administration. DMI failed to elevate FFA, even at 25 mg./Kg., in the reserpinized rat. These results indicated that DMI may mobilize FFA through its central stimulatory activity (adrenergic mechanisms) and these effects may have a different time course than the DMI inhibitory effects on FFA release. These parameters are currently being investigated and will be reported in detail at a later date.

CONCLUSION

The data obtained in this study show that DMI is capable of inhibiting lipolytic activity in adipose tissue slices, homogenates, and cell-free preparations and also exhibits this property *in vivo*. The results suggest that DMI exerts its primary inhibitory effects at a site beyond the adrenergic receptor, probably at the enzymic site although conclusive proof of this postulate is lacking at this time.

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Metabolic Study of 2-(Acetyl-imino)-3-[2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline in Chickens

Detection of an Active Metabolite, 5,6-Dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole

By FERNAND T. N. ALLEWIJN and PAUL J. A. DEMOEN

After oral treatment of chickens with antazonite (R 6438), six basic products have been found by thin-layer chromatography in extracts of feces, one of them being the parent compound. The most important metabolite (metabolite No. 3) was isolated by column chromatography, and its structure was elucidated by physicochemical methods and confirmed by synthesis and analysis. The new product, 5,6-dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole (antienite, R 8141) was about 4 times as active as the original compound. There is some evidence suggesting that the anthelmintic activity of R 6438 is due to this metabolite. Two other metabolites were identified by comparison with existing substances on thin-layer chromatoplates. Further synthetic and parasitological work has shown that chemical modification of R 8141 may result in still more active substances. The most interesting analog, 2,3,5,6-tetrahydro-6-phenyl-imidazo[2,1-*b*]thiazole (tetramisole, R 8299) has been chosen for further detailed parasitological and pharmacological investigation.

THIENPONT and co-workers (1, 2) assigned good anthelmintic activity to 2-(acetyl-imino)-3-[2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline (R 6438, antazonite). When given orally to chickens in a dose of 160 mg./Kg. body weight, all *Ascaridia* sp., all *Heterakis* sp., and about 30% *Capillaria* sp. are expelled within 48 hr.

In order to gather information about the metabolism of R 6438, serum, different organs, eggs, and the feces of hens treated with the product were analyzed. The samples were homogenized, extracted by conventional techniques, and the extracts were analyzed by thin-layer chromatography (TLC) on Silica Gel G. A modified Liebermann-Burchard reagent was used as a spray to reveal the spots.

In the feces of chickens, at least five metabolites of antazonite occurred, together with the parent compound. Metabolite No. 3 was found in the greatest concentration, accounting for about 20% of the administered dose. The sum of the other metabolites was less than 5% of the dose of R 6438 given.

To elucidate the structure of the different metabolites, a total amount of 6 Gm. of R 6438 was given to 8 chickens, each animal receiving 750 mg., divided in three oral portions of 250 mg. The doses were administered at intervals of 32 and 38 hr. The feces (2.5 Kg.), collected over a period of 4.5 days after the first treatment, were dried, homogenized, and suspended in dilute acid. The basic compounds were extracted and purified by liquid–liquid extraction, and the final solution was concentrated to a small volume. This concentrate was used for thin-layer and for column chromatography on silica gel.

The fractions of eluate containing the major metabolite were combined and concentrated. Small samples of it were used for measurement of ultraviolet and infrared spectra. Both absorption patterns suggested a structure containing a 2-substituted, nonconjugated thienyl ring, and a C=N double bond in a five-membered ring. It was presumed at that moment that the product could be 5,6-dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole. The remainder of the concentrate was treated with oxalic acid yielding an oxalate salt, which, after recrystallization, gave 190 mg. of a crystalline product. This salt was analyzed (basic equivalent, C, H, N, and S determination, U. V. and I. R. spectra, TLC) and shown to be $C_9H_8N_2S_2.C_2H_2O_4$. TLC showed that it was a pure product, whereas both spectra and analyses were in agreement with the proposed formula. Treatment of one hen with 100 mg. of the material (corresponding to 70 mg. of the base) showed that the metabolite possessed enhanced anthelmintic properties (1).

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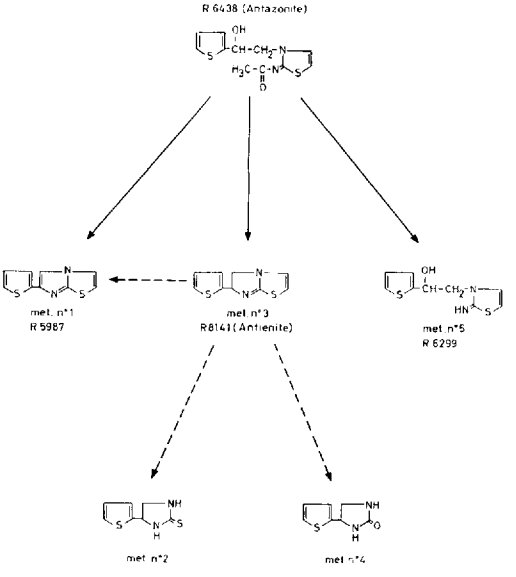
5,6 - Dihydro - 6 - (2 - thienyl) - imidazo[2,1 - *b*]-thiazole was prepared synthetically (3); the melting point of its oxalate salt and mixed melting point with the oxalate of the metabolite isolated from the feces proved the identity of the structures. TLC patterns, ultraviolet, and infrared spectra of both oxalates were also identical.

Two other metabolites were identified by comparison of their TLC patterns with those of known products related to R 6438. One of them, designated as metabolite No. 1, is an unsaturated analog of metabolite No. 3 and is 6-(2-thienyl)-imidazo[2,1-*b*]thiazole (R 5987) (3). The other identified product, designated as metabolite No. 5, is the deacetylated derivative of R 6438 and is 2-amino-3[-2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline (R 6299) (3). Both metabolites are practically devoid of anthelmintic properties (1). The remaining two metabolites (No. 2 and 4) could not be identified with certainty. However, it is supposed that both metabolites are degradation products of metabolite No. 3. This is supported by the fact that an analogous breakdown has been observed during metabolic studies on 5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole (R 8193), which is strongly related to R 8141. The structure of the metabolite No. 2 would then be 2-thio-5-(2-thienyl)-imidazolidine, whereas No. 4 would correspond to 2-oxo-5-(2-thienyl)-imidazolidine.

It is supposed that the anthelmintic activity of R 6438 is due to its major metabolite, R 8141. This is supported by the following facts.

(*a*) R 6438 is active as an anthelmintic in poultry, but not in rats (1). Its metabolite, R 8141, is active in both species. This metabolite occurs in feces of chickens and pigeons, but not in feces of rats. (*b*) About one-fifth of the R 6438 administered orally to chickens is found as R 8141 in feces. This metabolite is about 4 times as active as R 6438.

The metabolic degradation of R 6438 is represented in Scheme I.



Scheme I

TABLE I.—SOLUBILITY OF R 6438 AT ROOM TEMPERATURE

Solvent	pH	Solubility of R 6438, %
Water	6.4	0.065
0.01 *N* Hydrochloric acid	3.0	0.3
0.1 *N* Hydrochloric acid	2.1	2.5
Hexane	...	0.08
Chloroform	...	9.6
Methyl alcohol	...	6.9
Ethyl alcohol	...	4.1
Isopropyl alcohol	...	1.8
Diethyl ether	...	0.5
Acetone	...	5.4
Methyl isobutyl ketone	...	1.6
Ethyl acetate	...	2.4

TABLE II.—FECAL COLLECTIONS FOLLOWING TREATMENT

After First Treatment, hr.	Wt. of Feces, Gm. Fresh	Dried	Dry Material, %
32	1028	211	21.5
60	648	147	22.7
84	424	91	21.5
108	449	83	18.5
Total	2549	532	20.9

EXPERIMENTAL, RESULTS, AND DISCUSSION

Physicochemical Properties of Antazonite.—Antazonite, 2-(acetyl-imino)-3-[2-hydroxy-2-(2-thienyl)-ethyl]-thiazoline, has the structure as shown in Scheme I. It was synthetized by Raeymaekers (3). It occurs as a yellowish to brown-white, fine powder without odor or taste, with a melting point between 131 and 134°. The solubility of antazonite in water increases with decreasing pH (Table I). It is soluble in chloroform, methyl alcohol, ethyl alcohol, and acetone, sparingly soluble in isopropyl alcohol and methyl isobutyl ketone, and slightly soluble in diethyl ether.

The change in U.V. absorption upon variation in pH of the solutions indicates that antazonite has a pK value of about 3.6.

Principles of Extraction of R 6438.—The extraction of R 6438 is based on the fact that the compound forms a water-soluble salt, the solubility of which increases with decreasing pH. When the aqueous solution is made alkaline, antazonite precipitates, so the free base can be transferred into a water-immiscible organic solvent, such as ether, chloroform, or benzene.

Based on these principles, the first experiments were carried out on eggs of chickens, which were treated orally with one dose of 160 mg. R 6438/Kg. body weight. After extraction of these eggs and TLC of the extracts, four spots were found. The total amount of these different compounds was about 90–260 mcg./egg, within 48 hr. In order to elucidate the structure of these unknown products, further work was done on the feces of chickens.

Treatment of the Hens and Collection of Feces.—Eight white leghorn chickens, maintained each in a cage were given antazonite in No. 00 gelatin capsules, with invervals of 32 and 38 hr. Each bird was given 750 mg. of R 6438 and 600 mg. of sodium bicarbonate. They were given water and food *ad libitum*.

Fecal collections were made 32, 60, 84, and 108 hr. after the first treatment. (The results of each collection are given in Table II.)

Extraction of the Feces.—The feces of each collection were dried *in vacuo* at a temperature of 50°, whereafter the dried fecal output was homogenized in a Glencreston ball mill. (Afterward the drying process seemed to be unnecessary.) The homogeneous powder is suspended in 3 L. of 1 *N* hydrochloric acid and shaken for 1 hr. The suspension is filtered on a Büchner funnel, and the residue is treated again with 2 L. of distilled water. The filtrate, which is clear and deep-brown in color, is alkalized with 10 *N* sodium hydroxide (pH 10) and extracted with 4 + 3 + 3 L. of diethyl ether.

After concentration to about 1 L., the ether extract was extracted with 200 + 100 + 50 ml. of 0.1 *N* hydrochloric acid. The water layer was alkalized with 5 ml. of 10 *N* sodium hydroxide and extracted 3 times with 50 ml. of chloroform. The extract was concentrated by evaporation *in vacuo* to a volume of 20 ml., leaving a clear dark brown solution.

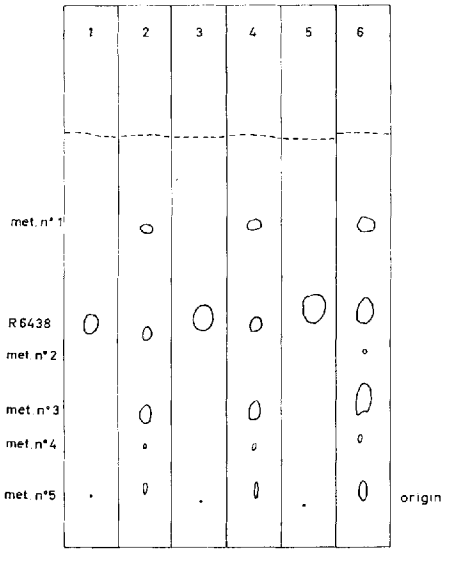


Fig. 1.—Thin-layer chromatogram of the concentrated fecal extract on Silica Gel G, 250 μ. Key: strips 1, 3, and 5: 1, 2, and 5 mcg. of R 6438; strips 2, 4, and 6: 1, 2, and 5 μl. of concentrated fecal extract.

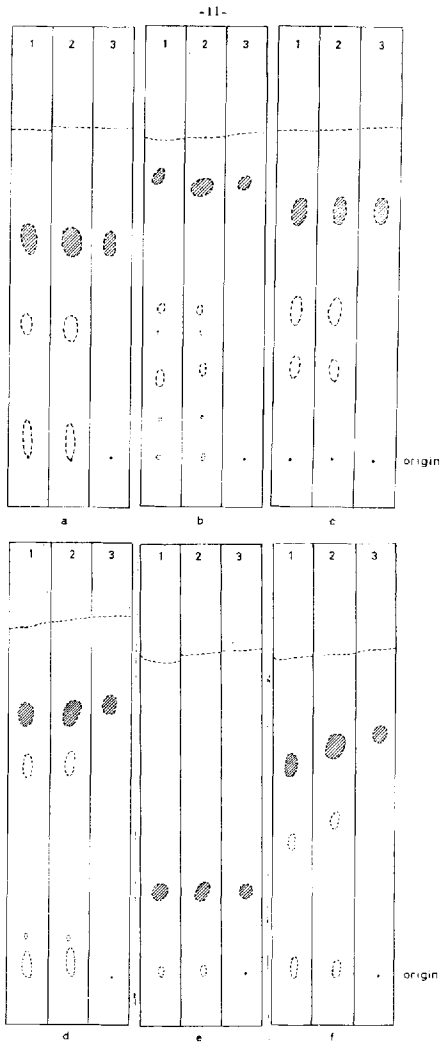


Fig. 2.—Identification of metabolite No. 1. Thin-layer chromatography of the concentrated extract on silica gel, 250 μ. Key: strip 1, 1 μl. of the concentrated extract; strip 2, a mixture of 1 and 3; strip 3, 1 mcg. of R 5987. Solvent systems are: a, methyl alcohol; b, chloroform–methyl alcohol (95:5 v/v); c, methyl alcohol–acetate buffer pH 4.7 (90:10 v/v); d, ethyl acetate; e, methylene chloride; f, methyl isobutyl ketone.

Thin-Layer Chromatography.—Small aliquots (1–5 μl.) of the concentrated extract were spotted on Silica Gel G chromatoplates (250 μ) and developed with a solvent system consisting of chloroform–methyl alcohol (95:5, v/v) (Fig. 1).

The air-dried chromatograms were sprayed with a modified Liebermann-Burchard reagent. This consists of ethyl alcohol–sulfuric acid, d. 1.84–acetic anhydride (80:10:10, v/v). Antazonite and its metabolites containing the thiophene ring system produce a blue color with this reagent after about 20 min. at a temperature of 110°.

At least six Liebermann-Burchard-active spots are found; two are very faint (metabolites No. 2 and 4).

Identification of Metabolites No. 1 and 5.—Metabolite No. 1 corresponds to 6-(2-thienyl)-imidazo[2,1-*b*]thiazole (R 5987) (Scheme I). This has been concluded after TLC on Silica Gel G with different moving liquids (Fig. 2): methyl alcohol, chloroform–methyl alcohol (95:5, v/v), methyl alcohol–acetate buffer pH 4.7 (90:10, v/v), ethyl acetate, methylene chloride, and methyl isobutyl ketone.

The R_f values of metabolite No. 1 correspond to those of the synthetically made R 5987.

Metabolite No. 5 was identified as deacetylated antazonite, 3-[2-hydroxy-2-(2-thienyl)-ethyl]-2-imino-thiazoline (R 6299). (See Scheme I.) The reference compound was chromatographed alone and co-chromatographed with the fecal extract. Three different moving liquids prove the structure of metabolite No. 5 (Fig. 3): methyl alcohol, ethyl acetate, and methyl alcohol–acetate buffer pH 4.7 (90:10, v/v).

Identification of Metabolite No. 3.—*Isolation Technique.*—This main metabolite was isolated by column chromatography on silica gel 0.05–0.20 mm. (E. Merck A. G., Darmstadt, 7734). An amount of 120 Gm. of the silica gel was suspended in methyl alcohol and poured in sections into a glass tube with an internal diameter of 3.0 cm. The column (35 cm. in length) was equilibrated at room temperature with about 200 ml. of methyl alcohol. The extract (about 15 ml.) was then placed on the column.

The chromatogram was first developed with methyl alcohol at a flow rate of 5 ml./hr., 10-ml. fractions being collected. After 1000 ml. of effluent, the developing solution was replaced by methyl alcohol containing 1% ammonia, of which 500 ml. was used.

Results of the Column Chromatography.—Each fraction of effluent was examined by thin-layer chromatography using 10-μl. quantities. The following separation was obtained: fractions 16 to 24, R 5987 and antazonite; fractions 23 to 64, the unknown metabolite (No. 3); fractions 106 to 124, deacetylated antazonite (R 6299).

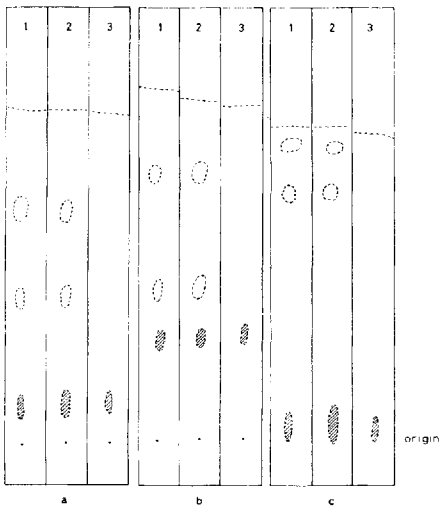


Fig. 3.—Identification of metabolite No. 5. Thin-layer chromatography of the concentrated extract on Silica Gel G, 250 μ. Key: strip 1, 1 μl. of the concentrated extract; strip 2, a mixture of 1 and 3; strip 3, 1 mcg. of R 6299. Solvent systems are: a, methyl alcohol; b, methyl alcohol–acetate buffer pH 4.7 (90:10 v/v); c, ethyl acetate.

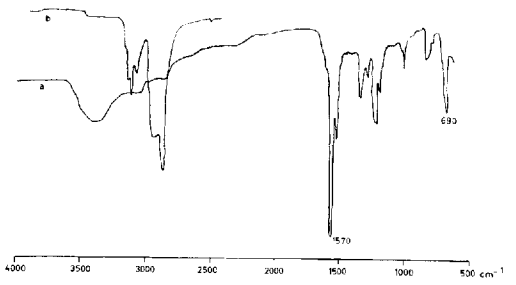


Fig. 4.—Infrared scan of one of the fractions 26–64 after evaporation. Key: a, KBr-disk; b, solution in carbon tetrachloride.

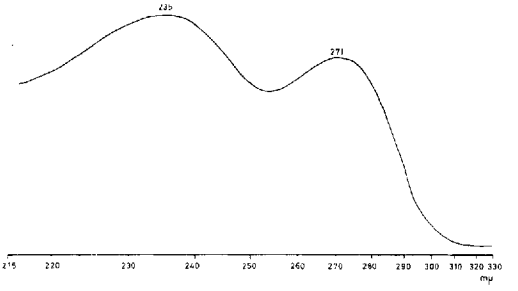
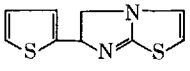


Fig. 5.—Ultraviolet scan of the same fraction (Fig. 4) in isopropyl alcohol.

Spectrophotometric Measurements.—U.V. and I.R. spectra of one of the fractions 26–64 were run. From the I.R. spectrum (Fig. 4) the following assignments could be made. The presence of —OH, =NH, and C=O is excluded. The 690 cm.$^{-1}$ band can be assigned to the γCH of the thiophene ring while the 1570 cm.$^{-1}$ band probably originates from a C=N group situated in a five-membered ring.

Examination of the U.V. spectrum (Fig. 5) revealed the presence of a nonconjugated thiophene ring (235 mμ). In this manner structure I



I

could be deduced (5,6-dihydro-6-(2-thienyl)-imidazo[2,1-*b*]thiazole).

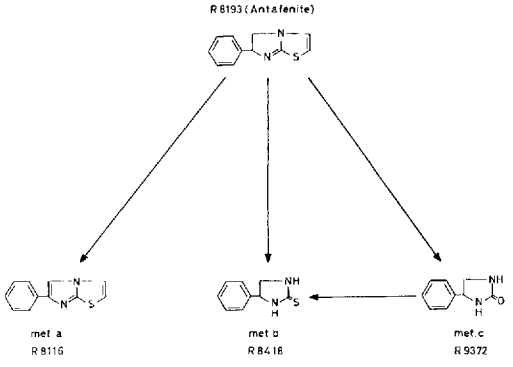
Crystallization and Analytical Results.—The fractions 26 to 60 were concentrated by evaporation *in vacuo*, and the remaining oily substance was crystallized as the oxalate salt from isopropyl alcohol.

The melting point after drying at 70° for 2 hr. was 191–192°.

Anal.—Calcd. for $C_9H_8N_2S_2.(COOH)_2$: C, 44.28; H, 3.38; N, 9.39; S, 21.50. Found: C, 44.05; H, 3.36; N, 9.55; S, 21.51.

The mixed melting point with the synthetically made oxalate (R 8025) was not depressed (192–193°). The ultraviolet spectrum (isopropyl alcohol) showed absorption bands at 237 mμ (ε 10,200) and 266 mμ (ε 9,320).

Possible Structure of Metabolites No. 2 and 4.—These two metabolites were not identified with certainty. Both metabolites could be degradation products of the major metabolite. This suggestion is supported by metabolic studies on 5,6-dihydro-6-phenyl-imidazo[2,1-*b*]thiazole (R 8193). The metabolic fate of R 8193 (antafenite) is represented in Scheme II.



Scheme II

Metabolites a and b were identified by thin-layer chromatography on Silica Gel G with several moving liquids. The spots were revealed with Dragendorff's reagent modified by Thies and Reuther (4).

Metabolite c was isolated by column chromatography on silica gel 0.05–0.20 mm. in the same manner as described above. The structure was elucidated by infrared spectrophotometry, and, after synthesis of the product, by thin-layer chromatography.

Comparison between the metabolic patterns of R 6438 (R 8141) and R 8193 suggests that analogous breakdown takes place, with the formation of analogous metabolites. The similarity between the R_f values of both groups of metabolites also confirms this suggestion (Fig. 6). It is concluded, therefore, that metabolite No. 2 could be 2-thio-5-(2-thienyl)-imidazolidine. Accordingly, metabolite No. 4 could be 2-oxo-5-(2-thienyl)-imidazolidine.

Anthelmintic Activity of R 8141 and R 6438.—One hen was treated with 100 mg. of the oxalate salt of metabolite No. 3 (R 8025). This corresponds to 70 mg. of the base. Examination of the feces showed the anthelmintic activity of the metabolite. In further parasitological studies the oxalate salt (R 8025) was replaced by the hydrochloride salt (R 8141).

The anthelmintic properties of the different substances in chickens can be summarized as follows.

(*a*) R 6438, 2-(acetyl-imino-3-[2-hydroxy-(2-thienyl)-ethyl]-thiazoline (antazonite). An oral dose of 160 mg./Kg. body weight has 100% effectiveness against *Heterakis* sp. and *Ascaridia* sp. Only 30% of *Capillaria* sp. are expelled.

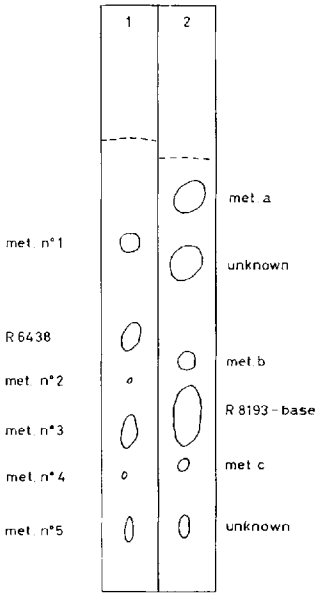


Fig. 6.—Comparative thin-layer chromatography of fecal extracts of chickens after oral treatment with R 6438, resp. R 8193. Key: strip 1, moving liquid–chloroform–methyl alcohol (95:5 v/v), spray reagent, Liebermann–Burchard; strip 2, moving liquid, chloroform–methyl alcohol (90:10 v/v); spray reagent, Dragendorff's reagent.

(*b*) R 8141, 5,6-dihydro-6-(2-thienyl)-imidazo-[2,1-*b*]thiazole (antienite). One oral dose of 40 mg./Kg. expels all *Heterakis* sp., *Ascaridia* sp., and *Capillaria* sp. from the chickens.

(*c*) R 8193, 5,6-dihydro-6-(2-phenyl)-imidazo-[2,1-*b*]thiazole (antafenite) has the same anthelmintic activity as R 8141, but the product is not so well tolerated.

(*d*) R 8299, 2,3,5,6-tetrahydro-6-phenyl-imidazo-[2,1-*b*]thiazole (tetramisole)[1] has been chosen for further parasitological and pharmacological work for several reasons. The dose required is less than 20 mg./Kg. body weight; the aqueous solution is sufficiently stable at room temperature; the product can be administered in several ways (orally, subcutaneously, or intramuscularly); and no side effects were observed (1).

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[1] Marketed under the trade names: Ripercol, Nilverm, and Citarin.

Diffuse Reflectance Studies of Solid–Solid Interactions II

Interaction of Metallic and Nonmetallic Adjuvants with Anthracene, Prednisone, and Hydrochlorothiazide

By MICHAEL BORNSTEIN* and JOHN L. LACH

Spectral information is presented on chemisorption-diffuse reflectance studies of metallic and nonmetallic adjuvant interaction with anthracene, prednisone, and hydrochlorothiazide. The systems investigated indicate that the primary mechanism responsible for the bathochromic, hyperchromic, and visual color changes is that of charge transfer chelation.

THIS COMMUNICATION is the second in a series in which diffuse reflectance spectra (DRS) of chemisorbed systems of pharmaceutical importance are examined. In the first report (1), the complexing properties of oxytetracycline, phenothiazine, anthracene, and salicylic acid have been investigated in order to verify the existence of such solid–solid interactions in pharmaceutical systems. Based on these data and theoretical considerations, it was felt that these interactions probably exist in other drug–adjuvant systems.

The purpose of this study was to continue this investigation in order that additional information be obtained concerning the nature of these interactions. Complexing data for anthracene, prednisone, and hydrochlorothiazide, with a number of metallic and nonmetallic adjuvants, is presented along with a discussion of probable mechanisms involved.

EXPERIMENTAL

Reagents.—Alcohol recrystallized anthracene, m.p. 218° (Eastman Organic Chemicals); prednisone U.S.P., m.p. 233–235° dec. (American Roland); hydrochlorothiazide, m.p. 273–275° (Merck, Sharp and Dohme); acid (anionotropic) alumina (Woelm); basic (cationotropic) alumina (Woelm); aluminum hydroxide, magnesium borate (K & K Laboratories); talc, attapulgite (colloidal) (Minerals and Chemicals Phillip); kaolin (colloidal), calcium carbonate, dibasic calcium phosphate, zinc stearate, stearic acid; anhydrous methyl alcohol.

Procedure.—The routine experimental procedure for preparing the complexes and method of analysis have been described in a previous report (1). All samples were prepared by aqueous equilibration in 25 ml. of distilled water for a period of 24 hr.

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RESULTS AND DISCUSSION

It has been shown in our previous publication (1) that large spectral shifts involved in the 400 mμ region, along with significant color changes observed, indicate that these interactions are of the donor–acceptor or charge transfer type. Spectral data dealing with anthracene, prednisone, and hydrochlorothiazide further indicate that these interactions are of a charge transfer variety.

ANTHRACENE–ADJUVANT SYSTEMS

The first group of systems to be discussed is the anthracene–adjuvant interactions. Anthracene was selected in that our preliminary studies, together with various literature reports, suggest that it undergoes various donor–acceptor interactions. For example, Aalbersberg *et al.* (2), in discussing complexes of aromatic hydrocarbons with strong Lewis acids, show electronic absorbance spectra from solution between basic anthracene (M) and such Lewis acids (A) as BF_3, PF_5, $SbCl_5$, and SO_3 and imply that these complexes may be covalent, MA, or contain M^+ ions. Solution MA spectra resemble those of MH^+, suggesting addition of A and H^+ at the same C atom in the —CH_2 bridges. Leonhardt and Weller (3), in studying proton–donor effects of hydrated cations with aromatic hydrocarbons, found that the order of proton donor capacity of metallic ions was as follows.

$Mg^{2+} > Ca^{2+} > Ba^2 > Li^+ > Na^+$. Perkampus and Kranz (4) studied anthracene complexes of Al_2O_3 with the aid of U.V. spectroscopy and found that the complexes do not fulfill the laws of normal electron donor–acceptor complexes and concluded that these were not charge transfer complexes. Rooney and Pink (5), in the EPR study of anthracene adsorption of a silica–alumina catalyst, compared the color of his complexes with spectra of anthracene dissolved in 98% H_2SO_4 and found that the color of the adsorbed species was identical to the corresponding H_2SO_4 solution, indicating that the adsorbed species is a radical resulting from the transfer of a single electron from the aromatic molecule to a hole in the surface, probably located at a Lewis acid site. Bhaltacharya (6) measured charge transfer interaction energies of I complexes with anthracene and other hydrocarbons in CCl_4 and found that the charge transfer energy was not

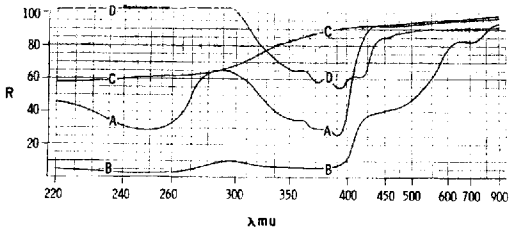

Fig. 1.—Diffuse reflectance spectra (DRS) of anthracene (100 mg.) and acidic alumina (3.50 Gm.). Key: A, control (physically mixed components); B, equilibrated sample; C, pure equilibrated acidic alumina, with no anthracene present; D, pure anthracene with no adjuvant present.

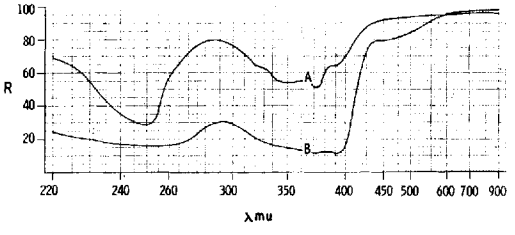

Fig. 2.—DRS of anthracene (100 mg.) and magnesium borate (2.00 Gm.). Key: A, control; B, sample.

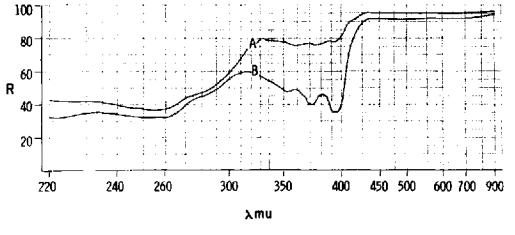

Fig. 3.—DRS of anthracene (100 mg.) and talc (2.00 Gm.). Key: A, control; B, sample.

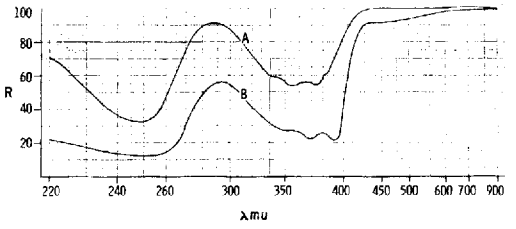

Fig. 4.—DRS of anthracene (100 mg.) and magnesium carbonate (2.00 Gm.). Key: A, control; B, sample.

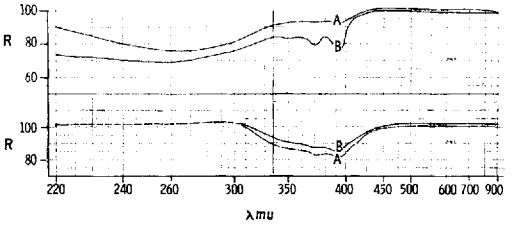

Fig. 5.—DRS of anthracene (100 mg.) and dibasic calcium phosphate (2.00 Gm.). Key: A, control; B, sample. (Top.) DRS of anthracene (100 mg.) and calcium carbonate (2.00 Gm.). Key: A, control; B, sample. (Bottom.)

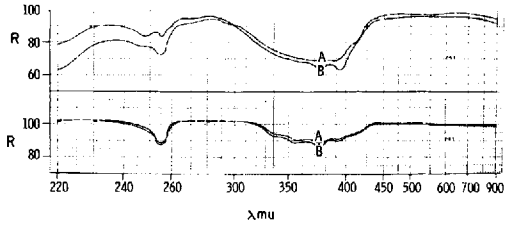

Fig. 6.—DRS of anthracene (50 mg.) and stearic acid (2.00 Gm.). Key: A, control; B, sample. (Top.) DRS of anthracene (200 mg.) and zinc stearate (0.750 Gm.). Key: A, control; B, sample. (Bottom.)

appreciably affected by the change in the dielectric constant of the medium, and, therefore, postulated that charge transfer must take place between the molecule, held together by forces other than electrostatic.

The adjuvants chosen for this study were selected on the basis of the metal fraction they contain or because of their usage in pharmaceutical formulation. Several of the adjuvants included in this study have also been investigated with respect to their effects on the absorption of various phenothiazine derivatives (7, 8).

This type of drug–adjuvant interaction, presented in Fig. 1, deals with the complex formation between anthracene and acid (anionotropic) alumina. It becomes apparent in examining this figure that Fig. 1,B, representing an equilibrated anthracene–alumina system, is significantly different from that of the physical mixture (1,A) and from the spectra of the individual components (1,C and 1,D). Upon interaction, a visual color change was evident; the physical mixture was white in color while the interacted sample acquired a reddish-tan hue. Along with the color change, a huge bathochromic shift is observed in the 400 mμ region along with a new shoulder formation and decrease in reflectance. It might also be pointed out that, in this system, the spectrum of the control is somewhat similar to that of pure anthracene, but the interacted sample loses some spectral fine structure in the region of 350–400 mμ. Furthermore, not only are significant spectral changes observed in the visible region, but a large change also occurs in the U.V. region of the spectrum. The absorbance peak observed at 290 mμ in the control is almost absent in the equilibrated sample, suggesting the complexity of this chemisorption phenomenon and the probable existence of a number of mechanisms. The individual spectrum of pure anthracene and pure alumina, in the region of 425–900 mμ, show no absorption while the interacted sample strongly absorbs in the visible region. The large bathochromic shift observed in the equilibrated sample occurs at a higher wavelength than that which would be produced by

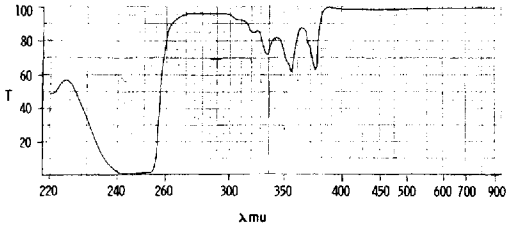

Fig. 7.—Transmittance spectrum of anthracene (5 mg./L.) in anhydrous methyl alcohol.

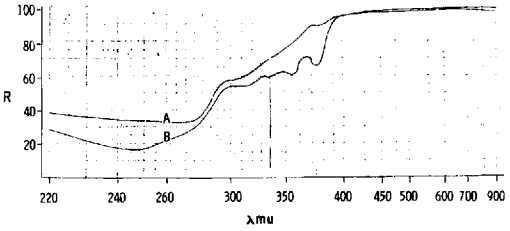

Fig. 10.—DRS of prednisone (100 mg.) and magnesium borate (2.00 Gm.). Key: A, control; B, sample.

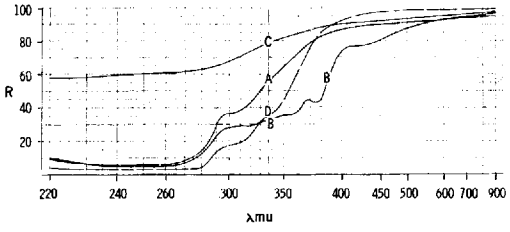

Fig. 8.—DRS of prednisone (100 mg.) and acidic alumina (3.50 Gm.). Key: A, control; B, sample; C, acidic alumina, 100%; D, prednisone, 100%.

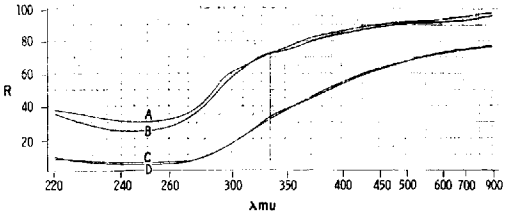

Fig. 11.—DRS of prednisone (100 mg.) with kaolin (2.00 Gm.) or with attapulgite (2.00 Gm.). Key: A, prednisone–kaolin control; B, prednisone–kaolin sample; C, prednisone–attapulgite control; D, prednisone–attapulgite sample.

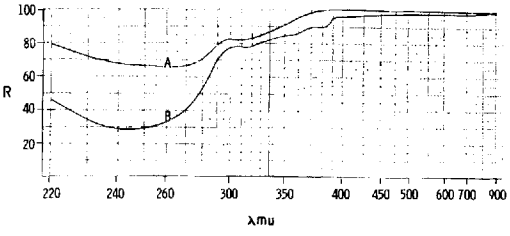

Fig. 9.—DRS of prednisone (100 mg.) and aluminum hydroxide (2.00 Gm.). Key: A, control; B, sample.

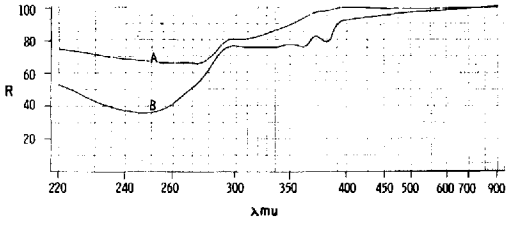

Fig. 12.—DRS of prednisone (100 mg.) and magnesium carbonate (2.00 Gm.). Key: A, control; B, sample.

a summation of the individual spectra of anthracene and alumina. Such a bathochromic shift along with the inability to elute the interacted anthracene sample with a large volume of water (1) further indicates the chemical nature of this strong interaction.

Although the spectra of an anthracene-basic alumina system are not shown due to space consideration, this system does exhibit similar spectral changes after equilibration, but to a lesser extent. Although a bathochromic shift and decreased reflectance were apparent, they were not as pronounced as the anthracene–acidic alumina system shown in Fig. 1. Since the shifts occur with both protophilic and protogenic excipients, it is suggested that anthracene may function either as an electron donor or acceptor, depending on the adjuvant available for interaction. Since greater shifts were observed in the acid alumina system, under the same experimental conditions, it may be reasoned that either less energy is required for the activation of the anthracene–acid alumina system or that fewer Lewis base sites were available for chemisorption with the anthracene. Similar changes were also observed when equilibrating anthracene with aluminum hydroxide. In this system, however, a white to orange visual color change was observed along with a slightly smaller shift in the visible region, but greater intensity increase in the area of 270–340 mμ.

In addition to the aluminum-containing systems mentioned above, a group of magnesium-containing adjuvants were also investigated. Results of magnesium trisilicate and magnesium hydroxide interactions have been previously reported (1). Figure 2 represents an anthracene–magnesium borate system. Here again, the usual bathochromic shift, loss of fine structure, and decreased reflectance associated with such an interaction, are found in the interacted sample. An anthracene–talc system may be found in Fig. 3. Talc, defined as native hydrous magnesium silicate, with a small portion of aluminum silicate, shows relatively little interaction affinity for anthracene, as seen in the small

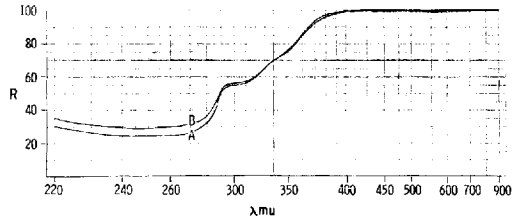

Fig. 13.—DRS of prednisone (100 mg.) and calcium carbonate (2.00 Gm.). Key: A, control; B, sample.

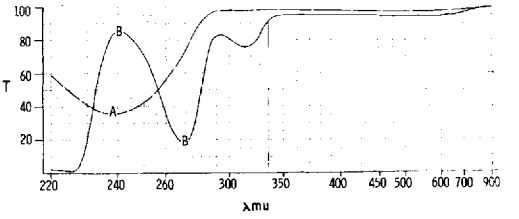

Fig. 16.—Transmittance spectra of prednisone and hydrochlorothiazide solutions. Key: A, spectrum of prednisone (10 mg./L.) in anhydrous methyl alcohol; B, spectrum of hydrochlorothiazide (10 mg./L.) in anhydrous methyl alcohol.

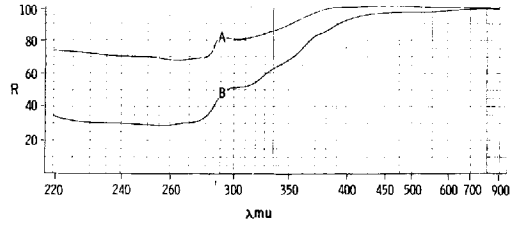

Fig. 14.—DRS of prednisone (200 mg.) and zinc stearate (1.00 Gm.). Key: A, control; B, sample.

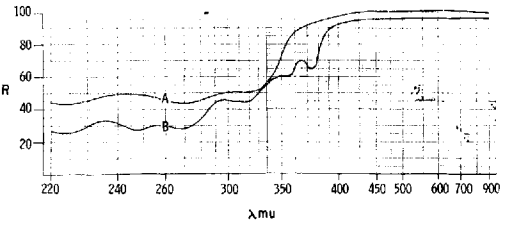

Fig. 17.—DRS of hydrochlorothiazide (100 mg.) and basic alumina (3.50 Gm.). Key: A, control; B, sample.

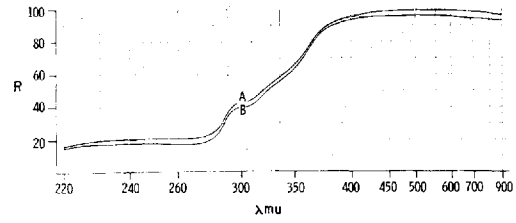

Fig. 15.—DRS of prednisone (100 mg.) and stearic acid (2.00 Gm.). Key: A, control; B, sample.

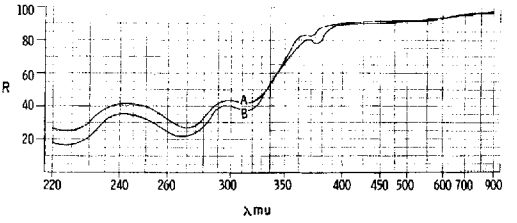

Fig. 18.—DRS of hydrochlorothiazide (100 mg.) and acidic alumina (3.50 Gm.). Key: A, control; B, sample.

spectral changes, both in the 220–300 mμ as well as in the 425–900 mμ regions. A larger change does occur, however, between 310–400 mμ. This change probably indicates some interaction of the metallic ion with the adsorbate since analogous changes were observed with attapulgite, which is hydrous magnesium aluminum silicate, and with kaolin which is hydrous aluminum silicate. The magnitude of anthracene–adjuvant change was found to be of the following order: kaolin > attapulgite > talc.

The difference in spectral changes of metal-containing anthracene–adjuvant systems may reside in the fact that although the magnesium or other ions may be present in talc and other metal-containing systems, they may be held tightly by the anionic fraction of the adjuvant and are, therefore, unavailable for interaction. This metal "attraction" difference exists between adjuvants since many surfaces are more saturated than others and the valency requirements of their surface atoms may be thought to be more fully satisfied by bonding with nearby atoms, therefore facilitating less chemisorption. Another cause for differences in saturation between metallic adjuvants may be the result of different amounts of hydration in adjuvant systems. For example, talc (primarily native hydrous magnesium silicate) may have a large portion of its active sites unavailable for charge transfer interaction since these sites are occupied by water molecules, resulting in the weaker electrostatic forces which may include hydrogen bonding.

An additional magnesium-containing system, found in Fig. 4, represents an anthracene–magnesium carbonate interaction. On equilibration, one again sees the usual bathochromic shift, along with decreased reflectance.

In addition to the aluminum and magnesium-containing adjuvants, anthracene interactions were also studied with calcium carbonate and dibasic calcium phosphate. The spectra of the anthracene–calcium carbonate system, shown in Fig. 5, indicate that the degree of interaction was minor, since the spectra of the equilibrated and nonequilibrated mixtures are comparable. The slight increased reflectance observed in curve 5,B (bottom) is attributed to the loss of small amounts of anthracene in the equilibrating procedure. The lack of any significant interaction with this calcium-containing adjuvant, as compared to the large changes observed in the magnesium and aluminum-containing systems,

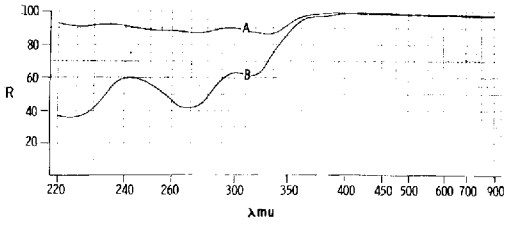

Fig. 19.—DRS of hydrochlorothiazide (100 mg.) and aluminum hydroxide (2.00 Gm.). Key: A, control; B, sample.

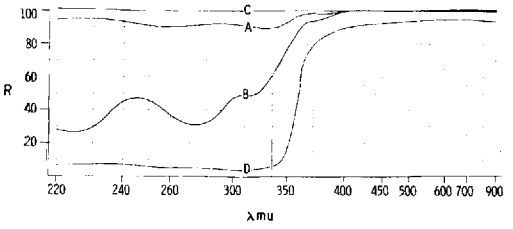

Fig. 20.—DRS of hydrochlorothiazide (100 mg.) and magnesium carbonate (2.00 Gm.). Key: A, control; B, sample; C, magnesium carbonate, 100%; D, hydrochlorothiazide, 100%.

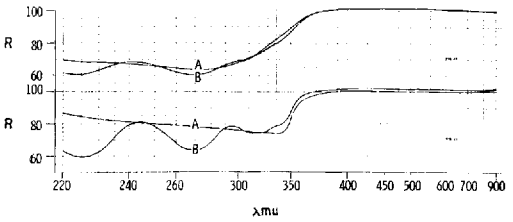

Fig. 21.—DRS of hydrochlorothiazide (100 mg.) and dibasic calcium phosphate (2.00 Gm.). Key: A, control; B, sample. (Top.) DRS of hydrochlorothiazide (100 mg.) and calcium carbonate (2.00 Gm.). Key: A, control; B, sample. (Bottom.)

suggests that the mechanism of paramount importance in this chemisorption process is one of chelation. This lack of calcium adjuvant–anthracene interaction is further illustrated in Fig. 5 (top), which again shows minor changes in the anthracene–$CaHPO_4$ system. However, since $CaHPO_4$ does show some decreased reflectance as compared to the $CaCO_3$ system, one may attribute this difference to proton site interactions, resulting from hydrogen-bond interaction rather than chelation. The lack of a color change in the calcium-containing adjuvant systems is in good agreement with the spectral data. As with the calcium-containing adjuvants, zinc stearate showed very little interaction tendency with anthracene, as illustrated in Fig. 6. Although other zinc-containing adjuvants were not studied, it is felt that the zinc ion would show low interaction tendencies with this anthracene system. It is further interesting to note that stearic acid itself shows no important interaction affinity for anthracene and the minor changes observed in the spectrum, Fig. 6, may be due to van der Waal or hydrogen-bond type of interactions.

With respect to the general mechanism of chemisorption, there is no specific agreement by what mechanism(s) this phenomenon proceeds. Weiss (9) suggested that all molecular complexes essentially have an ionic structure, B^+A^-. He points out that a low ionization potential for the base, B, and a high electron affinity for the Lewis acid, A, should give a stable complex. Here the color of molecular complexes, as in quinones or nitro compounds with unsaturated hydrocarbons, is described as being essentially of ionic character formed from the two components by an electron transfer from unsaturated hydrocarbon or its derivative (A), to the quinone or polynitro compound (B). Matsen and associates (10), in discussing charge transfer adsorption on metals, describe the metals as Lewis acids. In a similar manner, one might expect the unsaturated anthracene, due to its "basic" character, to transfer electrons to Al^{3+} or Mg^{2+} due to their acidic sites.

Mulliken (11) suggests that molecular complexes are a union of a Lewis acid (electron acceptor) and a Lewis base (electron donor) resulting in the formation of charge transfer complexes. Here, the electron of the adsorbate absorbs a quantum of radiation and is excited, not to a higher energy level of this molecule, but rather to one of the vacant high energy levels of the adsorbent. Therefore, in addition to absorption in the ground state (N), the molecule may combine to an excited state (E), resulting in an intense absorption band $\lambda N \rightarrow \lambda E$. This probability often accounts for the color changes observed when molecular complexes are formed. Similarly, in the anthracene–adjuvant systems presented in this communication, the large increase in intensity observed in the U.V. region (290 mμ), Fig. 1, may be attributed to a charge transfer complex between the Lewis base, anthracene, and the Lewis acid, Al^{3+}. Akamatu (12) has suggested, however, that in some anthracene chemisorption complexes, the metallic components transfer electrons to hydrocarbons, which in this case serve as acceptors rather than donors. This is probably the case when using the basic (cationotropic) alumina adsorbent discussed above.

It is interesting to compare Figs. 3, 4, and 6, containing anthracene equilibrated with magnesium carbonate, talc, and stearic acid, respectively; here bathochromic shifts and intensity differences also decrease respectively as one compares the three systems. These differences could be explained by

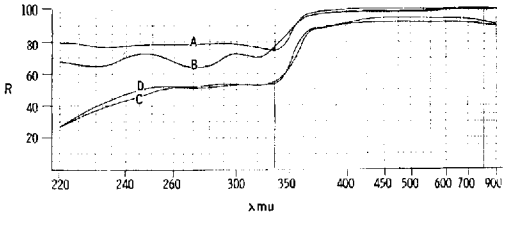

Fig. 22.—DRS of hydrochlorothiazide with zinc stearate or stearic acid. Key: A, hydrochlorothiazide (200 mg.) zinc stearate (0.750 Gm.) control; B, sample; C, hydrochlorothiazide (100 mg.)—stearic acid (2.00 Gm.) control; D, sample.

stating that the larger shifts are related to donor–acceptor forces which represent a charge transfer interaction along with the weaker hydrogen bonding, while in the talc and stearic acid, the weaker electrostatic forces predominate. It is also probable that some small spectral differences do result from regular reflection (1) and particle-size variations.

On further examination of the anthracene system, it is observed that aqueous equilibration of the anthracene–adjuvant mixture (Fig. 1,B) results in the disappearance of the band maxima at 290 mμ along with a loss of fine vibrational structure in the 350–400 mμ region. This band and fine structure also exist in anthracene solution spectra as seen in Fig. 7, as well as in the anthracene–adjuvant unequilibrated physical mixture (Fig. 1,A). In contrast to such a change, the equilibrated oxytetracycline–adjuvant system previously reported (1), show the formation of a new reflectance band at 320 mμ. It is further interesting to note that the maximum absorbance band is also present in transmittance spectra of oxytetracycline solutions but absent in the unequilibrated oxytetracycline–adjuvant mixture. Although no explanation is offered at this time, it nevertheless does illustrate the complexity of these interactions and the probability of a change of mechanism involved.

Based on the spectral data and visual color changes presented and already discussed, it is highly likely that the major mechanism involved in this anthracene chemisorption interaction, with various type adjuvants, is one of chelation since the metallic ions do show a specificity with respect to the degree of interaction. For example, aluminum and magnesium interactions were significantly larger than those observed with calcium and zinc-containing adjuvants. It is, therefore, difficult to make generalizations regarding such interactions in that the nature and the degree of interaction will vary with the type of drug and adjuvant employed.

PREDNISONE–ADJUVANT SYSTEMS

Since a number of reports have appeared in the literature dealing with the possible inactivation of prednisone by excipient-type materials used in solid dosage forms (13, 14) and since prednisone represents a polyfunctional steroidal molecule used in milligram quantities in therapeutics, an investigation of this drug with various adjuvants was undertaken. Although no color change was observed on equilibration of prednisone with acidic alumina, Fig. 8, an examination of the spectral data does indicate that prednisone undergoes a significant interaction. In addition to the usual bathochromic shifts observed, the presence of the equilibrated prednisone–adjuvant spectrum at a higher wavelength than that of the physical mixture or the pure prednisone, further supports this high degree of reactivity. Equilibration of prednisone with basic alumina results in similar spectral changes, but to a lesser extent.

A comparison of prednisone–aluminum hydroxide interaction, Fig. 9, with the above alumina system clearly illustrates the spectral changes possible with the use of different adjuvants. In an aluminum hydroxide system, no significant bathochromic shifts are observed, but a large hyperchromic change occurs in the U.V. region, suggesting the variety of mechanisms possible in such interactions.

This is further observed in magnesium-containing adjuvants, as illustrated in Fig. 10. In this prednisone–magnesium borate system, equilibration facilitates small intensity changes in the U.V. region, along with a new band formation between 350–400 mμ and a smaller bathochromic shift. The use of magnesium trisilicate as the adjuvant results in comparable spectral changes, accompanied by a larger intensity increase in the U.V. region.

Furthermore, even though ions may be present in such adsorbents as talc, attapulgite, and kaolin, their interaction tendencies with prednisone are minor, as illustrated in Fig. 11. As was previously pointed out in the anthracene–adjuvant systems, the degree of saturation of individual adjuvants varies, resulting in different reaction potentials. This difference in availability or reaction potential of these ions is shown in the prednisone–magnesium carbonate interaction, illustrated in Fig. 12. Here again, large intensity and bathochromic changes in the U.V. and near the U.V. region are observed.

A comparison of prednisone–calcium carbonate equilibration, Fig. 13, indicates a lack of interaction of this drug with calcium, as seen from the minor intensity changes in the U.V. region. Equilibration of prednisone with $CaHPO_4$ produced spectral changes of similar nature to the calcium carbonate system, except for a slightly greater hyperchromic effect. This change in reactivity between $CaHPO_4$ and $CaCO_3$ may again be attributed to proton site interactions possible in the $CaHPO_4$ system.

In addition to aluminum, magnesium, and calcium-containing adjuvants, prednisone was also equilibrated with zinc stearate. Spectral data presented in Fig. 14 indicate a strong reactivity of prednisone for this adjuvant. In the previously discussed anthracene system, however, the same adjuvant showed very little interaction tendency. This difference in reactivity of prednisone and anthracene for the zinc stearate adjuvant, along with the lack of any interaction of prednisone with stearic acid, shown in Fig. 15, illustrates the chemical specificity of these charge transfer reactions.

The above information presented on these prednisone–adjuvant systems again indicates that although no visible color differences are observed on equilibration, large spectral changes do suggest strong interactions with various characteristics, depending on the adjuvant studied. Although the control and equilibrated sample's reflectance spectra somewhat resemble the prednisone transmittance spectrum in solution (Fig. 16,A) solid–solid interaction often facilitates the elucidation of new vibrational structure in the near U.V. region, not present in the prednisone solution spectra. Furthermore, the spectral changes of prednisone complexes differ from anthracene systems since aluminum-containing adjuvants appear to facilitate a greater degree of interaction with anthracene, while zinc ions play an important role in prednisone–adjuvant chemisorption.

HYDROCHLOROTHIAZIDE–ADJUVANT SYSTEMS

This medicinal agent has been selected for the study of these drug–adjuvant interactions due to its unique polyfunctional chemical nature and because of its widespread use as a therapeutic agent.

As in the prednisone–adjuvant interactions, although no color change was observed on equilibration of hydrochlorothiazide with basic alumina, an examination of Fig. 17 indicates that this system does undergo important interaction.

A somewhat different spectral graph was obtained on equilibrating hydrochlorothiazide with acidic alumina, Fig. 18, as compared to that of the basic alumina, since the usual bathochromic shift was absent and an intensity change of only 5% was observed. A possible explanation for this lack of reactivity in the hydrochlorothiazide–acid alumina system may be due to the presence of stronger electron-donating sites in hydrochlorothiazide, represented by the dioxide or sulfamoyl groups. Since hydrochlorothiazide and acidic alumina both function as Lewis bases, their lack of significant interaction, Fig. 18, is reasonable. However, hydrochlorothiazide does react strongly with basic alumina (cationotropic) or aluminum hydroxide, Fig. 19, which function as Lewis acids.

In addition to the aluminum-containing adjuvants, hydrochlorothiazide was also equilibrated with a group of magnesium-containing excipients. Figure 20, B, dealing with a hydrochlorothiazide–magnesium carbonate interaction, indicates that although no visible color change was observed in this, or any other hydrochlorothiazide system investigated, large intensity changes are seen in the U.V. region; these hyperchromic changes are also accompanied by important shoulder formation, not found in the pure hydrochlorothiazide reflectance spectrum (Fig. 20,D), in the physical drug–adjuvant control (Fig. 20,A), in the pure adjuvant's spectrum (Fig. 20,C), but present in the solution transmittance spectrum (Fig. 16,B). Similar spectral changes were also seen on equilibrating hydrochlorothiazide with magnesium borate or magnesium trisilicate adjuvants.

In contrast to these adjuvants, talc, attapulgite, or kaolin produced only minor intensity changes on equilibration with hydrochlorothiazide, again indicating minor interaction tendency for this drug. Hydrochlorothiazide shows different spectral changes with calcium adjuvant equilibration, as seen in Fig. 21. An examination of this figure indicates that equilibration of hydrochlorothiazide with $CaCO_3$ facilitates the formation of new bands, although the usual bathochromic shift seen with aluminum and magnesium systems was absent. This drug was also equilibrated with dibasic calcium phosphate. In the anthracene and prednisone systems, $CaHPO_4$, functioning as the adjuvant, showed somewhat greater interaction tendencies than observed with calcium carbonate. However, the interaction of $CaCO_3$ in the hydrochlorothiazide system was found to be in reverse, Fig. 21, indicating the $CaCO_3$ functions as a stronger Lewis acid (electron acceptor) in this system.

The interaction data presented and discussed indicate that a donor–acceptor chelation mechanism is operative in these systems and is further supported in the zinc stearate, stearic acid interaction differences with hydrochlorothiazide, as shown in Fig. 22, again pointed out the greater interaction tendency of the metal-containing adjuvants.

CONCLUSION

Chemisorption interaction data have been presented for anthracene, prednisone, and hydrochlorothiazide with a number of metallic and nonmetallic adjuvant systems. Although minor spectral contributions may be attributed to van der Waal and hydrogen bond-type interactions, particularly in nonmetallic adjuvants studied, the large bathochromic and hyperchromic spectral changes observed on equilibration are primarily the result of strong charge transfer chelation interactions.

Results indicate that anthracene and prednisone function mainly as Lewis acids in alumina systems, while hydrochlorothiazide primarily exhibits Lewis base properties. The interactions observed are, therefore, highly dependent on the chemical properties of the adsorbate and the adsorbent.

Although these surface chemisorption phenomena exist as a unimolecular film and may be of no great consequence when physically adsorbed multilayers of the drug exist, particularly when the ratio of the therapeutic agent to adjuvant is high, they do become significant when the drug–adjuvant ratio is low. In such systems, where the medicinal agent is primarily chemisorbed as a unimolecular layer, one would expect to find large deviation in dissolution rates and blood levels in biological systems. It is of significant interest to point out here that one cannot overlook the distinct possibility of the excipient itself existing as a chemisorbed layer covering the surface of the drug resulting in similar alterations of the physical or biochemical behavior of the medicament in dosage forms where the drug–adjuvant ratios are high. Diffuse reflectance spectroscopy therefore provides a simple means of studying the possible existence of such interactions in solid dosage forms.

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Diffuse Reflectance Studies of Solid–Solid Interactions III

Interaction Studies of Oxytetracycline with Metallic and Nonmetallic Adjuvants

By JOHN L. LACH and MICHAEL BORNSTEIN*

Spectral data are presented for chemisorption studies of oxytetracycline and oxytetracycline HCl with a number of metallic adjuvants containing aluminum, calcium, magnesium, zinc, and sodium ions as well as for some nonmetallic adjuvants including stearyl alcohol, cetyl alcohol, stearic acid, silica gel, starch, tannic acid, tragacanth, and polyethylene glycol 6000. The interactions, studied with equilibration, compression, and moisture techniques, indicate that a metal ion or polyfunctional adsorbent molecule is necessary for these charge transfer interactions.

In previous reports (1, 2), Lach and Bornstein have demonstrated that several drugs undergo chemisorption with a number of pharmaceutical adjuvants. Using diffuse reflectance spectroscopy (DRS), it was shown that salicylic acid, phenothiazine, anthracene, prednisone, and hydrochlorothiazide form complexes with a number of adsorbents commonly used in pharmaceutical dosage forms. Furthermore, it was pointed out that these reactions may occur either by compression techniques or equilibration of the components of the complex in aqueous or nonaqueous media. Interaction properties of oxytetracycline with magnesium trisilicate and activated alumina were also investigated, and results indicate that DRS of equilibrated or compressed samples produce large bathochromic shifts and intensity changes accompanied by significant visual color variation, indicative of charge transfer interactions.

The purpose of this investigation is to further study this oxytetracycline interaction with various metallic and nonmetallic adjuvants. Information from this investigation would give additional information as to the possible nature of the mechanism or mechanisms involved in this interaction.

EXPERIMENTAL

Reagents.—Recrystallized oxytetracycline, m.p. 182° dec. (Pfizer & Co.), oxytetracycline HCl (Pfizer & Co.), calcium phosphate (dibasic), calcium phosphate (tribasic), calcium hydroxide, calcium carbonate, calcium stearate, cetyl alcohol, stearyl alcohol, stearic acid, magnesium stearate, magnesium oxide, magnesium hydroxide, magnesium trisilicate, magnesium chloride, magnesium silicate,[1] magnesium carbonate, magnesium sulfate, talc, zinc stearate, aluminum hydroxide, silica gel, polyethylene glycol 6000,[2] starch, tannic acid, acacia, tragacanth, and dehydrated ethanol.

Procedure.—The routine experimental procedure for preparing the complexes and method of analysis have been described in a previous communication (1). All samples have been prepared by equilibration in 25 ml. of distilled water for 24 hr., except when otherwise indicated (2). The following is a special technique adopted for this study.

Moisture Effects.—This study involves the exposure of dried, physically mixed drug–adjuvant components to humidity conditions. The control is prepared by triturating a mixture of drug and adjuvant which were first individually vacuum dried for 0.5 hr. at 115°; the DRS of this physical mixture is then measured.

The moist sample is prepared by transferring a portion of this physical mixture onto an evaporating dish and placing it in a wax sealed desiccator in which water is substituted for the desiccant; the material is then allowed to stand in this humidity chamber for a designated period, after which time the moist sample is removed and its DRS is measured.

The above moist sample may also be vacuum dried for 0.5 hr. at 115° prior to DRS reading, in order to eliminate spectral moisture effects.

Past Work.—The complexes of interest in these studies vary in degree between the weaker van der Waal variety discussed by Higuchi and Lach (3), whose energy of reaction is derived mainly from dipole–dipole interactions, to the stronger intermolecular electron exchange mechanism described by McGlynn (4). These stronger charge transfer transitions of donor–acceptor complexes are often found as absorbancee bands in the near U.V. or visible region of the electromagnetic spectrum. According to Andrews and Keefer (5), this is often the case when acceptors are π acids.

Other pertinent reports in this area include com-

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Previous paper: Bornstein, M., and Lach, J. L., *J. Pharm. Sci.*, **55**, 1033(1966).

[1] Marketed as Magnesol by the Waverly Chemical Co., Inc.

[2] Marketed as Carbowax 6000 by Carbide & Carbon Chemical Corp., New York, N. Y.

plex formation studies between adjuvants (6, 7) and various medicinal agents. Several examples may also be cited which deal with the complex formation of multivalent metallic cations, including Mg^{2+} and Ca^{2+}, with tetracyclines (8, 9). Other studies deal with the ability of magnesium, calcium, and aluminum (10–12) to alter the antibacterial action of various tetracyclines. In view of these developments, it is not surprising to find literature reports indicating significant gastrointestinal absorption differences between complexed and uncomplexed forms of tetracyclines (13, 14).

RESULTS AND DISCUSSION

Survey of Adjuvants with Oxytetracycline.—As has been pointed out, this investigation was undertaken to further study a number of oxytetracycline–adjuvant systems with respect to this interaction and the possible mechanism(s) involved. A number of representative spectral systems are presented and discussed. Other spectral data obtained in this study are not included in this report due to space consideration; however, they are included in the discussion.

Spectral data presented in Fig. 1 illustrate the interaction between oxytetracycline and aluminum sulfate. Here curve B represents an oxytetracycline–aluminum sulfate system, equilibrated in dehydrated ethanol, while the physical dry mixture (control) is represented by curve A. Spectra C and D (Fig. 1) represent curves of the individual aluminum sulfate and oxytetracycline components, respectively. An examination of these spectra reveals that the alcohol-equilibrated sample is significantly different from the physically mixed control and also from the spectra of the individual components. These differences include a large bathochromic shift of approximately 200 mμ in the visible region, accompanied by an intensity change in the U.V. region. Visual evidence of this interaction was also noted by a color change between the control and sample, where the physical mixture had a faint yellow color while the alcohol-equilibrated sample acquired an orange hue. These large spectral and physical differences are indicative of a strong charge transfer interaction (1, 2, 15). Similar spectral changes were also observed when equilibrating oxytetracycline with anionotropic and cationotropic alumina (1) in aqueous media. The nature of the solvent (aqueous or nonaqueous) apparently does not interfere significantly with this interaction, as has been previously pointed out (1), thus indicating a high degree of chemisorption. The presence of this strong interaction is further illustrated by the fact that the alcohol equilibrated oxytetracycline–aluminum sulfate sample (Fig. 1, B) produces a 200 mμ bathochromic shift, occurring at a higher wavelength than that for the reflectance spectrum of pure oxytetracycline (Fig. 1, D). This spectral observation further substantiates this strong interaction in that a summation of the drug-adjuvant spectrum would not result in this degree of change. It is of further interest to point out here that the addition of water to a mixture of oxytetracycline and alumina results in the release of a noticeable amount of heat along with the formation of bubbles, again indicating a large degree of interaction for this exothermic system.

Another aluminum-containing system, Fig. 2, represents spectra of aqueous equilibration of a dry physical mixture of oxytetracycline with aluminum hydroxide. Although the bathochromic shift is somewhat different from that of the aluminum sulfate interaction, the change in intensity is greater. This hyperchromic change in intensity is accompanied by a new band formation at 310 mμ. Possible reasons for this band formation have been previously discussed (1, 2).

In addition to the aluminum-containing adjuvants mentioned above, several calcium-containing systems were also investigated. These adjuvants also react with oxytetracycline producing the usual bathochromic shifts accompanied by significant hyperchromic changes, new band formation, as well as additional shoulder formation in the visible region. It should also be pointed out that visual color changes in calcium-containing systems were smaller than those described in the aluminum-containing adjuvants; here a color change from light yellow to a deeper more intense yellow was often noticed. Spectral data of oxytetracycline–calcium hydroxide equilibration, found in Fig. 3, point out some of these changes between the physically mixed control (A) and the equilibrated sample (B). An examination of this figure illustrates the fact that the bathochromic shift in this calcium hydroxide system is similar in magnitude to the aluminum hydroxide system found in Fig. 2. A difference between these two figures is evident, however, since the calcium-containing system has a smaller intensity change, especially in the region of 310–330 mμ where oxytetracycline–aluminum hydroxide band formation is more intense than that observed with the corresponding calcium hydroxide system. Nevertheless, calcium hydroxide does appear to react with oxytetracycline, as seen from various spectral changes in Fig. 3.

Other oxytetracycline systems producing similar spectral changes with aqueous equilibration to those described above include systems containing oxytetracycline with calcium carbonate, or calcium stearate, not shown in this communication due to space consideration.

Spectral data of aqueous equilibration of dibasic calcium phosphate or tribasic calcium phosphate with oxytetracycline are somewhat similar to that of calcium hydroxide with a bathochromic shift of 80 mμ and small band formation for oxytetracycline–$CaHPO_4$ while the oxytetracycline–$Ca_3(PO_4)_2$ system shows a 30 mμ bathochromic shift accompanied by intensity changes similar to those seen with the dibasic salt. Color changes from faint yellow to a deeper yellow were also observed in these calcium-containing systems. This interaction difference between $CaHPO_4$ and $Ca_3(PO_4)_2$ with oxytetracyline may be attributed to the fact that the former salt has a proton which could more easily provide an active site for interaction, because of its greater tendency to accept electrons from the adsorbate. It is also possible that a greater degree of hydrogen bonding is facilitated with $CaHPO_4$ systems as discussed in a previous report (2).

Another group of metal adjuvants investigated were those containing magnesium ions. Figure 4 represents spectra of an equilibrated and nonequilibrated oxytetracycline–magnesium hydroxide system. The changes observed after equilibration

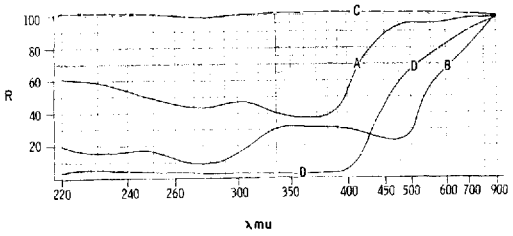

Fig. 1.—DRS of oxytetracycline (50 mg.) and aluminum sulfate (2.00 Gm.), equilibrated in dehydrated ethyl alcohol. Key: A, control (physical mixture); B, equilibrated sample; C, aluminum sulfate, 100%; D, oxytetracycline, 100%.

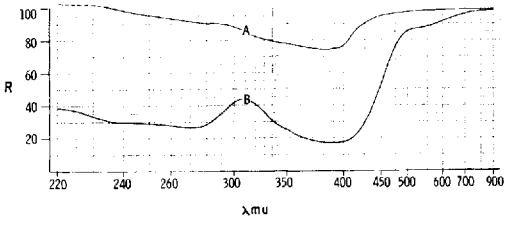

Fig. 2.—DRS of oxytetracycline (50 mg.) and aluminum hydroxide (2.00 Gm.). Key: A, control; B, sample.

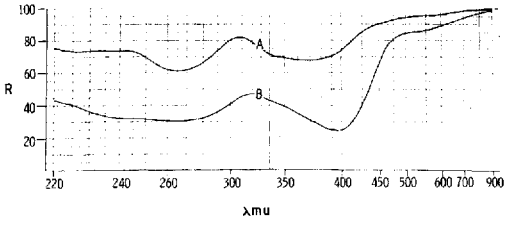

Fig. 3.—DRS of oxytetracycline (50 mg.) and calcium hydroxide (2.00 Gm.). Key: A, control; B, sample.

again include a bathochromic shift of about 50 mμ, a hyperchromic change, new band formation as well as a shoulder formation at 500 mμ. Similar spectral changes were observed with other oxytetracycline–magnesium-containing systems investigated. Included in this group are talc (native hydrated magnesium silicate), magnesium oxide, magnesium stearate, and synthetic magnesium silicate.[1] The interaction of oxytetracycline with synthetic magnesium silicate produces a bathochromic shift, intensity change, band formation, and shoulder formation to a greater degree than talc due to this material's high surface area, high porous structure, large internal surface, and high adsorption capacity. Similar changes were also observed on equilibrating oxytetracycline with zinc stearate, although the visual color change was not as pronounced.

Since data indicate that these interactions may be due primarily to the metallic ion in these systems, a study of nonmetallic adjuvants with respect to these interactions was undertaken. The interaction of oxytetracycline with stearyl alcohol or cetyl alcohol, shown in Fig. 5, illustrates this lack of interaction involved. An examination of this figure indicates that neither the stearyl alcohol, nor cetyl alcohol–oxytetracycline spectrum shifted to any appreciable degree after equilibration, as was expected. There is neither evidence of significant intensity variation, new band formation, shoulder formation, nor visual color change. The small variation in reflectance observed may be due to particle size difference, light scattering, or weak electrostatic attractions (2). Similar spectral results were also obtained on equilibrating oxytetracycline with stearic acid.

Since calcium stearate, magnesium stearate, zinc stearate, and other metal-containing adjuvants react strongly with oxytetracycline, as evidenced by large spectral changes, while stearyl alcohol, cetyl alcohol, and stearic acid do not, the interactions observed are primarily due to the metallic ion portion of the adjuvant. The other spectral and visible color observations previously indicated (1, 2) further suggest that this is a charge transfer chelation interaction.

It should be pointed out, however, that other nonmetallic adjuvants, containing polyfunctional groups, show spectral changes on equilibration with oxytetracycline, although these systems have inherently different spectral characteristics. An example of this type of change may be found in

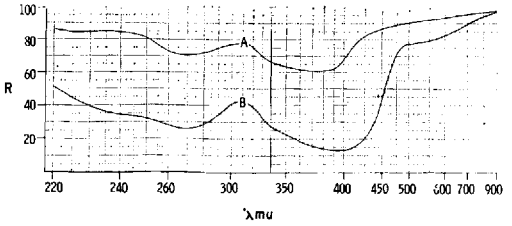

Fig. 4.—DRS of oxytetracycline (50 mg.) and magnesium hydroxide (2.00 Gm.). Key: A, control; B, sample.

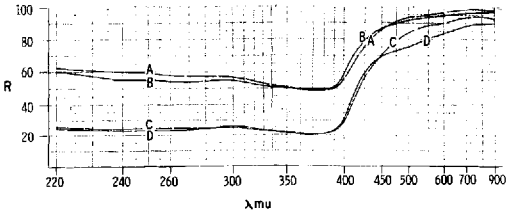

Fig. 5.—DRS of oxytetracycline (50 mg.) with stearic acid (2.00 Gm.) or with cetyl alcohol (2.00 Gm.). Key: A, oxytetracycline–stearic acid control; B, oxytetracycline–stearic acid sample; C, oxytetracycline–cetyl alcohol control; D, oxytetracycline–cetyl alcohol sample.

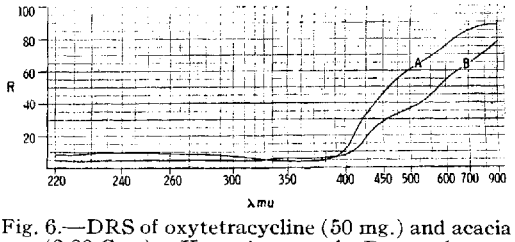

Fig. 6.—DRS of oxytetracycline (50 mg.) and acacia (2.00 Gm.). Key: A, control; B, sample.

Fig. 6, showing spectra of an equilibrated and non-equilibrated oxytetracycline–acacia system. Since acacia is a polyfunctional material, it is reasonable to assume that one or more of the functional groups may react with oxytetracycline by a donor–acceptor mechanism (16), producing the observed spectral change in the visible region. It should also be pointed out that other adjuvants, including tannic acid and starch, react with oxytetracycline to give similar changes; spectra of these systems are not presented due to space considerations. Figure 7 represents the spectra of an equilibrated and non-equilibrated oxytetracycline–polyethylene glycol 6000 system. The large bathochromic shift, hyperchromic effect, and visual color changes observed in equilibrating this system may again be attributed to an interaction between oxytetracycline and the ether linkage of the ethylene oxide units of the polyethylene glycol polymer. This observation is in agreement with published reports dealing with polyethylene glycol interactions in aqueous solution (3, 17). A bathochromic shift of similar magnitude, along with a visual color change from light yellow to a deep red-brown, was also observed on equilibrating oxytetracycline with silica gel.

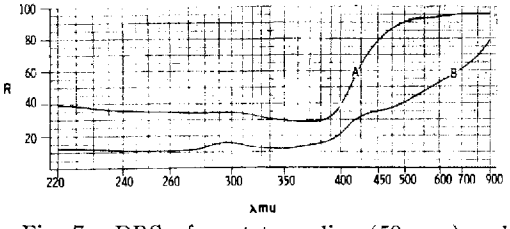

Fig. 7.—DRS of oxytetracycline (50 mg.) and polyethylene glycol 6000 (2.00 Gm.). Key: A, control; B, sample.

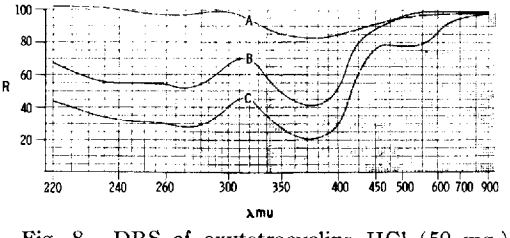

Fig. 8.—DRS of oxytetracycline HCl (50 mg.) and magnesium trisilicate (2.00 Gm.). Key: A, control (physical mixture); B, physical mixture left in moisture chamber for 24 hr. and vacuum dried; C, sample, equilibrated for 24 hr.

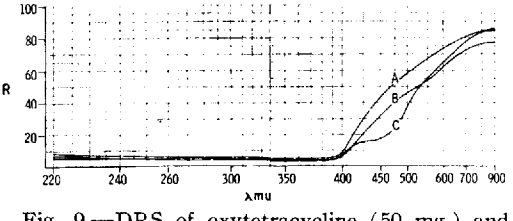

Fig. 9.—DRS of oxytetracycline (50 mg.) and sodium chloride (2.00 Gm.) study of moisture and drying effects. Key: A, control (physical mixture); B, physical mixture left in moisture chamber for 3 hr. and measured as the moist sample; C, spectrum of vacuum dried moist sample.

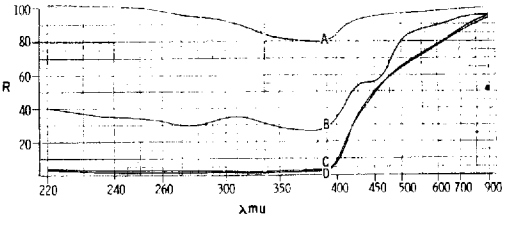

Fig. 10.—DRS of oxytetracycline (50 mg.) and magnesium sulfate (2.00 Gm.). Key: A, control; B, moist sample, remained in moisture chamber for 12 hr.; C, dry oxytetracycline, 100%; D, moist oxytetracycline, remained in moisture vessel for 12 hr., 100%.

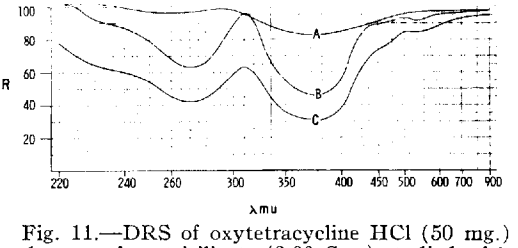

Fig. 11.—DRS of oxytetracycline HCl (50 mg.) and magnesium trisilicate (2.00 Gm.) studied with moisture effects and moisture effects followed by compression. Key: A, control (physical mixture); B, physical mixture left in moisture chamber for 30 hr. and measured as a moist sample; C, compression of the moist sample (B) at 30,000 p.s.i. for 5 min.

Particle-Size Variation.—Since particle size may produce variation in the reflectance spectrum (18), the authors' preliminary study dealing with this aspect indicates that some variation was obtained, although the changes are of a minor nature. For example, the spectrum of an equilibrated oxytetracycline–magnesium trisilicate complex was not altered to any significant degree when this equilibrated sample was subjected to reduction in particle size by homogenizing the powder for 0.5 hr. with the use of a Virtis blender. An examination of the filtered, vacuum dried sample's spectrum was comparable to that of the equilibrated control.

Although variation in particle size does have some effect on the regular reflection (1), the general characteristics of the control and homogenized sample spectra were essentially similar except for minor variations in the order of ±2%.

Moisture Effects.—Since solid pharmaceutical dosage forms involve the use of excipient materials such as fillers, binders, disintegrators, and lubricating agents, and since a certain percentage of moisture is necessary for the preparation of such dosage forms, as in the case of tablets, a preliminary investigation dealing with the effects of moisture was undertaken. Results presented thus far, dealing with drug–adjuvant interactions, were facilitated by compression or by equilibration techniques, either in aqueous or nonaqueous media. The present study involves the exposure of the dried, physically mixed drug–adjuvant components to humidity conditions for various time intervals.

Figure 8 illustrates this moisture effect with respect to spectral changes. Here curve A is the

diffuse reflectance spectrum of a dry physical mixture of oxytetracycline HCl and magnesium trisilicate and serves as the control. A portion of this dry physical mixture was then transferred onto an evaporating dish and placed in a wax-sealed desiccator in which water was substituted for the desiccant and allowed to stand for a period of 24 hr. This moist sample was then dried to eliminate moisture effects, and the DRS, represented by Fig. 8, curve B, was taken. Figure 8, C, represents the spectrum of physical mixture which has been equilibrated in water, filtered, and dried. An examination of curve B indicates that interaction does take place under moist conditions since this curve is comparable to the spectrum of the equilibrated sample (Fig. 8, C), except for an intensity change and the shoulder formation at 475 mμ. This greater intensity change, observed in the equilibrated sample, is probably due to a greater degree of interaction, since more surface area is exposed in equilibrating techniques. These spectral changes were accompanied by the usual color change from light yellow to a deep yellow color. It is of interest to note that the spectra of pure dry and moist oxytetracycline HCl or magnesium trisilicate, exposed to the same moist conditions, were similar in nature and did not vary by more than ±2%.

A similar effect is shown in Fig. 9, representing the interaction of oxytetracycline with sodium chloride. Spectrum A represents a physical mixture (control) of vacuum dried oxytetracycline and NaCl. A portion of this dried mixture was subjected to the moist humidity chamber previously described, and allowed to remain in this atmosphere for 3 hr. The DRS of this moist sample was then obtained and is represented by curve B. Figure 9, C, represents the spectrum of this moist sample which was vacuum dried. It is interesting to note that an interaction was observed with NaCl (Fig. 9, B), which cannot be totally attributed to a moisture effect, since drying of this moist sample does not cause a reversal in the spectrum back to the control, but instead, a higher degree of interaction results (Fig. 9, C). This may be explained on the basis that drying removes some of the surface water molecules from the adsorbent, producing additional reactive sites which facilitate the increased chemisorption or interaction.

Other adjuvant systems, investigated for oxytetracycline moisture effects, include anhydrous magnesium sulfate, magnesium chloride, and stearic acid. An examination of Fig. 10 again points out large spectral changes induced when a physical mixture of oxytetracycline and anhydrous magnesium sulfate is allowed to sit in a moisture chamber for 12 hr. Here curve A represents a physical mixture of individually dried oxytetracycline and magnesium sulfate, while curve B is the spectrum of a moist sample. These spectral changes along with an important color change from a whitish yellow to a deep yellow intensity are again indicative of a strong interaction. Similar spectral changes are also observed when subjecting an oxytetracycline–magnesium chloride mixture to a moist atmosphere. A difference between the sodium chloride and magnesium chloride reaction with oxytetracycline does exist, however, since the moisture exposed oxytetracycline–NaCl sample shifts, seen in Fig. 9, are of a magnitude of about 75 mμ while a bathochromic shift of about 150 mμ is observed with a corresponding oxytetracycline–magnesium chloride system. The greater spectral shift observed with the magnesium chloride system would be expected since magnesium chelates of oxytetracycline are known, and the interaction would be of a stronger variety than that produced by the sodium salt. It is interesting to point out again, however, that these changes are not observed between a physical mixture and moist sample of oxytetracycline and stearic acid. Spectral curves of the latter two systems have not been presented due to space considerations and chelation explanations for these differences have been discussed in previous sections.

Curves C and D (Fig. 10) represent spectra of dry and moist oxytetracycline, respectively. It is evident from these curves, and other adjuvant spectral data, that moisture effects of the individual components of these complexes are minor compared to the huge spectral differences when a physical drug–adjuvant mixture is allowed to remain in a moist environment.

The significance of such interactions can be easily applied to the area of tableting. The preparation of tablets usually requires that some moisture be present in the process of granulation, prior to tablet compression. Although such interactions, if they do occur, are already present in these granules, the effect of compression further accentuates this interaction. This effect is illustrated in Fig. 11, which represents the dry physically mixed control (curve A), the moist sample (curve B), and the compressed moist sample (curve C). An examination of the curves in this figure indicates that a greater degree of interaction may be obtained under compression pressures, as represented by the increased intensity and bathochromic change, even though the sample has already interacted due to moisture chamber equilibration. Furthermore, vacuum drying of these moisture equilibrated samples and subsequent compression does not destroy this interaction.

CONCLUSION

An examination of the large number of adjuvants which may react with oxytetracycline, together with the large spectral changes of specific systems, suggests the desirability of preliminary screening for the possible existence of such drug–adjuvant interactions in the formulation of dosage forms. Although no experimental data are available concerning the effect of these surface interactions with respect to dissolution and adsorption rates, it is felt that the possible existence of such interactions should be recognized. The literature does, however, contain information which strongly suggests that such interactions are responsible for the variation in blood level drug concentrations.

Since chemisorption primarily involves the surface area of the adjuvant, this type of interaction may not be of great therapeutic value when the drug to excipient ratio is sufficiently large, in that the chemisorbed unimolecular layer covering the surface of the adjuvant does not manifest itself until all the other physically adsorbed layers of the drug are removed. However, such drug–adjuvant interactions are extremely important when the ratio of the therapeutic drug to the amount of adjuvant or excipient is extremely low. In such ratios, the therapeutic agent primarily exists as the interacted

unimolecular layer and would be expected to significantly alter the dose response obtained. It is well to point out here that such interactions would not only manifest themselves in solid dosage forms, as in the case of tablets and capsules, but could also occur in suspensions and ointments, where drug–adjuvant ratios are usually low. It is of significant interest to point out here that one cannot overlook the distinct possibility of the excipient itself existing as a chemisorbed layer covering the surface of the drug resulting in similar alterations of the physical or biochemical behavior of the medicament in dosage forms where the drug–adjuvant ratios are high.

Data presented in this study further illustrate the possibility that donor–acceptor interaction of many varieties play an important role in these drug–adjuvant interactions. This has been illustrated by different magnitudes of spectral shifts observed among aluminum, calcium, magnesium, zinc, and sodium-containing adjuvants as well as nonmetal-containing excipients represented by stearyl alcohol, stearic acid, cetyl alcohol, acacia, tragacanth, tannic acid, and polyethylene glycol. The degree of interaction observed further depends on the nature of the drug and the type of adjuvant used. For example, calcium-containing adjuvants interact strongly with oxytetracycline but show very little interaction tendency for anthracene.

Studies are currently in progress in these laboratories dealing with the effects of such interactions on various aspects of drug dosage formulation and absorption.

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LSD Analogs

N-Methyl-*N*-*p*-(and *m*-)methoxyphenyl-β-alanine Derivatives

By KENNETH J. LISKA, JAMES L. JOHNSON, JAMES P. MASTRIAN, and MARIE L. STEENBERG

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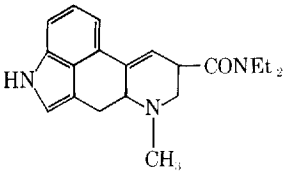
ATTEMPTS HAVE been made to elucidate an active psychotomimetic moiety in the lysergic acid diethylamide (LSD) molecule (1, 2). In the present work, the *N*-methyl-*N*-phenyl-β-alanine fragment of LSD was selected for study; an electron-rich methoxy group *para* or *meta* on the ring was intended to approximate the contribution made by the pyrrole nitrogen.

The ethyl esters (R = OEt in β-alanine moiety



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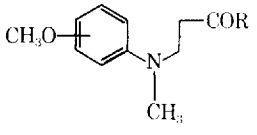

β-Alanine Moiety

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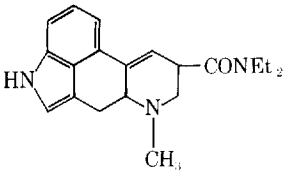
The ethyl esters (R = OEt in β-alanine moiety



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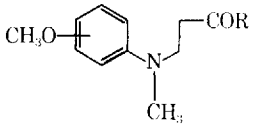

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TABLE I.—EFFECT OF COMPOUNDS I, II, AND VI ON RESPONSE OF ISOLATED RAT FUNDUS TO SEROTONIN

Compd.	Dose, mcg./ml.	No. of Expt.	Inhibition, % Mean ± S.E.
I	78.8	5	94.5 ± 2.3
	53.7	5	79.1 ± 4.5
	35.8	5	53.5 ± 4.7
	17.9	5	39.5 ± 4.4
II	28.4	4	95.4 ± 2.7
	14.4	4	83.6 ± 1.4
	5.7	4	38.9 ± 3.2
	2.8	4	5.0 ± 2.1
VI	7.0	3	95.7 ± 2.1
	2.8	3	78.5 ± 1.7
	1.4	3	20.0 ± 2.5
	0.7	3	0

structure), produced by addition of *N*-methyl-*p*-anisidine or *N*-methyl-*m*-anisidine to ethyl acrylate, were converted into the simple amides (R = NH_2) employing ammonia water at refrigerator temperatures. When the corresponding aminolysis employing the ethyl ester of *N*-methyl-*N*-*p*-methoxyphenyl-β-alanine and diethylamine was attempted at room temperature, at 75° for 72 hr., or at 160° for 22 hr., no amide formation could be detected by infrared spectral examination of the crude reaction products. Only unchanged ester appeared to be recovered. This diethylamine aminolysis was also attempted using catalytic amounts of water (at 110° for 64 hr.), sodium ethoxide (room temperature for 65 hr.), and calcium chloride (112° for 15 hr.). Again, only unchanged ester could be detected by infrared examination of the crude reaction products. Since the *N*,*N*-diethylamide in the *para* series could not be obtained by aminolysis, it and the *N*,*N*-diethylamide in the *meta* series were prepared by treating *N*,*N*-diethyl-3-bromopropionamide with either *N*-methyl-*p*-anisidine or *N*-methyl-*m*-anisidine. As a further proof of structure, the ethyl ester in the *para* series was hydrolyzed to the free acid, which was isolated as the amino acid hydrochloride salt.

Pharmacological testing for serotonin inhibitory activity was performed utilizing the isolated rat fundus. Five compounds were tested: the ethyl esters in both series, the simple amide in the *para* series, and the *N*,*N*-diethylamides in both series (compounds I, II, III, V, and VI. See under *Experimental*). Compounds I, II, and VI antagonized the action of serotonin on the isolated rat fundus. Compound VI appeared to be the most potent antagonist tested, showing inhibition at concentrations as low as 1.4 mcg./ml. and an ED_{50} of 2.2 mcg./ml. Compounds II and I were effective antagonists of serotonin, exhibiting approximately 50% inhibition at concentrations of 7.4 and 28.2 mcg./ml., respectively. Compound II also antagonized acetylcholine-induced contractions; 0.14 mg./ml. produced a 50% inhibition. Compounds I, II, and VI appear as relatively weak serotonin antagonists when their activities are compared to the activity of LSD on the mouse uterus (3). The most active compound (VI) appears to have a dual mechanism, antiserotonin and anticholinergic. These results are summarized in Table I. Compounds III and V were inactive.

EXPERIMENTAL[1]

N-Methyl-p-anisidine.—This was prepared from commercial *p*-anisidine according to the method of King and Tonkin (4), b.p. 139–141° (27 mm.). HCl salt, m.p. 121–123°. Major I.R. spectral peaks in $cm.^{-1}$: 3405 (m), 1506 (s), 1230 (s), 1030 (s), 815 (s).

N-Methyl-m-anisidine.—This was prepared from commercial *m*-anisidine following the directions of King and Tonkin (4) for the *para* compound, b.p. 119–120° (11 mm.), n_D^{24} 1.5670; picrate from alcohol, m.p. 147.5–149.5°. Major I.R. spectral peaks in $cm.^{-1}$: 3405 (m), 1600 (s), 1490 (s), 1204 (s), 1155 (s), 820 (m), 750 (m), and 680 (m).

N,N-Diethyl-3-bromopropionamide.—This was prepared according to the method of Gearien and Liska (5), b.p. 82–100° (0.5 to 1.0 mm.).

Ethyl 3-(N-Methyl-N-p-methoxyphenylamino)-propionate (I).—To a mixture of 52.0 Gm. (0.38 mole) of *N*-methyl-*p*-anisidine and 57.0 Gm. (0.57 mole) of ethyl acrylate was added 2 ml. of glacial acetic acid, and the mixture refluxed for 7 hr., then allowed to stand overnight. The product was taken up in ether, washed once with saturated sodium bicarbonate solution, once with water, dried over anhydrous sodium carbonate, the ether evaporated, and distilled. Discarding a forerun, the authors collected the fraction, b.p. 144–148° (0.1 mm.). The yield was 60.4 Gm. (67%) of pale yellow oil. Ester CO (str) 1720 $cm.^{-1}$.

Anal.—Calcd. for $C_{13}H_{19}NO_3$: C, 65.82; H, 8.02; N, 5.90. Found: C, 66.01; H, 8.10; N, 6.00.

The HCl salt could not be obtained in a solid form. Picrate, from alcohol, m.p. 126–129°.

Ethyl 3-(N-Methyl-N-m-methoxyphenylamino)-propionate (II).—A mixture of 12.2 Gm. (0.089 mole) of *N*-methyl-*m*-anisidine, 9.0 Gm. (0.089 mole) of ethyl acrylate, and 1 ml. of glacial acetic acid was heated under reflux for 5 hr. and then allowed to stand overnight. The workup was the same as for the *para* compound. Obtained was 12.0 Gm. (57%) of a pale yellow oil, b.p. 145–150° (0.1 mm.). Ester CO (str) 1720 $cm.^{-1}$.

Anal.—Calcd. for $C_{13}H_{19}NO_3$: C, 65.82; H, 8.02; N, 5.90. Found: C, 66.05; H, 8.16; N, 6.10.

The HCl salt could not be obtained in a solid form. Picrate, from alcohol, m.p. 104–106°.

3-(N-Methyl-N-p-methoxyphenylamino)-propionamide (III).—Ammonia water (25 ml. of 29% ammonia) was enclosed with 8.8 Gm. (0.037

[1] Melting points and boiling points are uncorrected. Infrared data from a Beckman IR-8. Analyses by Galbraith Laboratories and Weiler & Strauss.

mole) of ethyl 3-(*N*-methyl-*N*-*p*-methoxyphenylamino)propionate and stored with occasional shaking in a refrigerator for 10 days. The supernatant liquid was then decanted from the semisolid mass, a fresh 25-ml. portion of ammonia added, and the mixture allowed to stand an additional 3 days. The supernatants upon chilling to 0° yielded crude amide which was combined with additional amide obtained by cold ether extraction of the semisolid mass. Recrystallization from ligroin yielded fluffy white needles, m.p. 110–112°. Obtained 1.0 Gm. (13%). Amide CO (str) 1670 cm.$^{-1}$.

Anal.—Calcd. for $C_{11}H_{16}N_2O_2$: C, 63.46; H, 7.69; N, 13.46. Found: C, 63.24; H, 7.76; N, 13.23.

3 - (N - Methyl - N - m - methoxyphenylamino)-propionamide (IV).—This simple amide was prepared in 14% yield following approximately the same procedure used for the *para* isomer. Recrystallized from water, m.p. 94.5–96°. Amide CO (str) 1670 cm.$^{-1}$.

Anal.—Calcd. for $C_{11}H_{16}N_2O_2$: C, 63.46; H, 7.69; N, 13.46. Found: C, 63.66; H, 7.80; N, 13.27.

3 - (N - Methyl - N - p - methoxyphenylamino)-propionic Acid Hydrochloride.—The hydrolysis of ethyl 3-(*N*-methyl-*N*-*p*-methoxyphenylamino)propionate (10.0 Gm., 0.042 mole) was accomplished by refluxing with 50 ml. of 10% HCl for 4 hr. The volume of water was reduced to about one-fifth. The solution was allowed to stand overnight, whereupon 3.2 Gm. of pure, highly crystalline amino acid hydrochloride, m.p. 159–160°, was obtained. Evaporation of the remainder of the water and trituration of the solids with anhydrous ether yielded an additional 2.7 Gm. of pure product for a total yield of 57%. The salt recrystallizes well from absolute alcohol.

Anal.—Calcd. for $C_{11}H_{16}ClNO_3$: Cl, 14.43. Found: Cl, 14.19.

3 - (N - Methyl - N - p - methoxyphenylamino)-N′,N′-diethylpropionamide (V).—A solution of 10.2 Gm. (0.049 mole) of *N*,*N*-diethyl-3-bromopropionamide, 6.9 Gm. (0.049 mole) of *N*-methyl-*p*-anisidine, 5.3 Gm. (0.05 mole) of anhydrous sodium carbonate, and 15 ml. of benzene was heated at gentle reflux for 7.5 hr., and then allowed to stand overnight. The mixture was filtered, the solids washed with ether, the combined filtrates freed of solvents, and distilled. After a large forerun, the fraction, b.p. 183–186° (0.09 mm.), was collected as a yellow oil, amounting to 3.9 Gm. (30%). Tertiary amide CO (str) 1625 cm.$^{-1}$. n_D^{23} 1.5396.

Anal.—Calcd. for $C_{15}H_{24}N_2O_2$: C, 68.18; H, 9.09; N, 10.61. Found: C, 68.35; H, 9.20; N, 10.80.

The HCl salt was prepared crystalline but was very hygroscopic. The picrate could not be obobtained in a solid form. Methiodide, recrystallized from absolute ethanol, m.p. 121–124°, slightly hygroscopic.

3 - (N - Methyl - N - m - methoxyphenylamino)-N′,N′-diethylpropionamide (VI).—A mixture of 6.0 Gm. (0.044 mole) of *N*-methyl-*m*-anisidine, 9.1 Gm. (0.044 mole) of *N*,*N*-diethyl-3-bromopropionamide, 4.6 Gm. (0.044 mole) of anhydrous sodium carbonate, and 15 ml. of benzene was heated at gentle reflux for 5 hr. The mixture was cooled, filtered, the freed solids washed with benzene, and the combined benzene filtrates dried over sodium sulfate. After removal of the solvent, the residue was distilled and the fraction collected, b.p. 173–181° (0.09 mm.). Obtained 4.3 Gm. (37%) of a yellow oil, n_D^{23} 1.5448. Tertiary amide CO (str) 1629 cm.$^{-1}$.

Anal.—Calcd. for $C_{15}H_{24}N_2O_2$: C, 68.18; H, 9.09; N, 10.61. Found: C, 68.03; H, 8.98; N, 10.71.

The HCl salt could not be prepared in a solid state. The picrate was crystallized, after 5 days standing, from ethanol, m.p. 125–127°. Methiodide from absolute ethanol, m.p. 108–111°, hygroscopic.

PHARMACOLOGY

Wistar rats of both sexes (100–150 Gm.) were fasted for 24 hr., killed by a blow on the head, and the stomach removed. The fundus was removed and cut into a strip by opening the tissue along the lesser curvature and cutting to preserve the longitudinal muscle as described by Vane (6). The fundus strip was suspended in a 10-ml. muscle bath containing modified Ringer solution (7) (Gm./L.: NaCl, 9.0; KCl, 0.42; $CaCl_2 \cdot 2H_2O$, 0.06; $NaHCO_3$, 0.5; and glucose, 0.5) maintained at 37° and oxygenated with pure oxygen.

The strip was permitted to stretch in the organ bath for 1 hr., after which the minimum amount of serotonin required to give a response of 4–5 cm. was determined. This varied from 0.1–1.0 ng./ml. of bathing solution. This amount of serotonin was added to the bath at 6-min. intervals and the tissue washed twice between doses. Three equivalent responses to serotonin were followed by the addition of a known quantity of the drug, diluted in normal saline, 1 min. before the next addition of serotonin. If inhibition occurred, the tissue was washed until recovery was noted, and two equivalent responses to serotonin were recorded before the tissue was used for another test. Four different doses of each drug were selected so that the highest dose gave a complete blockade of serotonin and the lowest a 5–10% blockade. The volume of each dose was less than 0.4 ml.

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Effect of Ultrasound on Particle Size of Suspensions of Polyethylene Spheres

By M. A. SHEIKH, J. C. PRICE, and R. J. GERRAUGHTY

The effect of ultrasound energy on the particle size of polyethylene spheres in aqueous suspension, with and without additives such as surfactants and deflocculating agents, and using variable times of exposure to ultrasound, were studied. Particle size was determined microscopically and expressed as the mean diameter of the particles. It was found that ultrasonic waves, at a frequency of 100 kc., caused a significant reduction in particle size only if surfactants were present. It was also observed that surfactants having high HLB values were the most efficient of all additives. Increasing time of exposure to the ultrasound waves brought about a continued reduction of particle diameter up to 2 hr., but that after this time the particle size stabilized, and further exposure produced no significant decrease in the mean diameter of the polyethylene spheres.

IN RECENT YEARS, ultrasonic energy has been shown to cause particle size reduction in emulsions (1–4) and suspensions (5–9).

There are conflicting reports on the influence of the total time of exposure to the ultrasonic waves, however. Some authors stated that an optimum period was needed and that continued exposure could even cause an increase in particle size in emulsions (2, 3). Others found that after an optimum exposure time there is little or no change in particle size of emulsions (4). A similar observation has been made with suspensions (6). Another paper reports that the particle size reduction continued as the time of exposure was lengthened (8). It is very possible that the conflicting results of these studies might have been due to differences in the intensity and frequency of ultrasound that was used or to variance in the crystalline structure of the compounds used in the suspensions.

It was the aim of this investigation to correlate, if possible, the time of exposure to ultrasound to the degree of size reduction of suspension particles in a highly reproducible suspension under closely controlled experimental conditions; a second objective was to evaluate the effect of various concentrations of surfactants and deflocculants on the exposed suspensions. The third objective was to determine if the concentration of suspension particles is important.

A generator producing sound waves at a frequency of 100 kc. was selected. It was felt that higher frequencies could cause some side reactions to occur which might introduce other variables, and very high plate power is required to produce cavitation at higher frequencies.

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EXPERIMENTAL

Equipment and Materials.—The apparatus used to supply ultrasonic power for all experiments consisted of a 100-kc. generator,[1] and a bath equipped with a barium titanate transducer. A round copper coil, attached to a circulating constant-temperature bath, was inserted in the transducer bath in order to keep the temperature constant at 25 ± 1° during the investigation.

The instrument selected for the determination of the particle size of the suspensions was a Spencer microscope with an AO Spencer micrometer.[2]

A microfine polyethylene resin,[3] was selected to prepare the experimental suspensions. This substance offers several characteristics to commend it for this use: (*a*) the polyethylene is hydrophobic and cavitation occurs more easily at hydrophobic surfaces; (*b*) the particles are spherical and, therefore, easy to count; (*c*) there are two size ranges available, 3–20 μ diameter (Microthene 500) and 8–30 μ (Microthene 510); and (*d*) the material is inexpensive.

Procedure.—An 0.5% suspension of Microthene 500 or 510 was used as the basic suspension. Additives were used in varying concentrations at different times to study their effect. These included the surfactants polysorbate 20[4] and sodium lauryl sulfate, and the deflocculating agents Darvan,[5] Daxad,[5] and Marasperse.[6] The suspension to be exposed was placed in a 125-ml. flask, and this was immersed in the transducer bath so that the level of the suspension was always below the level of water in the transducer bath. Temperature was kept constant at 25°, and the generator plate voltage was kept at 1000 v. After varying intervals of time, representative samples of the suspension were withdrawn from the flask, particle sizes were determined microscopically by measuring the diameters of 200 particles for each sample, and the mean diameter was calculated for each sample. Later the concentration of Microthene 500 and Microthene 510 was varied from 0.5 to 4%. Poly-

[1] McKenna model 100 generator, McKenna Laboratories, Santa Monica, Calif.

[2] American Optical Co., Buffalo, N. Y.

[3] Marketed as Microthene by the U. S. Industrial Chemical Co., New York, N. Y.

[4] Marketed as Tween 20 by the Atlas Chemical Co., Wilmington, Del.

[5] Dewey and Almy Chemical Co., Cambridge, Mass.

[6] Marathon Chemical Corp., Division of American Can Co., Neenah, Wis.

sorbate 20 was added to all of these suspensions in a concentration of 0.1%.

RESULTS

The mean particle sizes of Microthene 500 and 510 suspensions containing varying concentrations of polysorbate 20 and sodium lauryl sulfate are shown in Tables I–IV. It is apparent that the addition of these agents facilitated a substantial reduction in particle size after 2 hr. exposure time, but that, usually, a plateau was reached at that time, and longer exposure caused little or no further reduction in the mean particle diameter.

It is also apparent that a 0.05 to 0.1% concentration of surfactant is satisfactory, and that higher concentrations do not increase the effectiveness of the ultrasound.

A study of the effect of the HLB (10) of surfactants was made by using mixtures of sorbitans[7] and polysorbates. The results are shown in Table V. It appears that there is little difference in effect on particle size reduction if the HLB of the surfactant mixture is greater than 10, but that as the HLB is reduced below that point, the effect of ultrasound is reduced.

There is no significant difference in the magnitude of the decrease between Microthene 500 and Microthene 510 if the decrease in particle size is computed on the basis of percentage of change of mean particle diameter.

Particle size reduction of Microthene suspensions in the presence of deflocculants such as Marasperse, Darvan, and Daxad was negligible, and practically the same as when no additive at all was used. These agents are charged particles which are adsorbed on the surface of the suspended particles preventing flocculation by electrical repulsion without appreciably affecting surface tension. Table VI, which shows the effect of ultrasound in a Microthene suspension in the presence of Darvan, is typical.

The effect of increasing the concentration of the suspended phase is shown in Tables VII and VIII. It is apparent that the effect of ultrasound in reducing particle size becomes less marked as the concentration of the suspended phase increases.

Table I.—Effect of Ultrasound Energy on Particle Size of Microthene 500 with Varying Concentrations of Polysorbate 20[a]

Polysorbate 20, %	Time of Exposure, min. 0	60	120	180	240
None	5.5	5.3	5.2	5.2	5.1
0.05	5.4	4.7	4.6	4.6	4.6
0.1	5.4	4.8	4.3	4.3	4.3
0.2	5.2	5.1	4.7	4.4	4.4

[a] All mean diameters are given in microns.

Table II.—Effect of Ultrasound Energy on Particle Size of Microthene 500 with Varying Concentrations of Sodium Lauryl Sulfate[a]

Sodium Lauryl Sulfate, %	Time of Exposure, min. 0	60	120	180	240
None	5.4	5.3	5.1	5.2	5.2
0.05	5.4	4.9	4.6	4.5	4.5
0.1	5.5	5.1	4.4	4.4	4.4
0.2	5.4	5.1	4.5	4.2	4.3
0.3	5.4	5.2	4.3	4.3	4.3

[a] All mean diameters are given in microns.

Table III.—Effect of Ultrasound Energy on Particle Size of Microthene 510 with Varying Concentrations of Polysorbate 20[a]

Polysorbate 20, %	Time of Exposure, min. 0	60	120	180	240
None	9.8	9.6	9.3	9.3	9.2
0.05	9.75	9.2	8.5	8.5	8.4
0.1	9.8	9.2	8.3	8.2	8.2
0.2	9.7	9.4	9.3	8.6	8.6

[a] All mean diameters are given in microns.

Table IV.—Effect of Ultrasound Energy on Particle Size of Microthene 510 with Varying Concentrations of Sodium Lauryl Sulfate[a]

Sodium Lauryl Sulfate, %	Time of Exposure, min. 0	60	120	180	240
None	9.8	9.5	9.3	9.2	9.3
0.05	9.7	9.0	8.4	7.9	7.8
0.1	9.8	9.2	8.2	8.0	8.0
0.2	9.7	9.1	8.3	7.9	7.9

[a] All mean diameters are given in microns.

DISCUSSION

In all of the experiments in which there was a substantial reduction in particle size of the suspended material, a plateau was noticed after an exposure time of about 2 to 2.5 hr., after which time no further significant reduction in size occurred. These results parallel the results reported by Singiser and Beal (4) for a liquid–liquid system although this study is concerned with a solid–liquid system. No reasonable explanation for this effect can be obtained from the data available, but it was seen consistently.

Substantial reduction in mean particle diameter occurred only in the presence of surfactants. This result can be explained partially from the character of the suspensions obtained. When surfactants having high HLB values were present, the particles were readily dispersed, and, therefore, the individual particles were completely surrounded by liquid; in the absence of surfactants the particles tended to remain clumped together and, therefore, not surrounded by liquid. Since the ultrasound waves and cavitation shock waves are transmitted to the particles through the liquid medium, a much reduced effect would be expected in the poorer suspensions.

It was observed that the deflocculating agents used did not lessen flocculation of the Microthene particles to a significant degree. The very small

[7] Marketed as Spans by the Atlas Chemical Co., Wilmington, Del.

TABLE V.—EFFECT OF HLB OF SURFACTANTS ON THE PARTICLE SIZE OF MICROTHENE 510 (0.5% SUSPENSION)[a]

Additives, %			Time of Exposure, min.				
Polysorbate 20	Sorbitan Monolaurate	HLB	0	30	60	90	120
75	25	14.6	10.0	9.6	8.8	8.3	8.2
50	50	12.6	9.9	9.4	8.9	8.2	8.3
50	50	10.8	9.8	9.6	8.9	8.5	8.5
20	80	10.0	10.0	9.4	8.9	8.3	8.4
10	90	9.5	10.0	9.7	9.2	8.8	8.8
5	95	9.1	10.0	9.8	9.6	9.6	9.5
0	100	8.6	9.9	9.7	9.6	9.6	9.6

[a] 0.05% total surfactant was used in all cases. All particle sizes are in microns.

TABLE VI.—EFFECT OF ULTRASOUND ENERGY ON PARTICLE SIZE OF MICROTHENE 500 WITH VARYING CONCENTRATIONS OF DARVAN[a]

Darvan, %	Time of Exposure, min.				
	0	60	120	180	240
None	5.5	5.5	5.3	5.3	5.3
0.05	5.5	5.5	5.2	5.2	5.2
0.1	5.6	5.5	5.2	5.3	5.1
0.2	5.5	5.3	5.0	5.1	5.1
0.3	5.6	5.2	4.9	5.0	5.0

[a] All mean diameters are given in microns.

TABLE VII.—EFFECT OF INCREASING CONCENTRATION OF MICROTHENE 500 IN SUSPENSIONS

Microthene 500, % in Suspensions[a]	Particle Size, μ	
	Before Exposure	After 2 hr. Exposure
0.5	5.6	4.5
1.0	5.6	4.4
1.5	5.7	4.6
2.0	5.6	4.8
2.5	5.5	4.8
3.0	5.7	5.0
3.5	5.5	5.1
4.0	5.7	5.3

[a] All suspensions contained 0.1% polysorbate 20.

TABLE VIII.—EFFECT OF INCREASING CONCENTRATION OF MICROTHENE 510 IN SUSPENSIONS

Microthene 510, % in Suspensions[a]	Particle Size, μ	
	Before Exposure	After 2 hr. Exposure
0.5	10.1	8.1
1.0	9.9	8.2
1.5	9.8	7.9
2.0	9.9	8.5
2.5	9.7	8.3
3.0	9.9	8.6
3.5	10.0	9.1
4.0	9.8	8.8

[a] All suspensions contained 0.1% polysorbate 20.

size reduction in these suspensions could be explained on the same basis as above, *i.e.*, the ultrasound waves and cavitation shock was not transmitted to the individual particles.

The effect of increasing the concentration of the suspended phase was quite predictable. In addition to the possibility of a greater degree of flocculation as more particles were present, it was noted that higher concentrations appeared to have a damping effect on cavitation by the ultrasound waves as evidenced by decreased agitation in the flask.

SUMMARY AND CONCLUSIONS

1. The purpose of this investigation was to study the effect of ultrasonic energy on the particle size of certain highly reproducible suspensions. It was observed that ultrasound waves had some reducing effect in all cases.

2. The effect of the time of exposure to ultrasonic energy appeared to be that the size changed significantly up to a period of 2 hr. and that after that a plateau was reached.

3. The use of deflocculating agents did not cause any significant change in the reduction of the particle size.

4. It was also noted, from this study, that a minimum concentration of a surfactant was necessary to obtain maximum effect on particle size reduction but that addition of surfactant above this concentration produced no noticeable change.

5. The HLB of the surfactant also has an effect on the reduction in the particle size. Surfactants with high HLB were found to be most effective, and if the HLB was less than 10, practically no effect was observed.

6. As the concentration of the suspended phase was increased, the change in particle size of suspension became less marked.

7. In view of the results of this study, it can be concluded that ultrasonic energy might be a useful tool for the reduction of particle size of certain pharmaceutical suspensions to make them more stable.

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Interaction of Various Phenethylamines with the Adrenergic-Adipose Tissue Receptor System, *In Vitro*

By K. F. FINGER and D. R. FELLER

The interaction of several phenethylamines with the rat adipose tissue adrenergic receptor system has been studied utilizing the release of free fatty acids as an index of the interaction. It has been established that structural modifications of the basic phenethylamine molecule produced marked changes in the affinity of the agonists for this system and, to a lesser degree, in the intrinsic activity constants. *N*-Substitution of large alkyl functions increased affinity most markedly. Hydroxylation of the β carbon of the ethylamino side chain also enhanced affinity, while changes in the ring substitution produced changes varying from complete loss of all activity to alterations in the affinity constants. Phenethylamine, tyramine, ephedrine, amphetamine, and metanephrine were found to be inactive in this system. The catechol nucleus appears to confer optimal activity upon the agonist molecule, but structural modifications, particularly upon the side chain nitrogen, markedly affect the ability of the molecule to release free fatty acids from adipose tissue, *in vitro*.

RECENT STUDIES have indicated the increasing importance of the mobilization of free fatty acids (FFA) in (*a*) normal body metabolism as a primary energy source (1, 2) and (*b*) as a factor or contributing factor in certain disease states (3). For these reasons, considerable research emphasis has most recently been placed on studies designed to elucidate the controlling mechanisms involved in the mobilization of FFA from triglycerides in adipose tissue.

The fact that the sympathetic nervous system and in particular the catecholamines play an important role in the mobilization of FFA has been recognized for a number of years (4, 5). Recent work has shown that the catecholamine-induced mobilization of FFA occurs along pathways similar to those elucidated by Sutherland and Rall (6) for the catecholamine-induced glycogenolytic processes of liver, *i.e.*, it appears that the catecholamines stimulate the conversion of ATP to 3′,5′-cyclic AMP, which, in turn, catalyzes the conversion of an inactive lipase to an active lipase (7, 8). The lipolytic enzymes thus activated catalyze the stepwise hydrolysis of triglycerides to yield free fatty acids.

Many studies have been reported concerning the effects of catecholamines on the release of free fatty acids both *in vivo* and *in vitro*. However, few, if any, have defined the structure-activity relationships involved in the interaction of catecholamines with the adipose tissue-adrenergic receptor or have provided a sufficient degree of quantitation to allow such relationships to be made. It is the purpose of this report to present findings with respect to the structure-activity relationships involved in the interaction of substituted phenethylamines with the rat epididymal fat tissue, *in vitro*. This knowledge is deemed important for an understanding of the nature of drug–receptor interaction in this tissue and for the development of highly selective agonists and antagonists of FFA release in the future.

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EXPERIMENTAL

Materials.—The chemicals[1] employed in this study and their source of supply are as follows: tyramine HCl, *d,l*-metanephrine HCl, dopamine HCl, *l*-phenylephrine HCl, *l*-isopropyl arterenol HCl, phenylpropanolamine HCl, and β-phenethylamine (Mann Research Laboratories); metaraminol bitartrate and ephedrine sulfate (Merck, Sharp & Dohme Laboratories); isoxuprine HCl (Mead Johnson Laboratories); nylidrin HCl (U. S. Vitamin Corp.); *l*-epinephrine bitartrate (Winthrop Laboratories); protochylol (Lakeside Laboratories); dextroamphetamine sulfate (K & K Laboratories); *l*-norepinephrine bitartrate and bovine albumin, fraction V (Nutritional Biochemicals Corp.). All concentrations expressed in this paper refer to the free base.

Methods.—Nonfasted, male Sprague–Dawley rats, weighing between 200 and 250 Gm., were maintained in their animal quarters at least 1 week prior to being employed in an experiment. The animals were sacrificed by stunning and decapitation. The anterior one-third of the epididymal fat pads were rapidly removed, placed in freshly prepared Krebs-Ringer bicarbonate buffer (pH 7.4), and minced with a small scissors to yield pieces weighing 5–10 mg. Tissue slices from six rats were pooled for each experiment.

Incubations of fat pads and the determination of the rate of FFA release as function of agonist concentration were conducted by procedures previously described (9).

[1] The authors thank their colleagues in the pharmaceutical industry for generously supplying many of the chemicals used in this study.

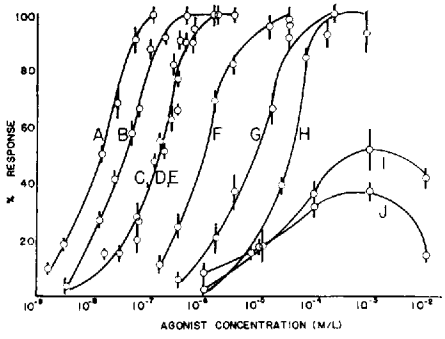


Fig. 1.—Dose-response curves for various phenethylamine agonists on mobilization of FFA from rat epididymal fat tissue, *in vitro*. Key: A, protochylol; B, isopropyl arterenol; C, epinephrine; D, norepinephrine; E, nylidrin; F, isoxuprine; G, metaraminol; H, dopamine; I, phenylephrine; J, phenylpropanolamine. From four to six determinations were made at each agonist concentration for all compounds presented in this paper. The values plotted represent the mean ± the standard error as indicated by the vertical lines.

The rates of FFA release, expressed in terms of μmoles of FFA released/Gm. of adipose tissue/hr., were calculated from the data obtained by serially sampling the incubation vessel at 0, 20, 40, and 60 min. after addition of the agonist to the media. In these studies, a maximal rate of FFA release of 18 μmoles/Gm./hr. was obtained and was found to be independent of the agonist employed. This maximal figure was employed to calculate the per cent response of the system in all studies described in this report.

RESULTS AND DISCUSSION

Dose - Response Relationships.—The dose-response relationships obtained for the active compounds employed in this study are presented in Fig. 1. It is apparent from these data, that with the exception of phenylephrine and phenylpropanolamine, all compounds were capable of producing a maximal release of FFA from adipose tissue slices, *in vitro*. The compounds varied, however, in their relative ability to mobilize FFA. Thus, in considering compounds A through H shown in Fig. 1, the rank order of compounds listed in order of decreasing potency is protochylol > isopropyl arterenol > epinephrine = norepinephrine = nylidrin > isoxuprine > metaraminol > dopamine. Phenylpropanolamine (compound J) and phenylephrine (compound I) possess properties which sharply differentiate them from the others. Thus, these latter two molecules show the property of auto-inhibition which manifests itself in a decrease in FFA release as the concentration of agonist is increased beyond a certain value.

Analysis of these data suggest that the catechol nucleus conveys optimal activity upon the molecule in agreement with the postulates of Belleau (10) regarding the interaction of catecholamines and ATP at the adrenergic receptor. Structural modifications of the catecholamine structure, however, produced pronounced alterations in the relative ability of these moieties to mobilize FFA from the adipose tissue slices. Norepinephrine and dopamine differ only in the presence or absence of the hydroxyl function on the β carbon of the side chain, yet these two compounds differ by a factor of about 160 in their activity in mobilizing FFA. Thus, side chain hydroxylation appears to increase FFA mobilizing activity.

Similarly, *N*-substitution of large alkyl functions enhanced activity as can be seen by comparing the dose-response curves for protochylol and isopropyl arterenol with those of epinephrine and norepinephrine. Indeed, the larger *N*-substitution found in protochylol enhanced its potency to a value approximately twice that of isopropyl arterenol, the compound previously found to be the most potent mobilizer of FFA in this system (9). Methylation

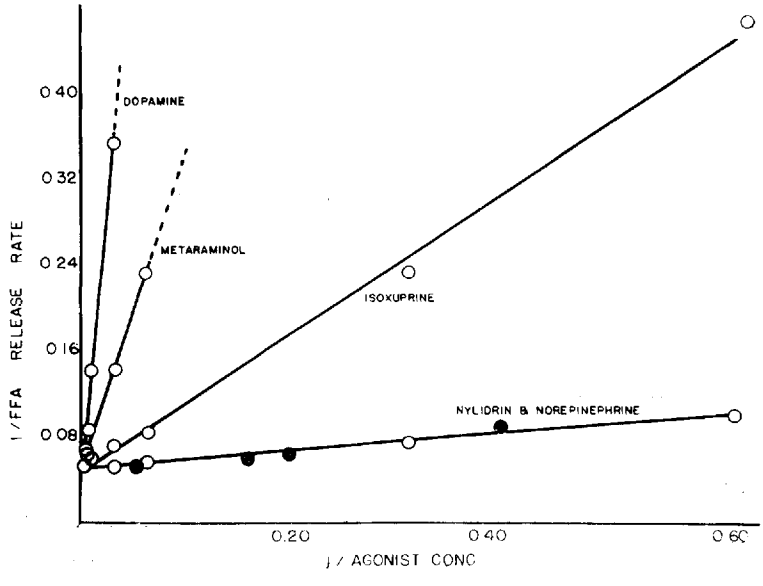


Fig. 2.—Double reciprocal plot illustrating effects of agonists on the rate of FFA release, *in vitro*. Concentrations of agonists were in terms of moles/L. × 10^{-7}.

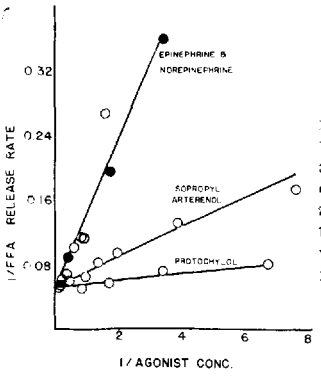


Fig. 3.—Double reciprocal plot illustrating effects of agonists on the rate of FFA release, *in vitro*. Concentrations of agonists were in terms of moles/L. $\times 10^{-7}$.

of the amino nitrogen did not, however, produce a statistically significant alteration in the dose-response relationship (compare epinephrine with norepinephrine).

The influence of *N*-substitution is also evident when one compares the dose-response relationships found for the noncatecholamines tested. Nylidrin, a compound lacking a catechol nucleus but possessing a rather large substituent on the side chain nitrogen, was found to be equipotent with epinephrine and norepinephrine while isoxuprine was found to be approximately one-fifth as potent as epinephrine or norepinephrine. The effect of this type of molecular alteration is also evident when comparing the dose-response relationships of nylidrin and isoxuprine with the other noncatecholamines shown in Fig. 1, namely metaraminol, phenylpropanolamine, and phenylephrine.

In contrast to the above-mentioned alterations in dose-response curves obtained by varying chemical structure, substitution on the α carbon of the side chain did not appear to alter biological activity in any significant manner.

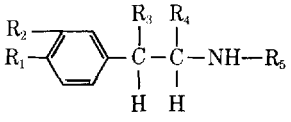
Double Reciprocal Relationships.—The equation described by Ariens (11) relating the relative response of a tissue to an agonist and the concentration of agonist employed has been modified slightly to yield

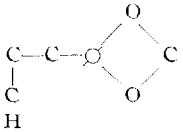
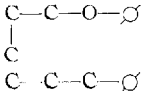
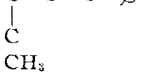
$$R = \frac{(I)(A)}{Ka + A}$$

where R is the response of the tissue measured as the rate of FFA release/Gm. of tissue/hr., I is the intrinsic activity constant or maximal response obtainable at an infinity concentration of the agonist, A is the concentration of agonist employed, and Ka is the apparent dissociation constant of the agonist–receptor tissue complex. Placing the above expression in the double reciprocal form yields

$$1/R = (Ka/I)(1/A) + 1/I$$

Table I.—Intrinsic Activity and Affinity Constants for Substituted Phenethylamines on the Mobilization of FFA from Rat Adipose Tissue



Compd.	Name	R_1	R_2	R_3	R_4	R_5	I^a	pD_2
1	Epinephrine	OH	OH	OH	H	CH_3	1.0	6.8
2	Norepinephrine	OH	OH	OH	H	H	1.0	6.8
3	Isopropyl arterenol	OH	OH	OH	H	$CH(CH_3)_2$	1.0	7.6
4	Protochylol	OH	OH	OH	H		1.0	7.9
5	Dopamine	OH	OH	H	H	H	1.0	4.4
6	Isoxuprine	OH	H	OH	CH_3		1.0	6.1
7	Nylidrin	OH	H	OH	CH_3		1.0	6.8
8	Phenylephrine[b]	H	OH	OH	H	CH_3	0.5	4.3
9	Metaraminol	H	OH	OH	CH_3	H	1.0	5.2
10	Phenylpropanolamine[b]	H	H	OH	CH_3	H	0.37	4.7
11	Phenethylamine	H	H	H	H	H	0.0	...
12	Tyramine	OH	H	H	H	H	0.0	...
13	Ephedrine	H	H	OH	CH_3	CH_3	0.0	...
14	Amphetamine	H	H	H	CH_3	H	0.0	...
15	Metanephrine	OH	OCH_3	OH	H	CH_3	0.0	...

[a] Intrinsic activity constant. [b] Compound shows properties of a dualist or partial agonist.

Plotting the data as $1/R$ *versus* $1/A$ yields a straight line with a slope numerically equal to Ka/I and an intercept of $1/I$. Analysis of the data in this manner provides an efficient means of calculating the intrinsic activity constant (I) and the affinity constant (the reciprocal of the apparent dissociation constant for the drug–receptor tissue complex, Ka) from the intercept and the slope of the line, respectively. The data obtained in this study and plotted in this manner are shown in Figs. 2 and 3. The data for norepinephrine have been included in both graphs for comparative purposes.

In Fig. 2, it is apparent from the steep slopes of the dopamine and metaraminol lines that these compounds are relatively weak agonists in this system as compared to isoxuprine, nylidrin, and norepinephrine. It is also apparent that all curves intercept the y-axis at a common point, indicating equal intrinsic activities.

In Fig. 3, a different scale for the x-axis was employed to permit the plotting of the data for the more active agonists in the double reciprocal manner. It is apparent in this graph that (*a*) the lines extrapolate to an intercept identical with that obtained in Fig. 2 indicating the same intrinsic activities for all compounds, and (*b*) in this system, the highest affinities were shown by protochylol and isopropyl arterenol, both compounds being clearly more potent than epinephrine and norepinephrine.

Structure–Activity Relationships.—The structure-activity relationships obtained in this study have been summarized in Table I. In this table, the intrinsic activity constants are expressed as ratios of the maximal response obtained with an agonist to the maximal response obtainable in the system (11), and the affinity constant is expressed as the pD_2 of Miller, Becker, and Tainter (12), defined as the negative logarithm of the agonist concentration required to produce a response equal to 50% of the maximal response obtainable in the system. These constants can be derived from either the dose-response relationships shown in Fig. 1 or from the intercept and slope value of the lines shown in Figs. 2 and 3. Inactive compounds have also been included in the table of data.

The data summarized in Table I indicate the effects structural modification of the basic phenethylamine structure had on the ability of the compounds to stimulate the mobilization of FFA from adipose tissue, *in vitro*. The parent compound, phenethylamine, was found to be completely inactive in this system, thus possessing zero intrinsic activity. Para-hydroxylation (tyramine), or α carbon methylation (amphetamine), did not increase activity to a measurable level, while β-carbon hydroxylation along with α-carbon methylation had variable results. (Compare the inactive ephedrine with the slightly active phenylpropanolamine.) Placing a hydroxyl function in the 3 position of the ring significantly increased FFA mobilizing activity (metaraminol). Ring hydroxylation in the *para* position coupled with N-substitution markedly enhanced FFA mobilizing activity (isoxuprine and nylidrin). The catechol nucleus appeared to confer optimal activity upon the molecule, however, as seen by the relatively high affinity constants possessed by these type compounds (epinephrine and norepinephrine). The affinity of the catecholamine can be enhanced, however, by N-substitution of large or bulky alkyl functions (isopropyl arterenol and protochylol) and diminished by removal of the β-hydroxyl group of the side chain (dopamine). Methylation of 3-OH group of epinephrine completely abolished FFA mobilizing activity (metanephrine).

CONCLUSION

It is concluded from these studies that (*a*) while the catechol nucleus appeared to confer optimal activity upon the agonist molecule, significant mobilization of FFA from rat epididymal fat tissue was achieved by monohydroxylated ring structures, (*b*) the hydroxyl function on the β carbon atom of the side chain played an important role in determining the affinity of the compound for the adipose tissue receptor system, (*c*) N-substitution of large alkyl functions greatly enhanced fat mobilizing activity, and (*d*) compounds whose action is mediated by release of endogenous catecholamines were not active in this system. Furthermore, from the information obtained in this study, it appears that both α- and β-type adrenergic stimulants are capable of stimulating the mobilization of FFA but that the β-type adrenergic stimulants are much more active in this regard than are the α stimulants.

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Solubility Profiles for the Xanthines in Aqueous Alcoholic Mixtures I

Ethanol and Methanol

By A. N. PARUTA* and S. A. IRANI

The solubilities of caffeine, theophylline, and theobromine were determined in two binary mixtures as a function of the dielectric constant. The binary mixtures chosen were water and two *n*-alkyl alcohols, ethanol and methanol. The alcohol was chosen on the basis of the dielectric constant range produced so that various "cuts" along the solubility curve could be obtained. The dielectric requirements (DR's) found in these systems could then be matched with previous findings. The DR's found in this study correlate to a fair degree with past work; however, a consistent new peak at a dielectric constant of about 40 was also found. These systems were experimentally designed so that solubility could be expressed in various concentration notations as well as mole fraction.

THE SOLUBILITY profile for the xanthine drugs in dioxane–water mixtures in terms of dielectric requirements has been given previously (1). It also had been shown the dielectric requirements (DR's) for salicylic acid (2) were relatively constant for a diverse spectrum of binary mixtures and that the magnitude of solubility at a given DR varied widely.

In a continuing effort to investigate the relative constancy of DR's in various binary mixtures, the present study on the xanthines was undertaken. In this case, the solubilities of the xanthines were determined in mixtures of two semipolar, *n*-alkyl alcohols with water. The two alcohols, ethanol and methanol, were chosen on the basis of giving a desired dielectric constant range so that isolation and limitation of a given number of the total DR's found in dioxane–water could be accomplished. The main purpose of this work was to determine if the DR's found with alcohol–water mixtures were the same as the DR's found in dioxane–water mixtures.

It should be noted that caffeine contains one more methyl group than the other xanthines, theophylline and theobromine, which are positional isomers. In the dioxane–water system, it was found that the first three DR's for caffeine were about 3–4 dielectric constant units below the first three DR's for the other xanthines. Whether this difference is due to the chemical difference noted above or experimental variation is not really known. However, it has been assumed that the latter was true and the xanthines show approximately the same DR's in dioxane–water mixtures. Whereas previous data were presented only in a mg./ml. convention, the experimental system in this study was so designed through density measurements to allow for presentation of solubility in various concentration notations as well as mole fraction. It has been shown (3) that the DR's are concentration notation dependent and it was felt that these systems should be similarly treated.

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EXPERIMENTAL

Solubility Determinations.—The protocol for solubility determinations has been described previously (4, 5). All runs were done at 25° and each of the three runs made were subjected to both spectrophotometric and gravimetric analysis. Internal averaging was performed and the results are reported for the three run average.

Materials.—Caffeine was obtained from Nepera Chemical Co., Inc., theophylline from Matheson, Coleman and Bell, 7094 TX450, and theobromine N.F. from Penick, lot NBT 4092. Ethanol was obtained from U. S. Industrial Chemical Corp., sealed absolute, methanol from Allied Chemical, reagent ACS code 1212. Distilled water was used throughout this study. All materials were used directly as supplied by the manufacturer.

Equipment.—A water bath and attendant controls were used as an equilibration environment at 25°. Twenty-four hours was the time allowed for equilibration. A Bausch & Lomb spectronic 505 was used for spectrophotometric analysis, and a vacuum desiccator was used to dry samples to constant weight.

RESULTS AND DISCUSSION

The solubility of caffeine at 25° in the various concentration notations given *versus* the dielectric constant of the binary mixtures studied is shown in Fig. 1.

In Figs. 2 and 3, the solubility of theophylline and theobromine at 25° in the mg./ml. of solution convention is illustrated as a function of the dielectric constant of the binary mixtures studied.

Since the solubility curves for theophylline and theobromine, in the mg./Gm. of solution and mole fraction conventions are analogous to those in Fig. 1 for caffeine they have been omitted from these figures. The variation of DR's with concentration notation has been summarized in Table II.

The DR's found for the xanthines in these alcohol–water mixtures have been summarized in Table I. The values of the DR's for the xanthines in dioxane–water mixtures are also shown in order to contrast the various binary mixtures used. For theobromine in the methanol–water system, a shoulder at a dielectric constant value of about 40 was not clear cut. Although slight shouldering can be seen at a dielectric constant value of about 38, the existence of this DR is questioned. There is some indication that shouldering occurs at a dielectric constant of about 42 on the mole fraction basis, but this also does not necessarily prove a DR existence with another concentration convention.

Although the DR's in these alcohol–water mixtures correlate well with the DR's previously found in dioxane–water mixtures (1), a new DR at a value of 42–44 was also found. No explanation for this new peak is given; however, were this peak to exist in dioxane–water mixtures, it would fall in the valley between the third and fourth DR's for these mixtures. It is possible that alcohol–water mixtures behave mechanistically different toward the xanthines, in so far as the extent of solvation and/or hydration is concerned relative to the cyclic ether, dioxane. This will be discussed in a latter portion of this communication.

These figures illustrate also a low degree of dielectric requirement sensitivity to concentration notation. A maximum change of about 2–5 dielectric constant units is found in going from the pharmaceutical convention to the mole fraction expression. The DR's found for the xanthines relative to concentration notation have been tabulated and summarized in Table II.

Since the solubility of the xanthines has been determined in several mixtures including dioxane–water mixtures (1), it was felt judicious to determine and compare the ratios of solubility of the xanthines to one another in each pure solvent and at the common dielectric requirements. It had been shown that the ratios of solubility for the xanthines (defining theobromine = 1) in dioxane–water mixtures at the DR's were closer to the ratios of solubility in water. This was felt to imply the importance of the aqueous hydration of the xanthines. The mag-

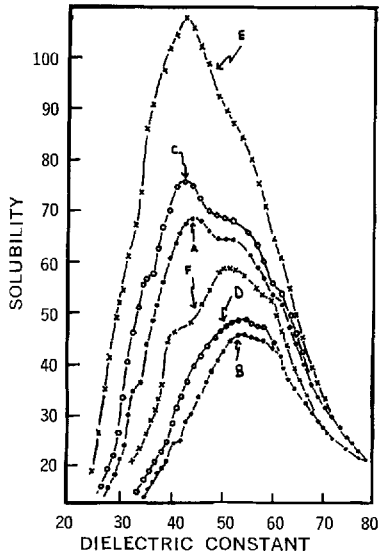

Fig. 1.—The solubility of caffeine at 25° as a function of the dielectric constant of ethanol–water and methanol–water mixtures. Key: A, aqueous ethanol; B, aqueous methanol; solubility expressed as mg./ml. of solution. C, aqueous ethanol; D, aqueous methanol; solubility expressed as mg./Gm. of solution. E, aqueous ethanol; F, aqueous methanol; solubility expressed as mole fraction $\times 10^4$.

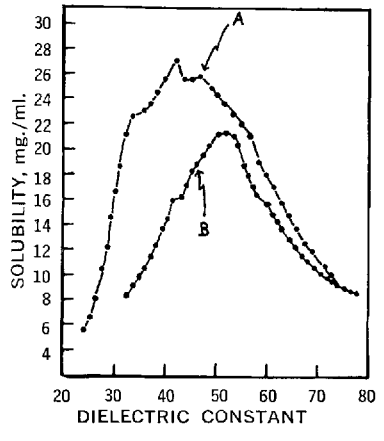

Fig. 2.—The solubility of theophylline at 25° in mg./ml. of solution as a function of the dielectric constant of ethanol–water mixtures (A) and methanol–water mixtures (B).

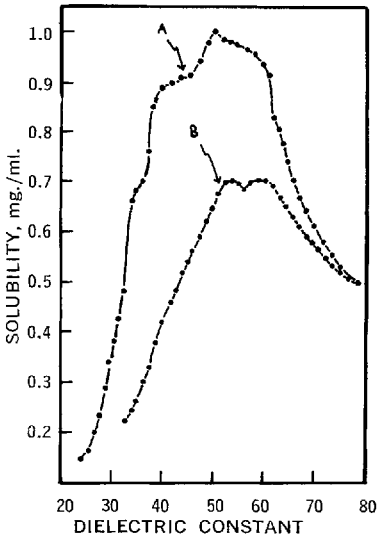

Fig. 3.—The solubility of theobromine at 25° in mg./ml. of solution as a function of the dielectric constant of ethanol–water mixtures (A) and methanol–water mixtures (B).

TABLE I.—SUMMARY OF THE DIELECTRIC REQUIREMENTS FOR THE XANTHINES IN ALCOHOL–WATER MIXTURES AND DIOXANE–WATER MIXTURES

System	Dielectric Constant Range	DR_1	DR_2	DR_3	DR_4	DR_5	DR_6
			Caffeine				
Dioxane–water	2.2–78.5	11	20	30	..	50	61
Ethanol–water	24.3–78.5	..	..	34	44	51	60
Methanol–water	32.4–78.5	..	..	..	42	54	60
			Theophylline				
Dioxane–water	2.2–78.5	14	20	34	..	50	61
Ethanol–water	24.3–78.5	..	..	34	41	48	58
Methanol–water	32.4–78.5	..	..	..	42	52	60
			Theobromine				
Dioxane–water	2.2–78.5	14	22	34	..	50	61
Ethanol–water	24.3–78.5	..	..	35	43	51	61
Methanol–water	32.4–78.5	..	..	..	38–42[a]	53	60

[a] See under *Results and Discussion.*

TABLE II.—SUMMARY OF THE DIELECTRIC REQUIREMENTS FOR THE XANTHINES AS A FUNCTION OF CONCENTRATION NOTATION

	Caffeine				Theophylline				Theobromine				
Concn. Notation	DR_1	DR_2	DR_3	DR_4	DR_1	DR_2	DR_3	DR_4	DR_1	DR_2	DR_3	DR_4	Solvent System
mg./ml.	..	42	54	60	..	42	53	60	..	40[a]	53	60	Methanol–water
	34	44	51	60	34	41	48	58	35	43	51	60	Ethanol–water
mg./Gm. of soln.	..	40	53	58	..	42	51	58	..	40[a]	51	60	Methanol–water
	35	42	49	60	34	40	48	58	35	43	50	60	Ethanol–water
mole fraction	..	42	52	58	..	42	50	59	..	42	53	59	Methanol–water
	33	43	50	60	34	40	47	58	33	43	51	62	Ethanol–water

[a] See under *Results and Discussion.*

TABLE III.—SUMMARY OF THE SOLUBILITIES AND SOLUBILITY RATIOS FOR THE XANTHINES AT THE OBSERVED DR'S AS WELL AS EACH PURE SOLVENT

Substance	Solubility in 2nd Component mg./ml.	Ratio	DR 30–34 mg./ml.	Ratio	DR 40–43 mg./ml.	Ratio	DR 50–55 mg./ml.	Ratio	DR 58–61 mg./ml.	Ratio	Solubility in Water mg./ml.	Ratio
					Dioxane–Water							
Caffeine	21	23	82	41	..	..	55.0	32	50.0	42	21.8	39
Theophylline	9	10	29	15	..	..	21.0	12	15.7	14	8.3	13
Theobromine	0.9	1	2.0	1	..	..	1.7	1	1.2	1	0.6	1
					Ethanol–Water							
Caffeine	6.4	47	36	51	69	77	65	65	54	60	21.5	44
Theophylline	5.3	38	23	33	27	30	25	25	22	24	8.3	17
Theobromine	0.14	1	0.7	1	0.9	1	1.0	1	0.9	1	0.5	1
					Methanol–Water							
Caffeine	10.2	47	..	..	25	50	47	67	44	66	21.5	44
Theophylline	8.3	38	..	..	16	32	21	30	16	23	8.3	17
Theobromine	0.22	1	..	..	0.5[a]	1	0.7	1	0.7	1	0.5	1

[a] See under *Results and Discussion.* This value of solubility was chosen at a dielectric constant of 40, although no shouldering was evidenced.

nitudes of solubility and solubility ratios have been summarized as shown in Table III.

These ratios have been plotted and are shown in Fig. 4. In the dioxane–water system, the ratios of solubility at the DR's found are seen to approximate the ratios in water. However, both the ethyl and methyl alcohol show different patterns. For theophylline, the ratios in going from pure water to pure ethanol or pure methanol increase linearly as the DR's decrease and approach the dielectric constant of the alcohol. For caffeine, the ratios in going from pure water to pure ethyl or methyl alcohol go through a maxima. Obviously, there is a potentiation effect for caffeine in the co-solvency of alcohol–water mixtures which does not occur with dioxane–water mixtures.

The linear increase for theophylline and the curve for caffeine may imply more effective and/or different hydration or solvation leading to the various hydrates or solvates having their own solubility characteristics. These effects may aid in explaining the multiplicity of peaks obtained in these co-

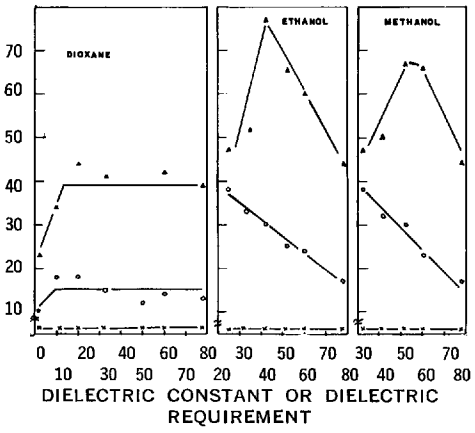

Fig. 4.—A plot of the solubility ratios (theobromine = 1) for the xanthines at the DR's found and in the pure solvents used. Key: ×, theobromine; O, theophylline; ▲, caffeine.

solvent mixtures and the newly observed peak at a dielectric constant value of about 40.

It was felt to be instructive to view these solubility curves from the point of view of co-solvency. Ideally, a co-solvent effect would deal with a solubility curve having only one maximum. In this case, co-solvency efficiency could be expressed as the ratio of the magnitude of solubility at the maximum to the magnitude of the solubility in either pure solvent.

Such, however, is not the case with the xanthines where a multiplicity of maxima occur. In order to determine the co-solvency efficiency of a given mixture, it would be necessary to determine the ratios of solubility at each dielectric requirement for a given xanthine relative to a pure solvent. Further, were one to compare the xanthines and solvent systems relative to one another, it would also be necessary to determine the co-solvency efficiency at each dielectric requirement relative to the pure solvent where the magnitude of solubility is the same, *i.e.*, water. In Table IV, the co-solvency efficiency for the xanthines in alcohol–water mixtures and dioxane–water mixtures is presented. The co-solvency efficiency has been defined as the solubility at a given dielectric requirement for each xanthine relative to the solubility in water for each xanthine. In other words, the co-solvency efficiency is the number of times the solubility of a given xanthine is increased over the solubility in pure water at a given dielectric requirement.

From inspection of Table IV, several approximate trends can be delineated. The efficiency of increasing solubility relative to water can be obtained for the solvent systems under consideration from Table IV. For a dielectric requirement of 30, common to dioxane–water and ethanol–water only, dioxane is seen to cause the greatest co-solvent effect. At a dielectric requirement of 40, common to ethanol–water and methanol–water mixtures, ethanol shows the largest co-solvent effect. At DR's of 50 and 60, common to all three solvent systems, ethanol has the highest efficiency at both values, whereas dioxane is better than methanol at DR 50 and slightly better than methanol at DR 60. Furthermore, at the common DR of 50 and 60, ethanol and methanol discriminate the xanthines to a larger extent. For example, at a DR of 50, the solubility ratios for the xanthines vary about 0.3 in dioxane–water mixtures, about 1.0 in ethanol–water mixtures, and 1.1 in methanol–water mixtures. This also indicates that dioxane increases the solubility of the xanthines to about the same extent at a given DR, whereas both ethanol and methanol increase the solubility to varying extents at a given DR.

As can be seen, as the dielectric constant of the second component (alcohol, dioxane) increases, the greatest co-solvency efficiency occurs at higher dielectric requirement values. In the dioxane–water system, the maximum co-solvency effect ($C.E._{max.}$) for the xanthines is seen to occur in the dielectric constant range of 20–30. By defining a term $DR_{max.}$, the dielectric constant of maximum co-solvency and taking the difference between this value and the dielectric constant of the second component, values of $DR_{max.} - \epsilon_2$ can be obtained. These values are simply the number of dielectric constant units above the dielectric constant of the second component where co-solvency efficiency is maximized. By taking the average $C.E._{max.}$ for the three xanthines in a given solvent system, values for $DR_{max.} - \epsilon_2$ are determined as shown in Table V.

It is obvious that for each of these systems, the average co-solvency efficiency is maximized at a dielectric constant value of 21–23 units greater than the dielectric constant of the pure second component. Furthermore, as the $DR_{max.}$ increases in value with an increase in the dielectric constant of the second component, the $DR_{max.}$ value gets closer and closer to the dielectric constant of the common component of these solvent systems, *i.e.*,

TABLE IV.—SUMMARY OF THE CO-SOLVENT EFFICIENCY [SOLUBILITY (DR, mg./ml.)/(WATER mg./ml.)] FOR THE XANTHINES IN VARIOUS BINARY MIXTURES

System	DR 11–14	DR 20–22	DR 30–34	DR 41–43	DR 50–55	DR 58–61	Substance
Dioxane–water	2.9	3.8[b]	3.8[b]	...	2.5	2.3	Caffeine
	4.0	4.5[b]	3.5	...	2.5	1.9	Theophylline
	3.0	3.3[b]	3.3[b]	...	2.8	2.0	Theobromine
Ethanol–water	...	...	1.6	3.2[b]	3.0	2.5	Caffeine
	...	...	2.8	3.1[b]	3.1[b]	2.7	Theophylline
	...	...	1.6	1.8	2.0	1.8	Theobromine
Methanol–water	...	...	...	1.2	2.2[b]	2.1[b]	Caffeine
	...	...	...	1.9	2.5[b]	1.9	Theophylline
	...	...	...	1.0[a]	1.4[b]	1.4[b]	Theobromine

[a] See under *Results and Discussion*. [b] These values have been used to determine the average co-solvency efficiency.

TABLE V.—SUMMARY OF THE MAXIMUM AVERAGE CO-SOLVENT EFFICIENCY ($DR_{max.}$) FOR THE VARIOUS BINARY MIXTURES STUDIED AND THE DIFFERENCE, $DR_{max.} - \epsilon_2$

System	$DR_{max.}$	ϵ_2	$DR_{max.} - \epsilon_2$	$DR_{max.} - \epsilon_1$
Dioxane–water	25	2	23	53
Ethanol–water	45	24	21	33
Methanol–water	55	32	23	23

TABLE VI.—SUMMARY OF THE $DR_{max.} - \epsilon_2$ FOR EACH OF THE XANTHINES IN THE BINARY MIXTURES USED

System	$DR_{max.} - \epsilon_2$		
	Caffeine	Theophylline	Theobromine
Dioxane–water	23[a]	18	26[a]
Ethanol–water	22	17	26
Methanol–water	22	20	24[a]

[a] Co-solvency maximum is equal at 2 DR's, thus average $DR_{max.}$ used.

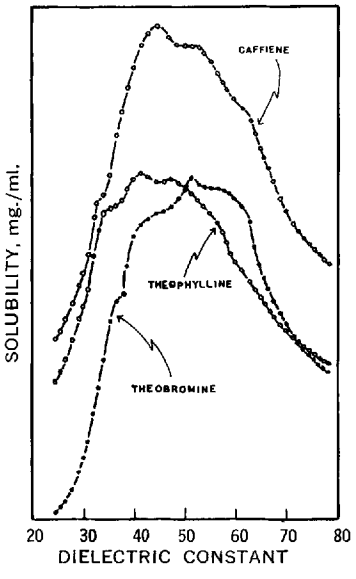

Fig. 5.—A plot of the solubility of caffeine, theophylline, and theobromine at 25° in mg./ml. *vs.* the dielectric constant of ethanol–water mixtures. Solubility magnitudes on overlapping uncommon scales. (See Figs. 1–3.)

water. Of course, these values should have different magnitudes, shown in column four, but they are proportionately related to the dielectric constant of the second component. Interesting enough, the average $DR_{max.}$ in methanol–water mixtures is equidistant between the dielectric constants of the pure components. It would be of more interest to view the xanthines in a comparative sense since in the xanthine drugs there exists a chemical difference (for caffeine) and theophylline and theobromine are positional isomers. Each xanthine in the three solvent systems under consideration can be handled separately relative to the dielectric constant of maximum co-solvency. In this case, the exact value of the DR (Table I) at maximum co-solvency has been used or when the co-solvency efficiency was equal at 2 DR's, an average value of the exact DR was used. Thus, the $DR_{max.} - \epsilon_2$ for each xanthine in the solvent systems used have been summarized in Table VI.

In order to illustrate the variation of $DR_{max.} - \epsilon_2$ found for the xanthines, a composite figure is given (Fig. 5) of the solubility of each xanthine in mg./ml. for ethanol–water mixtures.

Although caffeine on an individual basis is the same as the average value, *i.e.*, 21–23, theophylline falls below the average range, while theobromine falls above the average range. Furthermore, the variation of theophylline and theobromine from the average value is about the same, theophylline being 3–4 units below the average, whereas theobromine is 3–4 units above the average. The order of the xanthines with respect to the increasing magnitude of $DR_{max.} - \epsilon_2$ is theophylline, 18 < caffeine, 22 < theobromine, 25. As has been noted previously, the magnitude of the co-solvency efficiency (Table IV) for theophylline was equal to or greater than that of either caffeine or theobromine in the solvent systems studied.

The DR's obtained in this study for the xanthines in alcohol–water mixtures showed good correlation with the DR's obtained previously with dioxane–water mixtures. A consistent new peak at a dielectric constant of about 40 was also found for these alcohol–water mixtures. In this regard, the solubilities of the xanthines are being determined in a glycol ether (ethylcellosolve)–water mixtures to see if the DR of 40 is unique to the alcohols.

The xanthines show a low degree of DR sensitivity to concentration notation for the alcohol–water mixtures.

The solubility ratios, defining theobromine as unity, in the pure solvents and at the dielectric requirements found showed a linear trend for theophylline and a curve having a maxima for caffeine.

The co-solvency efficiency, defined as the ratio of the magnitude of solubility at a given DR to the magnitude of solubility in water, showed maximum efficiency on the average at about 20 dielectric constant units above the dielectric constant of the pure second component. For the individual xanthines, the $DR_{max.} - \epsilon_2$ was seen to be in the order, theophylline < caffeine < theobromine; however, the co-solvent efficiency for theophylline was equal to or greater than either caffeine or theobromine in all the solvent systems studied. This would imply that the solubility of theophylline is affected to a greater degree in contrast to caffeine or theobromine and the dielectric constant of maximum co-solvency may lie closer to the dielectric constant of the pure second component.

The solubilities of the xanthines are being studied in ethylcellosolve–water mixtures relative to the above points and the authors' results will be the subject of future communications.

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Solubility Profiles for the Xanthines in Aqueous Solutions of a Glycol Ether II

Ethyl Cellosolve

By ANTHONY N. PARUTA* and SHAPUR A. IRANI

The solubilities of the xanthines were determined in ethyl cellosolve–water mixtures as a function of the dielectric constant of these solvent mixtures. This mixture of a glycol ether with water was chosen in order to contrast the dielectric requirements found with those observed in alcohol–water and dioxane–water mixtures. The dielectric requirements found for the ethyl cellosolve–water system correlate to a good degree with previous findings. A dielectric requirement of 40 was also found which was not found in dioxane–water mixtures but was present in the alcohol–water mixtures. These systems were experimentally designed so that solubility curves could be expressed in various concentration notations as well as mole fraction.

IN A CONTINUING effort of investigating the relative consistency of dielectric requirements (DR's) for a given solute(s) in various binary mixtures (1–3), the present study was undertaken. The dielectric requirement is defined as the dielectric constant of maximum solubility in a given solvent mixture.

It had been found (2, 3) that the DR's for the xanthines in aqueous mixtures of a cyclic ether, dioxane, and normal alkyl alcohols, ethanol and methanol, were fairly consistent; however, in the aqueous alcoholic systems a new peak at a DR of about 40 was also found. It was felt important to see if this new peak at a value of 40 could be found in another aqueous mixture with a semipolar solvent. In this case, a glycol ether was chosen considering this to be sufficiently different in nature from a cyclic ether and a normal alcohol. The solvent chosen was ethyl cellosolve since it had a dielectric constant intermediate between dioxane and the alcohols and also had the property of being infinitely soluble in water. The dielectric constant range produced by these mixtures would be about 15–78, and it was expected that this range should accommodate 4 of the 5 DR's found in dioxane–water mixtures having values of about 20, 30, 50, and 60.

These systems were experimentally designed through density measurements so that solubility could be expressed in various concentration notations as well as mole fraction. It had been shown that the observed DR's are concentration notation dependent (3) and it was felt that these systems should be similarly treated.

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EXPERIMENTAL

Solubility Determination.—The protocol for solubility determinations has been described previously (1–3). All runs were done at 25°, and each of the three runs performed was subjected to both spectrophotometric and gravimetric analysis. Internal averaging was done for each run, and the results reported are for the three-run average.

Materials.—Caffeine was obtained from Nepera Chemical Co., theophylline from Matheson, Coleman and Bell, 7094Tx450, and theobromine N.F. from Penick, lot NBT 4092. Ethyl cellosolve was obtained from Union Carbide, 5753753. Distilled water was used throughout this study. All materials were used directly as supplied by the manufacturer.

Equipment.—A water bath with attendant controls was used as an equilibration environment at 25°. Twenty-four hours was the time allowed and found sufficient for equilibration. A Bausch & Lomb Spectronic 505 was used for spectrophotometric analysis and a vacuum desiccator was used to dry samples to constant weight.

RESULTS AND DISCUSSION

The solubility of caffeine in the various concentration conventions noted *versus* the dielectric constant of ethyl cellosolve–water mixtures is shown in Fig. 1. The concentration notation of mg./Gm. of solvent has been omitted from this figure and subsequent figures since the solubility curve coincides very closely to the mg./Gm. of solution solubility curve. In Figs. 2 and 3, the solubility of theophylline and theobromine plotted in the usual fashion are shown.

The DR's observed in the ethyl cellosolve–water system for the xanthines have average values of about 30, 40, 48, and 60. The DR's in ethyl cellosolve–water mixtures and dioxane–water mixtures (2) have been summarized in Table I. The DR of 40, which was observed in alcohol–water mixtures (3), was also found in ethyl cellosolve–water mixtures; however, this DR of 40 was not found in the dioxane–water system. Thus, this new DR is not unique to alcohol–water mixtures since it has also been found in ethyl cellosolve–water mixtures. To illustrate this point, a composite figure has been prepared showing the solubility

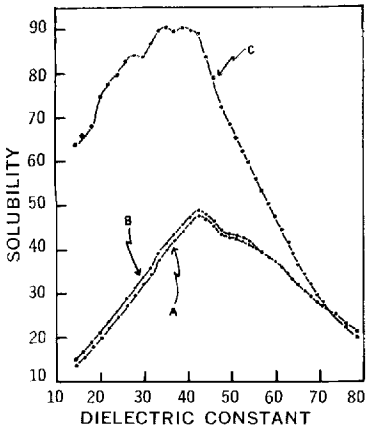

Fig. 1.—A plot of the solubility of caffeine at 25° *vs.* the dielectric constants of ethyl cellosolve–water mixtures. Key: A, solubility expressed as mg./ml.; B, solubility expressed as mg./Gm. of solution; solubility expressed as mole fraction (m.f. $\times 10^4$).

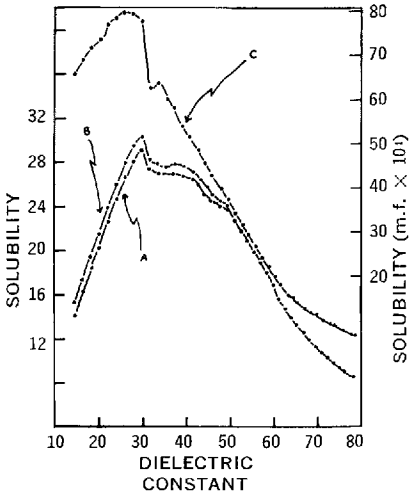

Fig. 2.—A plot of the solubility of theophylline at 25° *vs.* the dielectric constants of ethyl cellosolve–water mixtures. Key: A, solubility expressed as mg./ml.; B, solubility expressed as mg./Gm. of solution; C, solubility expressed as mole fraction (m.f. $\times 10^4$) using scale at right hand side of figure.

curves on a mg./ml. basis for caffeine in the four binary solvent systems studied. This is shown in Fig. 4. In the case of dioxane–water mixtures, a dielectric constant of 40 is seen to fall in a valley between the third and fourth DR's for this system. If a peak or shoulder does exist at about this value it would be, at best, difficult "to see" due to the nature of the curve and the inherent experimental variation involved. This figure also reveals another interesting point. At a dielectric requirement of 30, common to three binary mixtures, dioxane–water mixtures produce a strong peak, whereas both ethanol–water and cellosolve–water mixtures produce only a weak shouldering effect. At a dielectric constant of about 50, common to all four binary mixtures, aqueous dioxane and aqueous methanol produce definite peaks; whereas, aqueous cellosolve and aqueous ethanol again produce a shouldering effect.

There is a probablility that various hydrates/solvates (2), each with its own solubility characteristics, cause these complex individually overlapping solubility curves. It was thought that the production of a new peak (DR) in a given system would

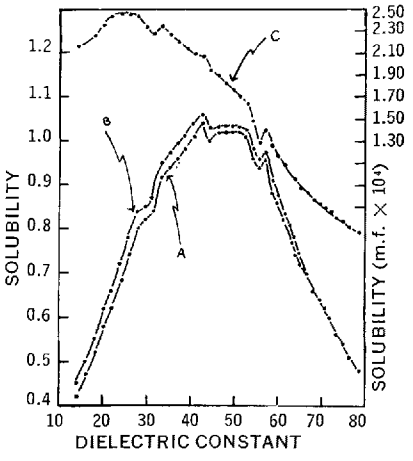

Fig. 3.—A plot of the solubility of theobromine at 25° *vs.* the dielectric constants of ethyl cellosolve–water mixtures. Key: A, solubility expressed as mg./ml.; B, solubility expressed as mg./Gm. of solution; C, solubility expressed as mole fraction (m.f. $\times 10^4$) using scale at right hand side of figure.

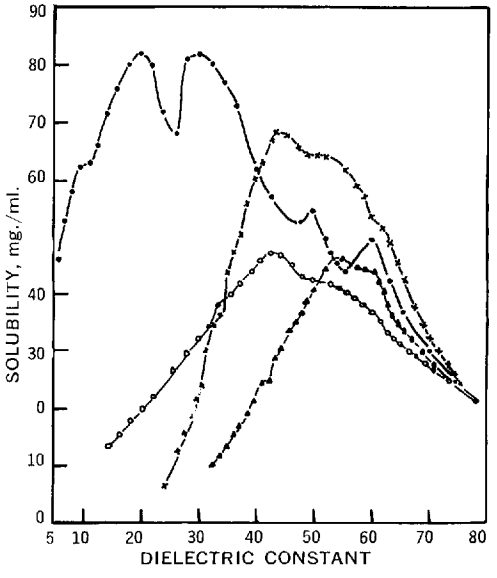

Fig. 4.—A plot of the solubility of caffeine at 25° in mg./ml. as a function of the dielectric constant of various binary mixtures. Key: ●, dioxane–water; ○, ethyl cellosolve–water; ×, ethanol–water, ▲, methanol–water.

TABLE I.—SUMMARY OF DR's FOR XANTHINES IN AQUEOUS ETHYL CELLOSOLVE AND AQUEOUS DIOXANE[a]

System	Dielectric Constant Range	DR_1	DR_2	DR_3	DR_4	DR_5	DR_6
			Caffeine				
Dioxane–water	2–78	11	20	30	..	50	61
Cellosolve–water	14–78	..	?[b]	32	43	50	62
			Theophylline				
Dioxane–water	2–78	14	20	34	..	50	61
Cellosolve–water	14–78	..	?[b]	30	37	46	61
			Theobromine				
Dioxane–water	2–78	14	22	34	..	50	61
Cellosolve–water	14–78	..	?[b]	32	42	48	58

[a] Data from *Reference 1*. [b] See under *Results and Discussion* and Table II.

TABLE II.—SUMMARY OF FIRST OR EXPECTED DR FOR XANTHINES IN BINARY MIXTURES STUDIED[a]

Systems	ϵ_2	DR	DR − ϵ_2	Peak or Shoulder Observed
Dioxane–water	2.2	13	10.8	Yes
Ethanol–water	24.3	34.5	10.2	Yes
Methanol–water	32.4	42.0	9.6	Yes
Ethyl cellosolve–water	14.5	20.7[b]	6.2	No

[a] Data from *References 2* and *3*. [b] Expected DR of about 20–22 found in dioxane–water mixtures.

be possible at the expense of DR's close to this new value. In other words, a given species could exist in two different solvent mixtures, depending on the composition of the mixtures and each component's contribution to the solvated state of that species. This does not seem to be the case in alcohol–water or cellosolve–water mixtures. Although a new DR of about 40 is found, the DR's of about 30 and 50 are also present which indicate that the DR of 40 is not a mixture of the two DR's surrounding it. However, it can also be seen that the strong peak at a DR of 30 in dioxane–water is reduced to a slight shouldering effect in aqueous ethanol and aqueous ethyl cellosolve. It would seem that this is partially due to the dielectric constant range of the solvent system chosen. The co-solvency efficiency has been discussed previously (3) and will be further elucidated in a latter portion of this communication.

Table I also shows that a DR of about 20 found in dioxane–water mixtures was not found in ethyl cellosolve–water mixtures. The dielectric constant range of aqueous cellosolve, *i.e.*, 14.5–78, certainly encompasses a dielectric constant of 20, but the solubility curves did not indicate any peaks or shoulders in this dielectric constant area. Upon re-examination of the data for the four binary mixtures studied, it was noted that the first DR observed starting from the second or semipolar component was a certain number of dielectric constant units above the value for the second component. Table II summarizes the first average DR found or expected and the difference between this value and the dielectric constant of the second component.

It is apparent that the first or expected DR resides at a definite value above the dielectric constant of the second component. The DR observed for the first three binary mixtures is seen to be about 10–11

TABLE III.—SUMMARY OF DR's FOR XANTHINES IN ETHYL CELLOSOLVE–WATER MIXTURES IN VARIOUS CONCENTRATION CONVENTIONS NOTED

	Caffeine				Theophylline				Theobromine				
Concn. Notation	DR_1	DR_2	DR_3	DR_4	DR_1	DR_2	DR_3	DR_4	DR_1	DR_2	DR_3	DR_4	DR_5
mg./ml.	32	43	50	62	30	37	46	61	..	32	42	49	57
mg./Gm. soln.	32	43	50	62	30	37	48	61	..	30	42	49	57
mole fraction	19	28	35	39	..	20	26	33	25	33	43	50	57

TABLE IV.—SUMMARY OF SOLUBILITY OF XANTHINES (IN mg./ml.) IN EACH PURE SOLVENT AND AT COMMON DR's AND SOLUBILITY RATIOS DEFINING THEOBROMINE AS UNITY

	Solubility, mg./ml.					
	Ethyl Cellosolve	DR 30	DR 40	DR 50	DR 60	Water
Caffeine	13.6	35	48	43	36	21.5
Theophylline	14.1	29	27	25	16	8.3
Theobromine	0.41	0.84	1.04	1.02	0.96	0.50
	Solubility Ratios					
	Ethyl Cellosolve	DR 30	DR 40	DR 50	DR 60	Water
Caffeine	33	42	46	42	37	42
Theophylline	34	35	26	24	16	17
Theobromine	1	1	1	1	1	1

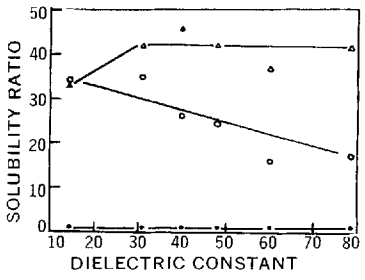

Fig. 5.—A plot of the solubility ratios for theophylline and caffeine relative to theobromine (unity) in each pure solvent and at the common dielectric requirements. Key: ●, theobromine; ○, theophylline; ▲, caffeine.

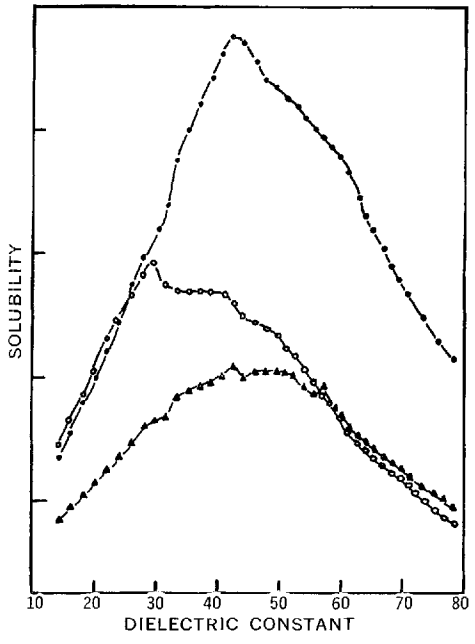

Fig. 6.—A plot of the solubility curves for caffeine (●), theophylline (○), and theobromine (▲) *vs.* the dielectric constants of ethyl cellosolve–water mixtures. The solubility scale does not describe magnitude since only the nature of the curves is being compared.

units above ϵ_2. Thus, a peak or shoulder may not be found unless the second component has a dielectric constant about 10–11 units below the expected DR. This approximate value of 10–11 units is, of course, only operative for the range and systems studied, and may only apply for the solutes under consideration.

It is interesting to note in this regard, that the suspected shouldering for theobromine at a dielectric constant of 38 in methanol–water mixtures (3) resides only 5.6 dielectric constant units above ϵ_2 and this probably indicates it cannot be seen.

Since there can be a variation of 3–4 dielectric constant units (2) in any given DR for these solutes, it is possible that theobromine falls below this approximate difference (DR $-$ ϵ_2) of 10–11 units. The very low solubility of theobromine also mitigates easily detecting shouldering in the solubility curve.

It has previously been shown (3) that the value of the DR was sensitive to concentration notation. In the case of succinic acid, there was greater DR sensitivity for dioxane (mol. wt. = 88) than for ethanol (mol. wt. = 46) in going from the pharmaceutical convention to mole fraction. For the xanthines (3), it was shown there was low DR sensitivity to methanol (mol. wt. = 32) and ethanol (mol. wt. = 46). In Figs. 1–3, it can be seen that there is high DR sensitivity for caffeine and theophylline, but low sensitivity for theobromine. It is felt that the very low solubility of theobromine and very small incremental changes of solubility with varying composition damps the possible shifting with a high molecular weight solvent such as cellosolve (mol. wt. = 90). This could also possibly account for the new DR at a value of 25, since a small deviation of a given value from a smooth curve could produce a shoulder or peak depending on the magnitude of the deviation. Assuming caffeine and theophylline to be typical, it would seem that DR sensitivity to various concentration notations depends upon the molecular weight of the second component. Since water is the other component common to all these binary mixtures, DR sensitivity to concentration notation would also depend upon the difference in molecular weight of the two components used.

The DR's found for the xanthines in ethyl cellosolve–water mixtures in the various concentration notations used are summarized in Table III.

Since the DR's found for the xanthines were relatively constant, it was felt that these solubility curves might be parallel to one another, indicating proportionality of the magnitude of solubility. Consequently, the ratios of the solubilities in each pure solvent and at the common DR's were taken defining the solubility of theobromine as unity. The solubility of the xanthines in mg./ml. for each pure solvent and at the common DR's and the solubility ratios have been summarized in Table IV. Previous results (2) indicated that the ratios in dioxane–water mixtures were closer to the solubility ratios in pure water, and this was felt to imply the importance of aqueous solvation. Other results (3) for the xanthines in aqueous methanol and aqueous ethanol showed a potentiated effect for caffeine and a linear rise for theophylline in going from the solubility ratios in pure water to pure ethanol and methanol. The solubility ratios in ethyl cellosolve–water mixtures have been calculated and are presented in Fig. 5. In this case, it can be seen that the solubility ratios for theophylline rise approximately linear going toward pure ethyl cellosolve and are similar to the results observed in alcohol–water mixtures. On the other hand, the solubility ratios for caffeine are close to the ratios in pure water, being similar to the results observed in dioxane–water mixtures. This would indicate that each of the xanthines interacts differently with the second component in terms of the nature of the hydrate/solvate formed.

In order to visualize the above, a composite figure has been prepared in which the solubility of the xanthines in mg./ml. has been plotted *versus* the dielectric constants of ethyl cellosolve–water mixtures (Fig. 6). Several things can be noted in this

TABLE V.—SUMMARY OF CO-SOLVENCY EFFICIENCY [SOLUBILITY, mg./ml., DR_x/SOLUBILITY, mg./ml., WATER] FOR XANTHINES IN ETHYL CELLOSOLVE–WATER MIXTURES AT COMMON DR'S FOUND

System	DR 30	DR 40	DR 50	DR 60	Substance
Ethyl cellosolve–	1.63	2.23	2.00	1.67	Caffeine
water	3.49	3.25	3.01	1.93	Theophylline
	1.68	2.08	2.04	1.92	Theobromine

TABLE VI.—SUMMARY OF DIFFERENCE OF MAXIMUM CO-SOLVENT EFFICIENCY ($DR_{max.}$) AND DIELECTRIC CONSTANT OF THE SECOND COMPONENT (ϵ_2) FOR INDIVIDUAL XANTHINES IN ETHYL CELLOSOLVE–WATER AND OTHER MIXTURES[a]

Substance	Ethyl Cellosolve $DR_{max.} - \epsilon_2$	$DR_{max.} - \epsilon_2$[a]
Caffeine	28	22–23
Theophylline	16	17–20
Theobromine	28	24–26

[a] Data from *Reference 3.*

figure. Theophylline shows a strong peak at a DR of 30, whereas both caffeine and theobromine indicate slight shouldering effects. Caffeine at a DR of 42 shows a peak and at a DR of 60 a shoulder in the solubility curve. However, theobromine shows weak peaks at both DR's of about 42 and 60. Variations of this type lead into a consideration of co-solvency efficiency. The co-solvency efficiency is defined as the solubility at a given dielectric requirement relative to the solubility in water for a given solute. In other words, the co-solvency efficiency is the number of times the solubility of a given solute is increased over the solubility in pure water at a given dielectric requirement. This term has been defined previously (3) and it was found that for previous systems studied the dielectric constant of average maximum co-solvency for the xanthines occurred at 21–23 units above the dielectric constant of the second component, ϵ_2. Furthermore, it was also found that on an individual basis for each xanthine, caffeine fell in the average of 21–23 units while theophylline fell below, *i.e.*, 17–20, and theobromine was above the average, *i.e.*, 24–26. The co-solvency efficiencies have been tabulated and are given in Table V. The values of $DR_{max.} - \epsilon_2$ on an individual basis for each xanthine in ethyl cellosolve–water mixtures have been summarized in Table VI. The values obtained for the previous binary mixtures studied (3) have been included to contrast the xanthines on an individual basis. It can be seen that both caffeine and theobromine deviate from the values found previously, but theophylline is about the same relative to $DR_{max.} - \epsilon_2$ in ethyl cellosolve–water mixtures.

Several things can be noted here; first, the magnitude of solubility of theophylline in pure ethyl cellosolve (Table IV) is higher than the solubility of caffeine. This is the first time that this reverse order has been found in the solvents studied. Normally, the order of solubility is caffeine > theophylline > theobromine. Second, the co-solvency efficiency of the present solvent system toward theophylline is greater than either caffeine or theobromine at the maximum, and this has been noted previously (2, 3) in dioxane–water and alcohol–water mixtures. The solubility ratios (Fig. 5) also indicated a mixed pattern for ethyl cellosolve–water mixtures relative to the patterns found in previous mixtures.

Although there seems to be a generally patternized response for the xanthines in diverse binary mixtures, more studies should be conducted to resolve many of the points brought out in these communications.

Some of the points intended for future study would include the possible dependency of DR's on the nature, type, and dielectric constant span produced by the second or semipolar component.

It may also be judicious to investigate the nature of the xanthine species involved when at common DR's a strong peak or weak shoulder is produced.

SUMMARY

The DR's obtained in this study for the xanthines in ethyl cellosolve–water mixtures showed good correlation with the DR's found in alcohol–water mixtures (3) and dioxane–water mixtures (2) except the former two systems showed a consistent new peak at a DR of about 40. The expected DR at a value of 20 was not found in ethyl cellosolve–water mixtures and this was felt to be due to the closeness of the expected DR to the ϵ_2 (Table II).

The xanthines, at least caffeine and theophylline, showed a high DR sensitivity to concentration notation and this is felt to be due to the high molecular weight of the second component.

The solubility ratios, defining theobromine as unity, showed a pattern for caffeine similar to that found with dioxane–water mixtures, whereas theophylline showed a pattern similar to that found with alcohol–water mixtures but dissimilar to that found in dioxane–water mixtures. The co-solvency efficiency and the $DR_{max.} - \epsilon_2$ showed relative consistency relative to order and magnitude.

In aqueous dioxane and aqueous alcohol (2, 3), the difference $DR_{max.} - \epsilon_2$ was 17–20 units for theophylline, whereas the difference in ethyl cellosolve–water mixtures was about 16 units.

For caffeine and theobromine (2, 3), the range of $DR_{max.} - \epsilon_2$ in the former mixtures was 22–23 and 24–26 units, respectively. In ethyl cellosolve–water mixtures $DR_{max.} - \epsilon_2$ for caffeine and theobromine were about 28 units. The order of $DR_{max.} - \epsilon_2$ in the solvent system studied were found to be theophylline < caffeine ≤ theobromine. The higher maximum co-solvency efficiency for theophylline in all these solvent systems (3) is relatively consistent, the order being theophylline > caffeine > theobromine except in ethanol where theophylline ≈ caffeine.

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Mechanism of Action of Starch as a Disintegrating Agent in Aspirin Tablets

By NATUBHAI R. PATEL* and RAYMOND E. HOPPONEN†

A study was made of the mechanisms by which starch may cause disintegration of tablets. Dried cornstarch was found to increase in volume by 78 per cent when suspended in water. In aspirin tablets where contact of starch grains is continuous in the interparticle spaces, disintegration is rapid and effective even when void spaces are eliminated. Where contact is not continuous, disintegration is slowed and appears to depend on the degree of contact between starch grains and aspirin particles and on the size of interparticle spaces. The primary mechanism appears to be a swelling action. Capillarity *per se* does not appear to have a disintegrating effect.

STARCH IS widely used as a disintegrating agent in tablets. It has been generally accepted that it acts as a disintegrating agent through a swelling action when exposed to water (1). Crisafi and Becker (2) have demonstrated that starch will absorb about 20% of its weight of water in 24 hr. when exposed to an atmosphere of 95% relative humidity at 27°. They make no mention of any volume changes involved in the process. Kerr (3), in his book on starch, states that in the natural state starch is insoluble in cold water but appears to absorb 25 to 30% and does not swell appreciably. Curlin (4) has suggested that the disintegrating effect is due to capillary action rather than swelling. The objective of this study was to investigate the mechanism by which starch functions as a tablet disintegrating agent.

EXPERIMENTAL

Swelling of Starch in Water.—Cornstarch (Argo brand) dried for 2 hr. at 110° was measured microscopically using a calibrated eye piece. Measurements were made along the longest dimension of the grain using high power (10× eyepiece and 45× objective). Slight adjustments of focus were made to get the sharpest image. Two-hundred grains were measured for each sample. Slides made of dry starch without a suspending medium gave an arithmetic mean diameter of 9.0 μ and a mean volume diameter of 10.8 μ. A light liquid petrolatum suspension gave values of 9.1 and 10.5 μ, respectively. When suspended in water at room temperature (23°), the arithmetic mean diameter increased within a few seconds to 11.2 μ, the mean volume diameter to 13.1 μ. The differences are statistically significant at the $P = 0.01$ level using the t test. Using the mean volume diameter and assuming a spherical shape, the increase in volume was calculated to be 78% of the original.

Mode of Disintegration of Tablets Containing Starch.—To eliminate variables other than the disintegrating agent, aspirin was chosen as the material for tableting. It was desired to note the effect of different sized spacings between the tablet particles on the mode and rate of disintegration and to eliminate as much as possible other spaces within the tablet. To this end, aspirin powder (U.S.P. grade, Merck) was recrystallized from acetone. The crystals were dried at 90° and screened into several sizes using a Fisher Ro-Tap sifter and standard screens (Tyler series). The crystal sizes were designated as $^{14}/_{20}$ (all crystals passing a No. 14 screen but not a No. 20), $^{20}/_{40}$, $^{40}/_{60}$, $^{60}/_{100}$, and below 100 (all crystals passing a No. 100 screen. The starch was dried at 110° for 2 hr. and stored in a tightly closed container until used. Tablets were compressed on a Colton model 3E single punch tablet press using concave 8.8-mm. punches. The material for each tablet (0.5 Gm.) was weighed out, placed manually in the die, and the machine turned slowly by hand to produce as much uniformity as possible in the tablets. The finished tablets were individually weighed and the thickness measured with a vernier caliper to insure uniformity in dimensions. Disintegration times were determined in a U.S.P.-type apparatus at 20 c.p.m. using distilled water at 23° and a No. 10 mesh screen on the bottom of the basket. Table I reports the results on aspirin tablets containing 10% by weight of starch. The results represent the averages obtained on 7 tablets at each crystal size.

The manner of disintegration changed between the $^{40}/_{60}$ and the $^{60}/_{100}$ tablets. The $^{14}/_{20}$, $^{20}/_{40}$, and $^{40}/_{60}$ tablets fell apart rapidly into a mass of crystals. The $^{60}/_{100}$ and below 100 tablets showed a progressive disintegration from all sides and a steadily shrinking core.

Samples of the tablets were examined microscopically to note the manner of distribution of starch. Transverse sections were made and scraped smooth with a razor blade. The sections were stained with iodine solution and examined after drying. The $^{14}/_{20}$ tablets showed a heavy concentration of starch grains in all the channels between the aspirin crystals. The $^{20}/_{40}$ and $^{40}/_{60}$ tablets showed a similar pattern but with decreasing amounts of starch in the channels. The $^{60}/_{100}$

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tablets displayed lines of starch granules around each crystal with many discontinuities where no starch was visible. In the below-100 tablets, starch grains were scattered in small groups among the aspirin crystals with few continuities in the channels.

Since the disintegration times of the tablets containing 10% starch were so short, a series was prepared with only 5% starch. Two additional crystal size designations were made, $^{100}/_{250}$ and below 250. The tablets were prepared in the same manner as previously. The screen on the disintegrating apparatus was replaced with a No. 6 screen to facilitate passage of the larger size crystals. The results, reported in Table II, represent the average disintegration times for five tablets.

The $^{14}/_{20}$ and $^{20}/_{40}$ tablets separated rapidly into large particles. The $^{40}/_{60}$ and $^{60}/_{100}$ tablets slowly broke up into large fragments which then disintegrated into crystals. The $^{100}/_{250}$ tablets separated in slices from the faces to the center, the slices then separating into granules. The below-250 tablets were not affected for some time, then separated into slices from the faces toward the center. The slices slowly separated into granules. Microscopic examination of the tablets revealed that the starch channels were continuous only in the $^{14}/_{20}$ and $^{20}/_{40}$ tablets.

TABLE I.—DISINTEGRATION TIMES OF ASPIRIN TABLETS CONTAINING 10% STARCH

Crystal Size	Time, sec.
$^{14}/_{20}$	60[a]
$^{20}/_{40}$	24[a]
$^{40}/_{60}$	12
$^{60}/_{100}$	18[b]
Below 100	35[b]

[a] The tablets fell into large particles in less than 10 sec. However, the particles passed the screen very slowly; thus, the recorded times are not a true measure of the time for the tablet to lose its shape and identity. [b] The tablets disintegrated toward the center from all sides retaining their shape during disintegration. The time indicated essentially marks the time of disintegration of the last of the tablet core.

TABLE II.—DISINTEGRATION TIMES FOR ASPIRIN TABLETS CONTAINING 5% STARCH

Crystal Size	Time, sec.
$^{14}/_{20}$	17
$^{20}/_{40}$	17
$^{40}/_{60}$	55
$^{60}/_{100}$	50
$^{100}/_{250}$	120
Below 250	>900

Penetration of Water into Tablets.—The change of the mode of disintegration of tablets containing 10% starch from the $^{40}/_{60}$ to the $^{60}/_{100}$ tablets and the appearance of discontinuities in the pattern of starch distribution in the $^{60}/_{100}$ tablets suggests a relationship between the two. Conceivably, the change in disintegration could be due to a change in the facility or the degree of penetration of water into the tablet. Samples of each crystal size group of the tablets were tested to determine to what extent water would penetrate into the tablet in a given time period. A series of flat-faced tablets with 10% starch were prepared for this purpose. The thickness of each tablet was measured with a vernier caliper. A 0.05-ml. drop of iodine solution, containing 50% alcohol to retard swelling of the starch, was placed in the center of a small watch glass coated with liquid paraffin to prevent spreading of the drop. The tablet was placed in contact with the drop and penetration allowed to proceed for 10 sec. The tablet was removed, blotted dry, and allowed to dry at room temperature. It was then carefully shaved with a razor blade to remove the colored portion, and the thickness of the remainder measured. The tablets showed the following per cent of penetration: $^{14}/_{20}$, 49%; $^{20}/_{40}$, 38%; $^{40}/_{60}$, 38%; $^{60}/_{100}$, 22%; and below 100, 9%.

Effect of Capillarity Alone.—To determine the effect of capillarity without expansion as a disintegrating effect, tablets of aspirin and dextrose were prepared and tested for disintegration time. Dextrose was chosen because it is a highly water-soluble material with a strong attraction for water. It has a chemical structure closely related to that of starch. It should be able to draw water into the spaces between the hydrophilic aspirin crystals without any concomitant swelling. Powdered dextrose (U.S.P. anhydrous, Mallinckrodt) was passed through a No. 80 screen and added in 10% concentration to $^{40}/_{60}$ aspirin crystals. Compression into 0.5-Gm. tablets was done with 8.8-mm. flat-faced punches. The material for each tablet was individually weighed and 5 series of tablets of increasing hardness were prepared by lowering the position of the upper punch for each succeeding series. When subjected to a disintegration test they showed the following disintegration times (average of four tablets): 140, 130, 191, 210, and 253 sec. The mode of disintegration in this case appeared to be a loss of cohesiveness of the tablet as the dextrose dissolved. The tablets gradually eroded away as fragments separated from the outer sides. The fragments for the most part were of the size of the aspirin crystals used. It appeared that the crystals simply fell away as the dextrose layer dissolved. There were no indications that disintegrating forces were acting in the interior of the tablet. A drop of

TABLE III.—VOID SPACES IN FLAT-FACED TABLETS OF ASPIRIN CONTAINING 5% STARCH

Crystal Size	Wt., Gm.	Vol., ml.	Void Space, ml.	Void Space, %
$^{14}/_{20}$	0.2999	0.2250	0.0110	4.89
$^{20}/_{40}$	0.2997	0.2242	0.0104	4.64
$^{40}/_{60}$	0.2993	0.2244	0.0109	4.85
$^{60}/_{100}$	0.2966	0.2208	0.0102	4.62
$^{100}/_{250}$	0.2942	0.2193	0.0095	4.34
Below 250	0.2950	0.2211	0.0106	4.77

dilute aqueous gentian violet solution placed on the surface of these tablets penetrated approximately 50% of all tablets in 4 min., except the softest in the series which was completely penetrated in less than 2 min.

The effect of capillarity was further checked by the use of aluminum hydroxide tablets. Aluminum hydroxide powder (dried gel, Reheis) was made into granules by slugging. Preparation of slugs in the usual manner was not possible because jamming of the machine occurred. Individual slugs were prepared by manually placing the aluminum hydroxide in the die and turning the machine by hand. Before preparing each slug, the punches and the die were lightly lubricated with magnesium stearate applied with a camel hair brush. The slugs were crushed and screened into granules of three sizes, $^{14}/_{20}$, $^{20}/_{60}$, and below 60. When compressed into tablets with 10% starch and tested, they disintegrated in times of 8, 18, and 20 sec., respectively. Microscopic examination of the tablets stained with iodine solution showed the distribution of starch to be continuous around the aluminum hydroxide particles. When placed in 95% ethanol, where penetration of solvent can still occur but no swelling of starch results, the tablets did not disintegrate. The penetration of the tablets by ethanol was checked by the same procedure as used to determine the penetration of aspirin tablets by an iodine solution. A measured drop of an ethanolic solution of iodine was used. In 10 sec. it penetrated the tablets to an average depth of 50% of the total thickness. This would seem to indicate that swelling is a necessary condition for disintegration but does not entirely eliminate the possibility of capillarity as a disintegrating force. The surface tension of ethanol is only about one-third that of water. In its simplest terms, the pressure produced by capillarity can be equated to $2\ \gamma/r$, where γ is the surface tension of the liquid, and r is the radius of the capillary. The difference in surface tensions of ethanol and water could very well be sufficient to prevent any capillary effect from being noted. The search for a liquid with a surface tension near that of water, capable of wetting starch, and not able to dissolve aspirin too readily nor too rapidly, suggested glycerin. It has a surface tension of 63 dynes/cm. compared to a value of 73 for water (5). It did not cause disintegration of $^{20}/_{40}$-aspirin tablets containing 10% starch even after 12 hr. of contact. The penetration of glycerin into the interior of the tablets was checked to insure that it did occur. A drop of glycerin (the volume was not measured because the viscosity makes such measurement almost meaningless) containing 0.6% iodine was placed on the surface of flat-faced $^{20}/_{40}$ tablets. It penetrated to an average depth of 30% in 2 min. These results again suggest that capillarity does not constitute a disintegrating force in this situation.

Relation of Void Space to Disintegration Time.—Since the volume of starch increased in water, the authors felt it would be of interest to investigate the relationship of the volume increase and the void-space volume of the tablets. In order to measure the volume, 8.8-mm. flat-faced punches were used to prepare the tablets. The dimensions of the tablets were measured with a vernier caliper. The densities of the aspirin crystals and of the starch were determined by the pycnometer method (6) using petroleum ether as the liquid. At 20° the density of aspirin was found to be 1.396 Gm./ml., that of starch 1.513 Gm./ml. Void spaces were determined as the difference between the volume calculated from the dimensions of the tablet and the volume of the tablet solids calculated from their densities.[1]

Table IV.—Influence of Compression Force on Void Space and Disintegration Time of Aspirin Tablets Containing 10% Starch

Pressure, lb./sq. in.	Void Space, %	Time, sec.
2000	7.0	7
4000	4.5	6
6000	2.3	8
8000	2.7	7
10000	2.0	8
12000	1.6	10
14000	0.1	10
16000	<0.1	10

Table V.—Influence of Compression Force on Void Space and Disintegration Time of Aspirin Tablets Containing 5% Starch

Pressure, lb./sq. in.	Void Space, %	Void Space as % of Starch Vol.	Time, sec.
2000	3.36	75	50
4000	3.20	71	60
5000	2.95	66	30
7000	1.95	43	53
10000	1.78	39	63
13000	0.97	21	64
15000	0.46	10	60

Tablets prepared from equal weights of different sizes of crystals and compressed to an equal volume should exhibit the same amount of void space, although the size of individual pores may differ substantially. A good uniformity of void volume was found in such a series prepared with 5% starch. The results are reported in Table III representing the averages of four tablets in each group.

Since the void volumes are substantially the same, the size of the openings between particles must get smaller as the crystal sizes get smaller and the number of crystals per tablet increases. This would lead either to the presence of interparticle spaces too small to permit ready entry of water or to discontinuities between starch grains in the spaces. Either circumstance would explain the longer disintegration time and the change in mode of disintegration for tablets of small crystal size.

The average volume of starch in the tablets was 0.00984 ml. which on swelling by the 78% indicated in water suspension would increase by 0.00768 ml. The increase is less than the void volume but, since disintegration requires only that the aspirin

[1] The void space calculated in this manner may not accurately represent the spaces between crystals since crushing of crystals during compression may lead to formation of new spaces in which no starch will be found. However, it will represent a good first approximation of the space into which water can penetrate.

particles be forced apart from each other, the effect could be exerted by starch grains properly located between aspirin particles even though empty spaces remained in the tablet structure. This suggests that an optimum ratio of starch volume to void space may exist at which disintegration would be most effective. In an attempt to find such a ratio, a series of tablets was prepared using 10% starch and $^{40}/_{60}$ aspirin crystals. Flat-faced tablets were prepared using a Carver hydraulic laboratory press to supply the compression force. The material for each tablet was individually weighed, placed in the die, and compressed. The pressures recorded were the dial readings of the press. The tablets were weighed and measured to determine void spaces and their disintegration times then determined. The averages obtained on six tablets at each pressure are reported in Table IV.

The disintegration times were too close together to detect any real minimum due to starch–void ratios. It was surprising to find that even at the highest pressures used, when void spaces were substantially eliminated, a rapid disintegration was still effected. This would indicate that as long as the starch grains were in continuous contact with each other their affinity for water would draw it into the tablet without regard to pore size. The three hardest tablets in the series (those with times of 10 sec.) showed a change in the mode of disintegration. They disintegrated from the outer sides toward the center.

If an optimum ratio of starch volume to void space did exist, it should be most readily detected when the channels were not completely filled with starch grains. To check this point, a series of flat-faced tablets was prepared from $^{40}/_{60}$-aspirin crystals with 5% starch. The results on six tablets at each pressure are recorded in Table V.

The decrease in disintegration time at 5000 lb. of pressure would seem to indicate the most favorable combination of conditions for this particular series. In each case the calculated increase in starch volume is larger than the total void space.

DISCUSSION

The increase in volume of starch in water strongly suggests that this is the principal mechanism for its action as a disintegrating agent. The disintegration of starch-containing tablets of aluminum hydroxide in water but not in ethanol and of aspirin tablets in water but not in glycerin adds support to this view. Where contact of starch grains in the interparticle spaces is continuous, disintegration is most rapid and is only slightly affected by changes in the amount of interparticle void space. In this situation, even when void space is substantially eliminated, starch is capable of drawing water into the interior of the tablet and producing rapid disintegration. When contact of starch grains in the interparticle channels is not continuous, the situation changes. The time for disintegration increases and the mode of disintegration changes. The appearance of a minimum disintegration time produced in aspirin tablets containing 5% starch in discontinuous contact might be attributed to two factors under the experimental conditions used. Berry and Ridout (7) have suggested that decreases in disintegration time would be produced by increasing contact between tablet particles and starch grains. This appears to be the case until a minimum time is reached. At this point, the second factor, a limit to the pore size which will allow entry of water into the tablet, begins to operate. A smaller pore size which could be occluded by air would hinder entry of water as was demonstrated by Wurster and Seitz (8). This would be particularly true in the case of a hydrophobic substance such as aspirin. In this situation, disintegration of the tablet would be slowed but could continue, either as the pores are enlarged by expanding starch grains near the surface of the tablet, or as diffusing water vapor progressively reaches and swells starch grains deeper in the interior of the tablet. Capillarity *per se* does not appear to have a disintegrating effect, although it may be a factor in aiding the entry of water into the tablet when the pore size is favorable.

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Interaction of Nonionic Hydrophilic Polymers with Phenols I

Interaction of Phenol and Hydroxyphenols with Certain Macromolecules

By B. N. KABADI* and E. ROY HAMMARLUND

There have been numerous studies of the interactions of certain phenols with nonionic macromolecules—particularly those dealing with a loss of antimicrobial properties of the phenols when combined with nonionic polymers. Relatively little attention, however, has been given to the possible disruption of the solubilizing and stabilizing properties of the polymers themselves when in combination with certain phenols. Miscibility titration studies were made by interacting the following polymers: PEG, PPG, PVP, and methylcellulose with the following phenols: catechol, pyrogallol, resorcinol, hydroquinone, tannic acid, and phenol. Photometric turbidimetric titration studies of interacting polyoxyethylene ethers, polyoxyethylene monostearates, and polysorbates with tannic acid were also carried out. Dextrose altered the complexing tendency of tannic acid by decreasing the solubility of tannic acid in PEG 6000 and increasing its solubility in PVP. Since tannic acid was found to interact strongly with nonionic hydrophilic polymers, its possible influence upon the stability of various pharmaceutical formulations containing nonionic hydrophilic polymers should not be overlooked.

INVESTIGATION has indicated that phenolic materials interfere with various nonionic hydrophilic polymers which are employed frequently in pharmaceutical and cosmetic formulations as solubilizing, stabilizing, or emulsifying agents (1–6). The interactions of various nonionic hydrophilic polymers and phenols have been the concern of many further studies (7–14), usually undertaken to explore the mechanisms of the various interactions and thereby make possible the prediction of their probable occurrence. Most prior work has focused upon the interference of the complex with the antimicrobial properties of the phenol rather than upon its interference with the solubilizing and stabilizing properties of the polymer. The present inquiry investigated the nature of the inactivation of certain nonionic polymers by various widely occurring hydroxyphenols which may be found in many pharmaceutical formulations that contain plant products. Methods employed were visual turbidimetric titration and photometric turbidimetric titration. Results are presented in the form of phase diagrams.

EXPERIMENTAL

Materials.—The nonionic polymers used were: polyethylene glycol (PEG) 1500, 1540, 4000, 6000, and 20,000; polyvinylpyrrolidone (PVP); polypropylene glycol (PPG) 400 and 1200; polysorbate 20, 40, 60, and 80;[1] polyoxyethylene monostearate;[2] polyoxyethylene ethers;[3] alkyl phenoxy polyethoxy ethanol;[4] methylcellulose 15 cps. All polymers were commercial samples. The phenols employed were phenol, resorcinol, tannic acid, catechol, pyrogallol (all reagent grade), and hydroquinone (technical grade).

Visual Turbidimetric Titration Method.—The experimental technique was similar in principle to that used by Higuchi and co-workers (11, 12), Ahsan *et al.* (15), Marcus *et al.* (16), and Karabinos *et al.* (17) in their studies of the interaction of phenols with macromolecules. One to 10 ml. of the polymer solutions was added to a series of 50-ml. tubes and each volume was adjusted to 10 ml. with distilled water. The phenol solution was added dropwise to the continuously stirred polymer solution until a distinct turbidity marked the end point of the titration. The same operation was repeated on each tube containing various dilutions of the polymer solutions.

The phenol and hydroxyphenol solutions were 6% w/v and the tannic acid solution was 1% w/v, except for the methylcellulose titration in which 0.1% tannic acid was used. The concentrations of the polymer solutions were: polyethylene glycol[5] 1500, 1540, 4000, 6000, and 20,000, 20.0% w/v; polyvinylpyrrolidone,[6] 4.0% w/v; methylcellulose, 15 cps., 1.0% w/v; polypropylene glycol,[7] 1200, 0.2% w/v; and polypropylene glycol,[7] 400, 20.0% w/v.

From the concentration and volume of the poly-

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[1] Marketed as Tween 20, 40, 60, and 80 by Atlas Chemical Industries, Inc., Wilmington, Del.

[2] Marketed as Myrj 51 and 52 by Atlas Chemical Industries, Inc., Wilmington, Del.

[3] Marketed as Brij 35, 56, 58, 76, 78, 96, and 98 by Atlas Chemical Industries, Inc., Wilmington, Del.

[4] Marketed as Triton X-100 by Rohm and Haas Co., Philadelphia, Pa.

[5] Marketed as Carbowax by Union Carbide Chemical Co., New York, N. Y.

[6] Marketed by K & K Laboratories, Inc., Plainview, N. Y.

[7] Marketed by J. T. Baker Chemical Co., Phillipsburg, N. J.

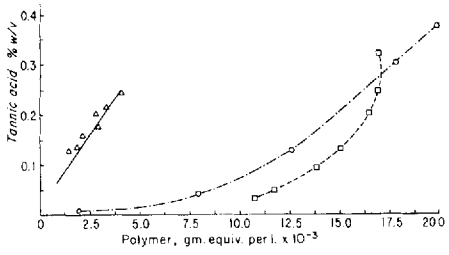

Fig. 1.—Influence of several polymers on the solubility of tannic acid. Key: △, PVP; ○, PEG 6000 □, PPG 400.

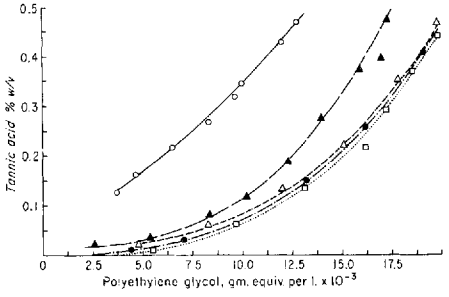

Fig. 2.—Influence of several PEGs on the solubility of tannic acid. Key: ○, PEG 20,000; ▲, PEG 1540; △, PEG 4000; ●, PEG 6000; □, PEG 1500.

mer solutions used the corresponding number of monomer-gram equivalents of polymer per liter was calculated (except for methylcellulose) and this value was plotted *versus* the final concentration of the phenolic solution in the mixture at the end point. The effect of dextrose on the interaction of PEG 6000 and PVP with tannic acid was likewise determined. The procedure used was the same as above except that the polymers were dissolved in 1 *M* aqueous dextrose solutions instead of in water. The data thus obtained from the titrations of the various polymers and phenols are plotted in the form of phase diagrams (Figs. 1–6).

Photometric Turbidimetric Titration Method.—The nonionic polymer solution was added slowly to a constantly stirred 10-ml. volume of 0.1% w/v tannic acid solution in a 50-ml. flask. At the first sign of turbidity an aliquot solution was removed, and its absorbance was measured at 625 mμ in a Bausch & Lomb Spectronic 20 colorimeter. The 0.1% tannic acid solution was used as the blank. Following this initial reading the aliquot was returned to the titration vessel, and more polymer solution was added to the mixture in 0.2-ml. increments. Absorbance readings were repeated until the solution in the titration flask became transparent (zero absorbance) which took about 0.5 hr. This titration procedure was carried out for each of the nonionic polymers tested. The solution absorbance was plotted *versus* the total volume of the polymer solution added. Figures 7–9 show the data thus plotted.

RESULTS AND DISCUSSION

Figures 1–6 show the phase diagrams of the complexation reactions between hydroxyphenols

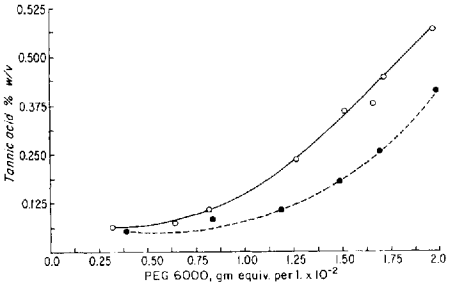

Fig. 3.—Effect of dextrose on the solubility of tannic acid in PEG 6000. Key: ○, PEG 6000 in water; ●, PEG 6000 in dextrose (1 *M*).

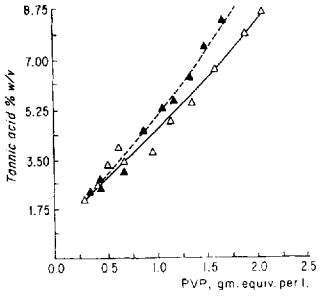

Fig. 4.—Effect of dextrose on the solubility of tannic acid in PVP. Key: △, PVP in water; ▲, PVP in dextrose (1 *M*).

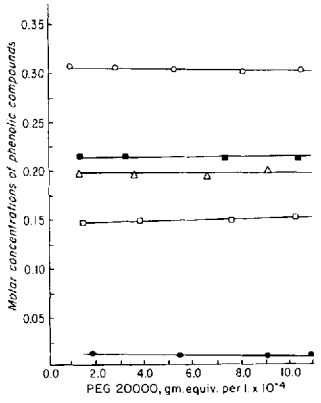

Fig. 5.—Effect of PEG 20,000 on the apparent solubility of phenol and polyhydroxy phenols. Key: ○, pyrogallol; ■, catechol; △, hydroquinone; □, resorcinol; ●, phenol.

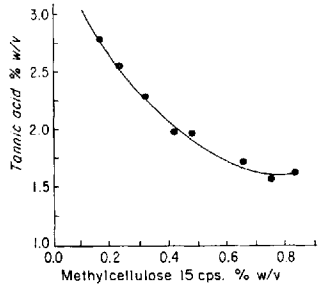

Fig. 6.—Influence of methylcellulose on the solubility of tannic acid.

and various hydrophilic polymers. In all these diagrams the points at low concentrations were less precise due to difficulty in discerning the end point. A heterogeneous system consisting of the white, oily complex dispersed throughout the aqueous phase existed at concentrations above any given curve. The mixtures were clear below each curve. The graphs in Figs. 1–5 have positive slopes which indicate that the insoluble complex was solubilized by an excess of uncomplexed polymer solution. In Fig. 6 the negative slope in the phase diagram of the methylcellulose was identical to that obtained from the interaction between phenol and PPG 1200 by Guttman and Higuchi (11). Likewise, the graph was similar to those which show interaction between phenol and certain surfactants below their critical micelle concentrations (CMC) (15, 16). For precipitation to occur, any decrease in the concentration of one reactant requires a corresponding increase in the concentration of the other. The negative slopes in this type of phase separation appear to be characteristic of polymers with negative temperature coefficients of solubility.

Since dextrose occurs as one of the products of tannic acid hydrolysis, its effect on the solubility of tannic acid in hydrophilic polymers was studied (Figs. 3 and 4). Dextrose was found to decrease the solubility of tannic acid in PEG 6000 and to increase the solubility of tannic acid in PVP.

The order of phenolic concentrations required for precipitation by PEG 20,000 (Fig. 5) was found to be phenol < resorcinol < hydroquinone < catechol < pyrogallol. Guttman and Higuchi (11) reported the sequence with PVP to be resorcinol < hydroquinone < phenol < catechol. No simple explanation appears to be evident for the discordant position of phenol in these two series.

In Figs. 7 and 8 there is a sharp increase in absorbance at a critical polymer concentration for all of the systems. This is due undoubtedly to a sudden increase in aggregation of the tannin–polysorbate complex above the CMC of the surfactant solutions. The absorbance reached a maximum and then began to decrease as the oily complex which formed became solubilized in the excess of polymer solution. A rapid decrease in the absorbance of the system resulted. Although the concentration of the surfactant at the point of the sudden increase in absorbance differs from its CMC values, the marked change in turbidity may be related to micelle formation in the complex system.

Figure 7 shows that each of the polysorbates produced a turbidity upon the addition of tannin. Figure 8 shows the absorbance breaks at different fixed concentrations of polysorbate 20 upon titration with tannic acid solution. Because of the marked differences between the graphs of the separate polysorbates, the photometric turbidimetric titration method employing tannic acid was found to be a useful method for distinguishing qualitatively between these surfactants. However, with a series of polyoxyethylene ethers having the same aliphatic chain length but with different size glycol chain lengths, there appeared to be no correlation between the concentration of the surfactants at their absorbance discontinuities and the monomer content of the surfactants.

The shapes of the titration curves of the polyoxyethylene ethers were nearly identical to those of the polysorbates which are shown in Figs. 7 and 8—although the sudden absorption increase at some critical point was not so sharp with the ethers as with the polysorbates. Moreover, aqueous solutions of polyoxyethylene ethers (No. 30, 42, 52, and 72) were too turbid initially to determine

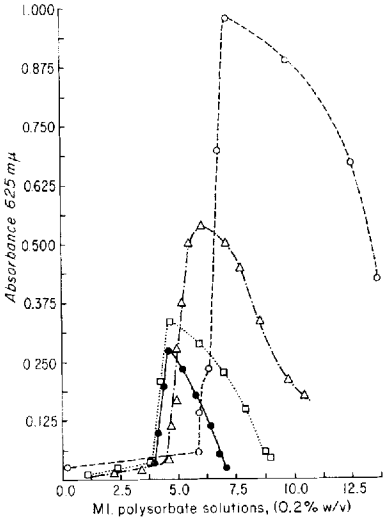

Fig. 7.—Photometric turbidimetric titration curves of polysorbates interacted with tannic acid. Key: O, polysorbate 20; △, polysorbate 40; □, polysorbate 60; ●, polysorbate 80.

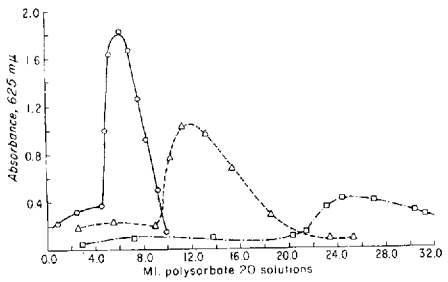

Fig. 8.—Photometric turbidimetric titration of tannic acid with various concentrations of polysorbate 20. Key: O, 0.4% w/v; △, 0.2% w/v; □, 0.1% w/v.

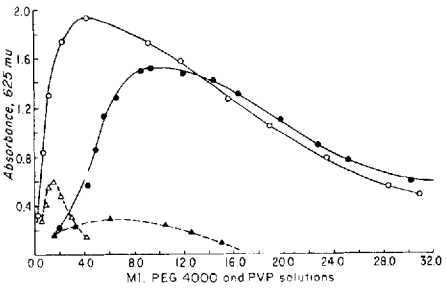

Fig. 9.—Photometric turbidimetric titration of tannic acid with PEG 4000 and PVP. Key: O, 0.2% w/v PEG 4000; ●, 0.5% w/v PEG 4000; △, 0.4% w/v PVP; ▲, 0.6% w/v PVP.

any further change in their absorbance properties. The polyoxyethylene monostearates and alkyl phenoxy polyethoxy ethanol did not show any abrupt increase in absorbance at a critical point above their CMC because they produced uniformly turbid mixtures. This was due possibly to a lack of purity of the commercial products. Polyethylene glycol and PVP polymers, which do not exhibit a CMC, gave smooth curves as expected without any breaks. Their graphs are shown in Fig. 9.

Although the interaction or binding of tannin as such may not necessarily be detrimental to pharmaceutical products, the fact that tannin occurs in numerous pharmaceutical formulations of natural origin could cause frequent unexpected incompatibilities if surfactants were added or whenever these products were mixed with other preparations containing surfactants. The decrease in the concentration of the stabilizers or solubilizers through their binding with tannin will obviously decrease the effective concentration of the stabilizing agent which may lead to the ultimate breakdown of some liquid pharmaceutical products. Except by complete separation of the ingredients, the only way that this incompatability may be overcome is by the addition of excess dispersing agent (18). Allawala and Riegelman (19) have pointed out that for optimum conditions of preservative effectiveness, the ratio of phenol to surfactant should be at a minimum. Therefore, in preserved tannin-containing pharmaceuticals this incompatibility with surfactants should be considered.

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By B. N. KABADI* and E. ROY HAMMARLUND

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ALTHOUGH THE interactions of nonionic polymers, e.g., polyvinyl pyrrolidone (PVP) and polysorbate 80, with various phenolic derivatives have been investigated rather extensively, the analogous reactions of polyethylene glycols (PEG) with phenols have received much less attention (1–4). Higuchi and co-workers (5, 6) have studied the interaction of PEG's and barbiturates and iodine in potassium iodide solution. They demonstrated that at a high cosolute concentration a complex formed which had a stoichiometric ratio of 2 base moles of ethylene oxide units (ETO) of the glycol to each cosolute molecule.

Various attempts to determine the mechanism of such interactions and the stoichiometric relationships of the complexes formed by interaction

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Abstracted in part from a thesis submitted by Balachandra Kabadi to the Graduate School, University of Washington, Seattle, in partial fulfillment of Doctor of Philosophy degree requirements.
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Previous paper: Kabadi, B. N., and Hammarlund, E. R., *J. Pharm. Sci.*, **55**, 1069(1966).

any further change in their absorbance properties. The polyoxyethylene monostearates and alkyl phenoxy polyethoxy ethanol did not show any abrupt increase in absorbance at a critical point above their CMC because they produced uniformly turbid mixtures. This was due possibly to a lack of purity of the commercial products. Polyethylene glycol and PVP polymers, which do not exhibit a CMC, gave smooth curves as expected without any breaks. Their graphs are shown in Fig. 9.

Although the interaction or binding of tannin as such may not necessarily be detrimental to pharmaceutical products, the fact that tannin occurs in numerous pharmaceutical formulations of natural origin could cause frequent unexpected incompatibilities if surfactants were added or whenever these products were mixed with other preparations containing surfactants. The decrease in the concentration of the stabilizers or solubilizers through their binding with tannin will obviously decrease the effective concentration of the stabilizing agent which may lead to the ultimate breakdown of some liquid pharmaceutical products. Except by complete separation of the ingredients, the only way that this incompatability may be overcome is by the addition of excess dispersing agent (18). Allawala and Riegelman (19) have pointed out that for optimum conditions of preservative effectiveness, the ratio of phenol to surfactant should be at a minimum. Therefore, in preserved tannin-containing pharmaceuticals this incompatibility with surfactants should be considered.

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of phenols and PEG derivatives have resulted in the appearance of contradictory statements in the literature (7–10). These are due largely to the many experimental difficulties encountered and the insufficiency of reproducible experimental evidence. Some of the difficulties which have prevented the determination of a definite stoichiometry of the formed complexes are: (*a*) the oily insoluble nature of the precipitate prevents the use of the conventional solubility titration method; (*b*) commercial nonionic surfactants are not pure products; (*c*) above their CMC's surfactants form micelles, and because of their solubilizing potential, these micelles would interfere with the isolation of a complex for study.

To avoid most of these difficulties this current study was limited to the PEG's because they are more homogeneous than the surfactants and they do not form micelles in water.

The object of this study was to investigate the general nature of the interaction between phenol and PEG and to attempt to determine the stoichiometry of the resulting complex by correlating equilibrium dialysis data of the reaction mixtures with NMR data obtained from the isolated oily complex. A literature search failed to reveal that NMR data had been employed previously in this type of an investigation on PEG–phenol complexes.

PEG 20,000 was chosen for this study for two reasons: (*a*) previous reports indicated that the low molecular weight PEG's did not form complexes, whereas the higher molecular weight PEG's did (5, 9, 11, 12); (*b*) PEG's with molecular weights of 6000 and less permeated cellulose dialyzing membranes, whereas PEG 20,000 did not, thus equilibrium dialysis could be used as an analytical tool (1).

EXPERIMENTAL METHODS AND RESULTS

Reagents

Phenol, *p*-chlorophenol, pyrogallol, phloroglucinol, and resorcinol (all reagent quality); PEG 300, 400, 1500, 6000, and 20,000 (commercial products).

Preparation of PEG 20,000 for Dialysis.—A 4.0% w/v commercial PEG 20,000 polymer solution was placed in Visking,[1] seamless, cellulose, dialyzing tubes which were then tied securely at both ends and dialyzed at room temperature against a large volume of distilled water. The dialysate was replaced with fresh distilled water at 4-hr. intervals, and the dialysis was continued until there was no frothing in the dialysate solution. This procedure usually took about 5 days. The dialyzed PEG 20,000 solution was dried under reduced pressure. The dried residue was powdered and this was employed in the equilibrium dialysis study. The yield was approximately 90%.

[1] Union Carbide Corp., Visking Division, Chicago, Ill.

Equilibrium Dialysis Method.—The equilibrium dialysis technique used in the present study was essentially the same as that used by Patel *et al.* and others (1, 3, 13). The phenols were estimated quantitatively by using a Beckman DU spectrophotometer. The wavelengths used for the various assays were: phenol, 270 mμ; resorcinol, 275 mμ; pyrogallol, 264 mμ; phloroglucinol, 267 mμ; and *p*-chlorophenol, 272 mμ.

Twenty milliliters of 0.5% w/v of the glycol polymer solution under study was placed in a series of cellophane bags. Each was then tightly tied and placed in a 125-ml. glass-stoppered flask containing 20 ml. of various concentrations of phenol solution ranging from quite dilute to saturated solutions. The control contained water in place of PEG solution. The flasks were agitated for 18 hr. at a constant temperature of 5°. Preliminary experiments had shown that equilibrium was reached under these conditions. The mixtures in the dialysis bags developed various degrees of cloudiness ranging from slightly turbid to extremely opaque, oily emulsions. The amount of phenol in the dialysate solutions was determined spectrophotometrically from its molar absorptivity, and the bound phenol

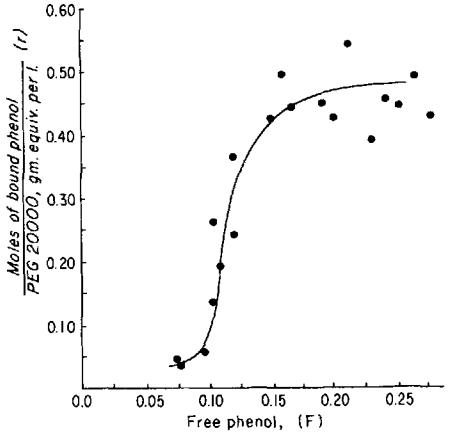

Fig. 1.—Interaction of phenol and PEG 20,000.

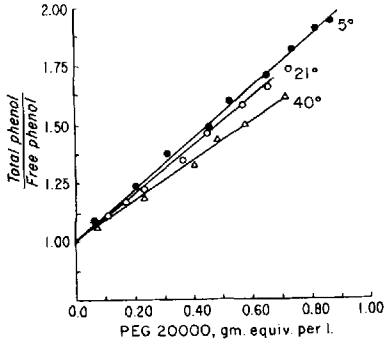

Fig. 2.—Effect of temperature on the binding of phenol and PEG 20,000.

concentration was calculated by difference from the control blank.

The data for the various concentrations of phenol bound to PEG 20,000 are plotted in the form of a Langmuir curve as shown in Fig. 1. At the lower concentrations the data were fairly reproducible; however, at the saturated concentration of phenol the points designating the amount of bound phenol were scattered. This same experiment was repeated using *p*-chlorophenol, and its Langmuir isotherm was found to be of the same shape as that of phenol in Fig. 1.

Effect of Temperature on Binding of Phenol by PEG.—The equilibrium dialysis experiment was carried out at different temperatures by keeping the phenol concentration constant and varying the concentration of the polymer. The results are plotted in Fig. 2 which shows that an increase in temperature decreased the degree of binding only slightly. This was due possibly to disruption of hydrogen bonding between the phenolic hydroxyl and the ether oxygen of the glycol.

Binding of PEG and Certain Hydroxyphenols.—The equilibrium dialysis method was used to study

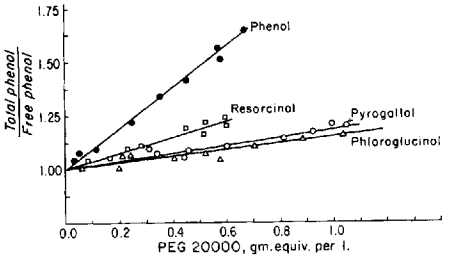

Fig. 3.—Binding of certain phenols with PEG 20,000.

the binding of certain hydroxy phenols with PEG. The data are plotted in Fig. 3. One might expect that as the number of the phenolic hydroxyl groups increased with a corresponding increase in the acidity, the phenols and PEG would interact more strongly. However, the amount of interaction in this case was found to be less, indicating that some others factors were involved. It appears likely that not all of the hydroxyl groups of the phenols are involved directly in complexation with polyglycols, and it is also possible that the degree of hydration of the various phenols may influence the complex formation. Furthermore, phloroglucinol, unlike the other phenols studied, did not precipitate PEG solution in any concentration indicating a lack of "squeezing out" action of the phloroglucinol–PEG complex from water.

Nuclear Magnetic Resonance Studies

The complexes of the various phenols and PEG 20,000 were difficult to work with because of their sticky nature and low solubility in water. The lack of light absorption of the PEG prevented the use of this analytical technique. Furthermore, the quantitative dialysis method could not be used to study the interaction of the low molecular weight

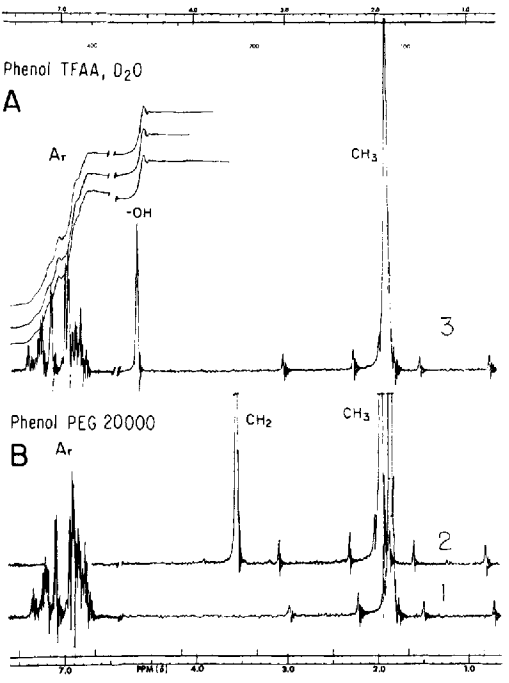

Fig. 4.—NMR spectra of phenol and PEG 20,000 in acetonitrile containing 1% TMS. Key: B1 and A3, phenol; B2, PEG 20,000.

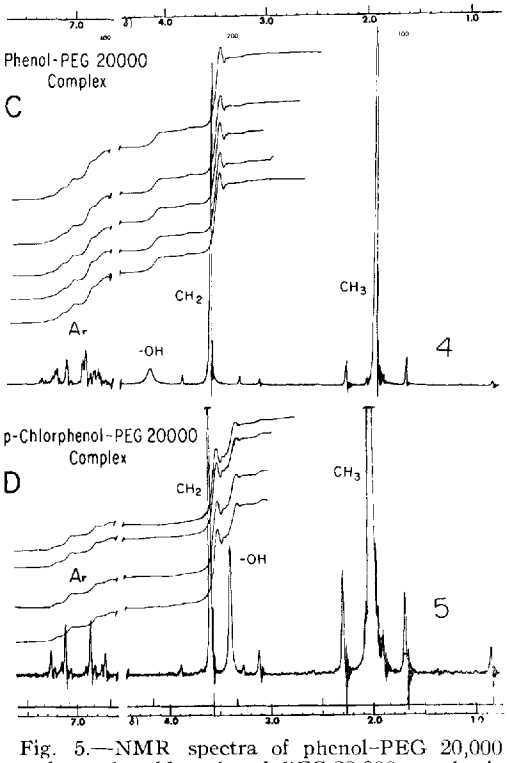

Fig. 5.—NMR spectra of phenol–PEG 20,000 complex and *p*-chlorophenol–PEG 20,000 complex in acetonitrile containing 1% TMS. Key: C4, phenol–PEG 20,000 complex; D5, *p*-chlorophenol–PEG 20,000 complex.

PEG's because they permeated the membranes. These difficulties were overcome by utilizing nuclear magnetic resonance (NMR) analyses to ascertain the ratio of PEG protons to phenol protons in the oily complex.

Preliminary Work on Pure Samples.—The NMR spectra were obtained using an analytical high resolution NMR spectrophotometer (Varian Associates A-60). Preliminary NMR spectra of pure phenol in acetonitrile containing 1% tetramethylsilane (TMS) did not indicate a hydroxyl proton, Fig. 4, B1. The addition of traces of D_2O and trifluoroacetic acid (TFAA) to the sample to catalyze the exchange of hydroxyl protons with D_2O produced the desired phenolic hydroxyl proton signal. The spectrum of this mixture is shown in Fig. 4,A. PEG 20,000 was added to this mixture and the NMR data were obtained. The signal from the phenol and PEG hydrogens did not overlap and could be integrated successfully as shown in Fig. 4,B. However, similar studies on phenol–PVP and the various phenol–polysorbate[2] mixtures did not produce conclusive results because of the complexity of their NMR spectra.

Examination of the spectra of several known ratios by weight of phenol and PEG upon integration disclosed that the ratios of the aromatic protons of phenol to the methylene protons of glycol were close to the calculated values within experimental limits. This spectrum is shown in Fig. 5,C. The lowest field signal which appeared as a multiplet centered at about $\Delta = 7$ and was due to the protons attached directly to the aromatic nucleus. The middle signal which appeared as a sharp singlet at $\Delta = 3.56$ was from the protons of the polyoxyethylene chain. The high field signal at $\Delta = 1.9$ was from the methyl protons of the solvent, acetonitrile. The position of the signal of the hydroxyl protons varied between 3 and 5 Δ values depending upon the water content of the sample.

Analyses of Oily Complexes of PEG and Phenol.—The oily complex mixtures formed by the interactions between a high concentration of phenol individually with PEG 6000 and PEG 20,000 each were collected from within the cellophane dialysis bags which had been agitated slowly for about 18 hr. at 5° in a shaker bath. The NMR spectrum of the oily portion was obtained in acetonitrile containing traces of TMS, D_2O, and TFAA, and was found to be identical in all respects to the spectrum shown in Fig. 5,C. Therefore, in this investigation the NMR spectrometry was found to be applicable as an analytical tool for determining the ratio of bound phenol to polymer. The actual structure of the phenol–polymer complex or any changes in its structure in the elected solvent, acetonitrile, have not been investigated. The integrated ratios of the aromatic protons of phenol to the methylene protons of PEG in the oily complex obtained from the different concentrations of phenol were not constant. However, following a thorough washing of the oily complex with distilled water, fairly reproducible ratios were obtained and are shown in Table I. The ratio of the methylene protons to the aromatic protons was found to be between 1.5 and 1.6.

[2] Marketed as Tweens by Atlas Chemical Industries, Inc., Wilmington, Del.

In a study of the low molecular weight PEG's, 1% w/v solutions of the PEG's were added to equal volumes of 7% phenol solutions and kept overnight in a separator. Oily complexes formed in the lower layer in each funnel and the NMR spectra of the complexes were obtained as previously described both before and after washing with water. It was found that the ratio for PEG 1500 before washing varied considerably from its predicted value (Table I). Likewise, the values for PEG 300 and 400 after washing were not as expected. The remaining ratios came out close to the predicted values. In a similar manner PEG 6000 and 20,000 were interacted with *p*-chlorophenol, and its oily complex was washed with water and gave proton ratios close to the predicted value as seen in Table I.

The water content of the insoluble material varied considerably from sample to sample. The integrated ratio of the water protons was always less than 3 moles considering the ratio of protons of 1 mole of phenol or 2 ETO units of glycol. However, the presence of any water associated with the insoluble complex was not investigated extensively.

TABLE I.—INTEGRATED RATIOS OF METHYLENE PROTON TO AROMATIC PROTON NMR PEAK AREAS FOR PHENOL– AND *p*-CHLOROPHENOL–PEG COMPLEXES

	Methylene Protons/Aromatic Protons (8/5)		
PEG Complexed with Phenol	Before Washing	After Washing	Predicted[a]
200,00	1.509	1.592	1.6
6000	1.470	1.524	1.6
1500	1.24	1.558	1.6
600	...	1.558	1.6
400	...	1.118	1.6
300	...	0.780	1.6
PEG Complexed with *p*-Chlorophenol			(8/4)
20,000	...	2.2	2.0
6000	...	1.7	2.0

[a] Assuming that there were 2 base mole units of ethylene glycol (8 protons) to 1 mole of phenol (5 protons) or *p*-chlorophenol (4 protons).

DISCUSSION

The Langmuir isotherm in Fig. 1 indicated that at low concentrations of free phenol the binding tendency for phenol is rather small. At somewhat higher concentrations of free phenol, the amount of binding depended upon the phenol concentration, and this portion of the curve shows a rapidly increasing slope. At still higher phenol concentrations, the results were not satisfactory because the points were highly scattered. The binding of phenol and PEG as shown by the dialysis data was similar to the binding of phenol by PVP (1) or cetylpyridinium chloride by methylcellulose and polysorbate 80 (14). Deluca and Kostenbauder (14) considered that isotherms such as these sometimes resulted when adsorption of a critical quantity of cosolute caused changes in the configuration of

the tightly coiled polymer molecules resulting in the availability of additional binding sites.

Although the stoichiometric relationship between the ETO of the PEG and phenol was not clearly defined in the dialysis experiments, the behavior of the polymers in the presence of increasing concentrations of phenol were meaningful. Since phenols have a greater tendency for hydrogen bonding than do water molecules (15), the addition of a small amount of phenol to the PEG would lead to the displacement of a few water molecules per polymer. At these low concentrations of phenol, the binding tendency of phenols seems to be comparatively small, and the complex remains in solution. As the ratio of phenol to glycol is increased, phenol would be expected to alter the orientation of the polyphenol complex, and some hydrogen bonded water molecules will be displaced. At the same time the water solubility of the phenol also is reduced by the interaction of the phenolic hydroxyl group with the ETO chains because the hydroxyl group is no longer available for hydrogen bonding with water molecules. This combination of the reduction in solubility of the phenol and the disorganization of the hydrated polymer could explain the precipitation of a hydrated phenol–polymer complex at a critical point. This was found to occur at a very low concentration of phenol.

At the point where the precipitation began to occur, the formation of this precipitate would change convoluted linear polymer chains into a more compressed configuration (16). This rearrangement apparently was initiated by the binding of relatively few phenol molecules which sterically made available many more binding sites for the attachment of additional phenol because this change in state was accompanied by a significant increase in phenol binding, *i.e.*, there was binding of phenol to PEG beyond the point of initial precipitation. Thus, the tendency for an individual phenol molecule to attach itself to a polyether molecule was relatively small, but when several phenol molecules combined with an ether chain, a favorable environment for additional binding was created. As a result more phenol molecules were bound and were held on the complex in the same fashion as with the nonionic surfactant micelles. This phenomenon was seen in the very high phenol region of the dialysis experiment. In this region the oily complex which separated out of the aqueous phase might possibly bind more and more phenol molecules, or the oily colloidal phase may act as a new separate phase which has a considerable attraction to the phenol molecules, and there will be partitioning of phenol between this complex and the aqueous phase. An alternative explanation would be that the oily complex possibly interfered in the equilibrium process of dialysis which caused the points on the graph in Fig. 1 to be scattered over a wider region (17).

Kato (18) has shown the presence of a "micelle-like" structure in simple aqueous solutions of a number of glycols. Thus, the attachment of the aromatic moiety to the water-soluble polyglycol molecules appears to have conferred hydrophobic characteristics to the association complex in proportion to the amount of phenol complexed (19). The solubility of the complex thus would be altered due to binding of phenol to a polymer and the insoluble complex would increase the turbidity which ultimately resulted in the separation of an oily phase when the phenol concentration was high.

Although the commonly used equilibrium dialysis method could not be applied effectively to determine the stoichiometric composition of the complex, a relatively new experimental procedure to this field—the NMR spectrometric method—has been utilized.

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Thin-Layer Chromatography of the Selective Rat Toxicant, Norbormide

By CASIMIR A. JANICKI, RONALD J. BRENNER, and BARBARA E. SCHWARTZ

Norbormide has been reported to be a mixture of stereoisomers. A technique for the qualitative identification and quantitative determination of the major isomers of norbormide has been developed. Norbormide is separated into its isomers on thin-layer plates consisting of 99 per cent Silica Gel G and 1 per cent colloidal alumina, using a solvent mixture of ethyl acetate–chloroform (7:3). The isomers are extracted into 0.1 *M* hydrochloric acid–ethanol (7:1). The isomers are assayed by U. V. absorption or by the use of fluorescence. Some ultraviolet and fluorescence characteristics of the isomers are presented. Data are presented to show the accuracy and precision of the TLC–U.V. method.

NORBORMIDE,[1] 5-(α-hydroxy-α-2-pyridylbenzyl)-7-(α-2-pyridylbenzylidine)-5-norbornene-2,3-dicarboximide, has been found to be a selective rat toxicant (1, 2). As currently synthesized it consists of a mixture of stereoisomers which were separated by a variety of paper and thin-layer chromatography methods (3). These isomers were observed to vary greatly in their toxicity toward rats (4). The isomers have been classified as *cis* (S, T, W, Y) and *trans* (R, X, U, V) and further as endo (U, V, W, Y) and exo (R, S, T, X) (3). Because of this difference in biological activity, it was necessary to develop a method which would separate the isomers so that a quantitative assay of each could be carried out. Thin-layer chromatography was chosen because of the speed and sharpness of separations possible.

A thin-layer separation technique is described which separates norbormide into three isomers, designated as X, W, and Y, a mixture of two isomers Z, and a by-product in the synthesis, 7-(α-2-pyridylbenzylidine)-5-norbornene-2,3-dicarboximide (McN-1392). The mixture of the two isomers known as Z is separated further into its two isomers U and V by a second thin-layer chromatographic step. As much as 1 mg. of norbormide has been quantitatively separated into five of the eight possible racemates. Spectrophotometric and spectrofluorometric assays of the separated isomers are described.

EXPERIMENTAL

Apparatus.—The Desaga-Brinkmann complete basic equipment No. 600[2] for TLC was used for the preparation of plates. Glass plates were 2 × 8 in. A Beckman DK-2A was used for the ultraviolet absorption measurements and an Aminco-Bowman spectrophotofluorometer No. 4-8106 for fluorescence measurements.

Reagents.—Analytical reagent grade or equivalent grade reagents were used in the study. Silica Gel G was obtained from Brinkmann[2] and colloidal alumina,[3] technical grade, from E. I. Dupont de Nemours and Co., Inc.

Preparation of Plates.—A slurry of 4 Gm. of colloidal alumina and 100 ml. of water was prepared. The slurry was diluted 10 to 70 ml. with methanol. To the methanol slurry was added 40 Gm. of Silica Gel G, and the resulting slurry mixed to a uniform consistency. The glass plates were covered with a 250-μ layer of adsorbent using a fixed thickness spreader. The plates were air-dried for an hour and then at 70° for 30 min. The plates were stored in a storage rack at room temperature, 50% relative humidity, and used without any prior activation. The addition of the colloidal alumina in the silica gel provides an excellent binding with the glass surface. The use of colloidal alumina as a binder has recently been published (5).

Standard Solutions.—Solutions of the isomers of norbormide and McN-1392 were prepared by dissolving accurately weighed amounts in a 1:1 chloroform–methanol solution, and taking to a known volume. Standard solutions for the spectrophotometric analyses of the isomers were prepared by dissolving accurately weighed amounts in ethanol, and taking to volume with 0.1 *M* hydrochloric acid. The final solution was 7:1 of 0.1 *M* hydrochloric acid–ethanol.

Sample Solutions.—Samples of norbormide, norbormide isomers, and isomer mixture Z were dissolved in a 1:1 chloroform–methanol solution to obtain a concentration of 8 mg./ml. McN-1392 was dissolved to give a concentration of 2 mg./ml.

Thin-Layer Method.—Solutions of the isomers of norbormide, McN-1392, and samples of commercial norbormide were applied to the silica plate about 2 cm. from the starting edge using a Hamilton syringe pipet. For the qualitative studies from 20 to 100 mcg. of sample was applied to the plate, using a gentle stream of air to keep the spots less than 5 mm. in diameter. A maximum of 5 spots was applied across a single plate. In the quantitative studies from 800 to 1000 mcg. of norbormide was applied in a series of spots resulting in a band whose width did not exceed 5 mm.

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[1] Norbormide has been designated the American Standards Association common name.

[2] Brinkmann Instruments, Inc., Westbury, N. Y.

[3] Trademarked as Baymal.

The silica plates were developed using the ascending technique in glass jars. No paper wicks were used in the jars. Development was allowed to continue until the solvent front reached within 1 in. of the top of the plate. The plates were removed, air-dried for 5 min., and placed back into the jars for another solvent pass with the same solvent. The solvent system for the initial separation of norbormide was chloroform–ethyl acetate (7:3). For the separation of the isomer mixture Z into isomers U and V, a solution of ethyl acetate–butyl ether–acetic acid (15:5:1) was used for a total of six solvent passes. *n*-Butyl acetate can be substituted for ethyl acetate with identical results.

The separation of isomer mixture Z into isomers U and V was checked by paper chromatography (3). The separated isomers were spotted on Whatman No. 1 paper which was then sprayed with the aqueous phase of the mixture *n*-butanol–*n*-butyl-acetate–hydrochloric acid–water (75:25:6:100). The chromatogram was placed into a tank, equilibrated with the aqueous phase, and developed for 40 hr. with the organic phase of the solvent mixture.

Detection of the Isomers.—Shortwave ultraviolet light was used to locate the position of the stereoisomers on paper or silica plates. Iodine vapor or Dragendorffs' reagent can be used to detect the isomers visually on the silica plates.

RESULTS

Qualitative Analyses of the Stereoisomers.—Using the solvent system given previously for the initial separation of norbormide, commercial samples of norbormide were found to separate into 5 spots. In the order of increasing distance from the origin they were: mixture Z, isomer Y, isomer W, McN-1392, and isomer X. Further development runs did not separate mixture Z into its components. When a solvent system of chloroform–ethyl acetate (7:3) was used and 6 to 8 solvent passes, Z did separate into the isomers U and V. As the concentration of Z increased from 50 to 300 mcg. the front running spot tailed into the back spot finally resulting in a single spot. The mixture Z was separated using the butyl ether, ethyl acetate, acetic acid solvent mixture described previously. The order of separation in increasing distance from the origin was U, V. Table I lists the average distances traveled from the origin by the isomers separated from norbormide. R_f values were not calculated because of the multiple solvent pass technique. The values of the distances traveled represent the average of 40 separations of various batches of norbormide. The values given for U and V are those obtained from the separation of isomer mixture Z. Isomers R, S, and T were not found in any significant amounts in norbormide. Isomers R, S, and T could not be separated from the other isomers by the given TLC method but were separated on cellulose plates prepared with microcrystalline cellulose superfine[4] using the organic phase of the mixture *n*-butyl alcohol–hydrochloric acid–water (100:6:100) as the solvent system (3). The order of separation in increasing distance from the origin was W, V, U, and Y, R, and S, T, X.

TABLE I.—RELATIVE DISTANCES FROM THE ORIGIN OF THE ISOMERS OF NORBORMIDE AND MCN-1392 IN mm.

Sample	Isomer	Relative Distance, mm.
Norbormide	X	91 ± 4
	McN-1392	85 ± 4
	W	80 ± 4
	Y	73 ± 3
	Z	62 ± 4
Isomer mixture Z	V	78
	U	53

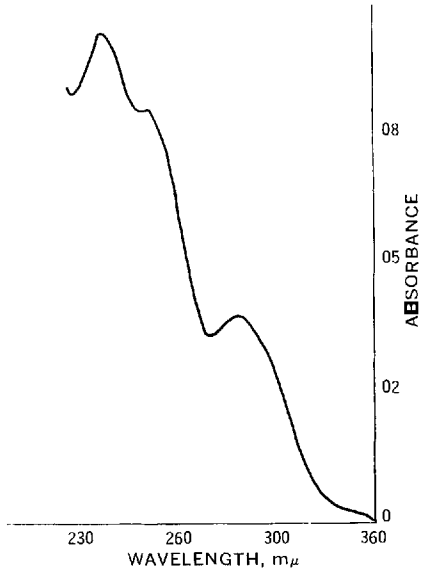

Fig. 1.—Ultraviolet absorption spectrum of norbormide in 0.1 *M* hydrochloric acid–ethanol (7:1).

Quantitative Analyses of Norbormide.—With successful resolution of norbormide into its isomers and McN-1392, the investigations were extended to studies of the resolution of mixtures for quantitative analyses. A solution containing known amounts of isomers was chromatographed, examined under ultraviolet light, and the bands outlined carefully with a sharp stylus. The adsorbent within the outlined area was scraped with a spatula onto glassine paper. The area was dry-washed with a small amount of silica gel, adding the wash to the sample. The adsorbent was transferred to a 1-oz. bottle fitted with a screw cap containing a plastic liner. An amount of 0.1 *M* hydrochloric acid–ethanol (7:1) solution was pipeted into the bottle, to obtain an approximate concentration of 0.02 mg./ml. The samples were shaken on a mechanical shaker for 1 hr., centrifuged, and the clear supernatant liquid transferred to a clean container.

Commercial precoated plates of Silica Gel G which did not contain the colloidal alumina were tried. The quantitative separations of both norbormide and norbormide isomer mixture Z were very unsatisfactory. No attempt was made to prepare TLC plates of Silica Gel G without the colloidal alumina.

Spectrophotometric Assay. The ultraviolet absorption spectrum of each sample was recorded from 360 to 230 mμ in 1-cm. cells using 0.1 *M* hydrochloric acid–ethanol (7:1) as the reference solution.

[4] Marketed as Avicel by American Viscose Division, FMC Corp., Marcus Hook, Pa.

TABLE II.—SOME ULTRAVIOLET CHARACTERISTICS FOR THE NORBORMIDE ISOMERS AND McN-1392 IN 0.1 *M* HYDROCHLORIC ACID–ETHANOL (7:1)

Sample	Max., mμ	mcg./ml./ΔA	ε	Max., mμ	ε	Absorbance Characteristic Near 260 mμ
R	295		5600	236	15,800	A peak
S	291		5200	232	13,700	Plateau
U	303	78.622	6700	239	20,800	Break
V	302	69.268	7000	239	19,300	Peak
W	300	65.329	8500	238	19,100	Break
X	295	90.073	6000	238	19,500	Break
Y	300	86.595	6400	236	13,400	Peak
Z	302	76.284	6900	238	19,500	Plateau
McN-1392	302	46.394	7500	238	17,200	Continuous

For each isomer an ultraviolet absorption curve was recorded. Approximately the same amount of Silica Gel G as in the sample was added to a volume of standard isomer solution identical to the volume of the sample. For a 1-mg. sample of norbormide 8, 4, 10, 16, and 4 ml. of the solvent was used for isomers W, X, Y, mixture Z, and McN-1392, respectively. The silica was added to the standards to match the composition of the sample solutions as closely as possible. The concentration of the isomers and McN-1392 in per cent by weight was determined by the following equation:

$$\% \text{ isomer} = \frac{(\text{mcg./}\mu\text{l./}\Delta A)_{\text{std.}} \times \Delta A_{\text{sample}} \times \text{dilution factor} \times 100}{\text{sample wt., mcg.}}$$

where ΔA, the corrected absorbance, is the absorbance of the maximum near 300 mμ minus the absorbance at 350 mμ. The ultraviolet absorption spectrum of norbormide at a concentration of 0.025 mg./ml. (1-cm. cells) in 0.1 *M* hydrochloric acid–ethanol (7:1) is given in Fig. 1. Absorption maxima are located at 300 and 238 mμ. The spectra of the individual isomers of norbormide are of the same general nature as that shown in Fig. 1. The maximum near 300 mμ was chosen to measure the concentration of norbormide since it is least affected by impurities one may encounter in a silica plate. The ultraviolet background absorbance of silica in the hydrochloric acid–ethanol solvent is linear from 360 to about 280 mμ. Since the background may vary, the absorbance near 300 mμ may be corrected for the background by subtracting the absorbance at 350 mμ.

For the purposes of identity a summary of some of the major ultraviolet absorption characteristics in 0.1 *M* hydrochloric acid–ethanol (7:1) is presented in Table II. The exo isomers, R, X, and S, have lower ε values for the higher wavelength maxima which are near 295 mμ than the endo isomers, U, V, W, and Y, at their maxima near 300 mμ. The values obtained for the factor mcg./ml./ΔA for some of the isomers are also given in Table II. They were used in the actual calculations for determining the isomer percentages given in other tables.

To test the accuracy of the assay method, three samples containing weighed amounts of the isomers W, X, Y and isomer mixture Z were assayed. The results of the assays are presented in Table III. Mixture *3* was assayed 3 times. In each case the recovery of X from mixture *3* was high. In general the recoveries of the isomers are good. Standard deviations were calculated in the usual manner.

Two samples of different lots of norbormide were assayed 6 times to test the precision of the method. The data are presented in Table IV. The 95% confidence intervals, based on the Student *t* are given for each result.

Twenty-five lots of norbormide were assayed over extended periods of time by several investigators. The mean results and 95% confidence intervals were calculated and are as follows: isomer W, 18.3% ± 4.3%; isomer X, 10.1% ± 4.3%; isomer Y, 28.7% ± 5.1%, and isomer mixture Z, 38.9% ± 4.9%. The results do not indicate significant differences in the isomer content of the different lots of norbormide.

Separation of Isomer Mixture Z.—The mixture of isomers Z was assayed by the quantitative TLC procedure already described. The composition of Z, which had been isolated and recrystallized to a constant melting point material (3), is given in Table V. Mixture Z is approximately 60% isomer V. Samples of Z separated by TLC from the other isomers in a norbormide sample were also assayed and the data given in Table V. The TLC method of separating Z from the norbormide isomers W, X, and Y has been described. However, instead of adding

TABLE III.—RECOVERY OF THE ISOMERS OF NORBORMIDE FROM STANDARD MIXTURES

Mixture *1*, mcg.	Isomers W	X	Y	Z
Taken	31	29	88	106
Recovered	37	33	86	112
Mixture *2*, mcg.				
Taken	47	44	133	159
Recovered	51	44	125	159
Mixture *3*, mcg.				
Taken	100	105	287	290
Recovered	103 ± 4	115 ± 3	274 ± 5	288 ± 16

TABLE IV.—THE COMPOSITION OF SOME NORBORMIDE SAMPLES WITH 95% CONFIDENCE INTERVAL

Sample	% W	% X	% Y	% Z
A	19.0 ± 2.8	10.1 ± 6.9	30.3 ± 2.5	34.2 ± 2.8
B	17.0 ± 4.9	12.7 ± 4.1	26.7 ± 4.4	37.3 ± 4.1

TABLE V.—COMPOSITION OF ISOMER MIXTURE Z

Sample	% U	% V
Mixture Z	36 ± 2	62 ± 3
Mixture Z[a]	37 ± 1	61 ± 2
Mixture Z[b]	40 ± 1	59 ± 1
Z[a]	14.0 ± 1.9	29.6 ± 2.4

[a] Separated by TLC from norbormide.

TABLE VI.—MOLAR FLUORESCENCE VALUES OF THE NORBORMIDE ISOMERS

Sample	Molar Fluorescence (Arbitrary Fluorescent Value Divided by the *M* Concn.)
Isomer R	3.33×10^9
Isomer S	2.49×10^9
Isomer U	5.85×10^9
Isomer V	6.51×10^9
Isomer W	2.40×10^9
Isomer X	3.47×10^9
Isomer Y	2.74×10^9
Isomer mixture Z	6.27×10^9
McN-1392	0.82×10^9

the 7:1 mixture of 0.1 *M* hydrochloric acid–ethanol, 25 ml. of methanol was added and the sample shaken for 1 hr. on a mechanical shaker. The sample was centrifuged and exactly 20 ml. of the clear solution was evaporated to dryness without the aid of heat. The residue was quantitatively taken up in methanol and streaked on the silica plates. The silica plate was developed as previously described.

Paper chromatography was used to determine the sharpness of separation of U and V. If the silica plates were not kept at 25° and 50% relative humidity there was from 5 to 10% tailing of the front running band V into the slower band U. The data given for Z separated from the other isomers by TLC represents separations from 5 different lots of norbormide. The standard deviations are also given in the table. Isomer mixture Z, obtained by recrystallization techniques, was found to contain U and V in the ratio of about 4:6 while, mixture Z separated from samples of norbormide by TLC contained them in the ratio of about 1:2.

Fluorescence Assay.—The norbormide isomers were also assayed by measurement of their fluorescence in acid. All the isomers have an activation maximum at 320 ± 5 mμ and a fluorescence maximum at 460 ±5 mμ in a 1:7 ethanol–0.1 *M* hydrochloric acid solvent. When arbitrary fluorescence units were plotted against concentration, a straight line relationship was found for each isomer in the concentration range of 1 to 10 mcg./ml. The method of assay is the same used for the spectrophotometric assay except that the sample dilution is increased. Standards containing approximately the same amount of silica as the samples are taken through the sample extraction procedure with 1:7 ethanol–0.1 *M* hydrochloric acid. Because of the care needed to avoid traces of impurities anywhere in the fluorometric assay, the spectrophotometric assay is preferred. Good agreement has been observed between the fluorescence and spectrophotometric assays for samples assayed by both methods. The molar fluoresence, arbitrary fluorescence units divided by the molar concentration, is given in Table VI for the major isomers of norbormide.

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Ion-Pair Extraction of Pharmaceutical Amines II

Extraction Profile of Chlorpheniramine

By TAKERU HIGUCHI and K. KATO

The extractability of chlorpheniramine in its ion-pair form has been investigated under several conditions to determine the suitability of the process for separation and isolation of the drug in analytical samples. Chlorpheniramine, chosen as an example of a drug having two basic centers per molecule, exists in aqueous solution as a mixture of uncharged, singly charged, and doubly charged species. Data are presented to show that the drug can be extracted as the chloride, bromide, maleate, trichloroacetate, picrate, etc. The extraction-pH profiles of both the picrate and the bromide correspond closely with the theoretical relationship. As with the monoacidic amines, extraction into the organic phase requires the presence of proton donating species. Experimental data suggest, for example, that the extracted species is coordinated with 5 molecules of chloroform.

THE EFFECTS of various anions and the dependence on solvating agents present in the organic phase on the formation of ion pairs extractable into lipoidal solvents were previously presented (1), with dextromethorphan as an example of a monoprotic amine. The current work deals with the behavior of a diprotic organic base, chlorpheniramine.

It has been found that, in addition to showing a marked dependency on the masking or complex-

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ing agent in the organic phase, the extent of ion-pair formation by the chlorpheniraminium cation is a function of the drug species in solution. The studies indicate that the singly protonated form of the drug can give rise to ion pairs, but none were detected with the doubly protonated form. This species specificity would allow separation of chlorpheniramine from monoacidic organic bases simply by ion-pair extraction at two appropriate pH values.

RESULTS AND DISCUSSION

Extraction as a Function of the Anion and Solvating Agent Concentrations. Studies of the partitioning behavior of ion pairs formed from the chlorpheniraminium cation with a number of inorganic and organic anions were carried out as reported previously on dextromethorphan (1). The nature of the partition data in this instance is evident in a typical set of data illustrated in Fig. 1. This particular plot shows the results obtained with varying concentrations of bromide ion in systems containing different concentrations of chloroform as the masking agent in cyclohexane. The pH of the aqueous layer was adjusted to pH 3.5 with citrate buffers. All data are corrected for the amount of the free base which could be extracted in the absence of ion-pair forming anions.

The system can be represented by the following relationship:

$$BH_{aq.}^{+} + X_{aq.}^{-} \overset{K_e}{\rightleftharpoons} [BH^{+}X^{-}]_{org.} + nM \overset{K_c}{\rightleftharpoons} [BH^{+}X^{-}\cdot M_n]_{org.} \quad \text{(Eq. 1)}$$

where $BH_{aq.}^{+}$ represents the monoprotonated chlorpheniraminium cation in the aqueous phase and $X_{aq.}^{-}$ the anion in the aqueous layer, in this case bromide; $[BH^{+}X^{-}]_{org.}$, the ion pair in the organic phase, and $[BH^{+}X^{-}\cdot M_n]_{org.}$, the solvated ion pair; K_e represents the classical extraction constant (2) and K_c, the stability constant for the complex formation.

Although from subsequent data it will be shown that BH^{++}, the doubly protonated chlorpheniraminium cation does not appear to be extractable in ion-pair form for the anionic species studied so far, the expression for the apparent partition coefficient must consider its presence in the aqueous phase, therefore:

$$PC_{app.} = \frac{[BH^{+}X^{-}]_{org.}}{[BH^{+}]_{aq.} + [BH^{++}]_{aq.}} \quad \text{(Eq. 2)}$$

It is readily apparent from Fig. 1 that $PC_{app.}$ is highly dependent on the concentration of chloroform. The data again suggest that the chloroform interacts with the ion pair in the organic layer and apparently renders it more compatible with the organic solvent by masking the anionic ion. As shown earlier at constant bromide ion concentration, $PC_{app.}$ is proportional to $(CHCl_3)^n$, where n is the number of molecules of chloroform reacting or associating with each ion pair, a plot of log $PC_{app.}$ *versus* log $[CHCl_3]$ yielding a straight line with a slope equal to n. Figure 2 shows such a plot for the chlorpheniramine–bromide system at constant bromide concentrations but varying chloroform concentrations. Evaluation of the slopes yields a coordination value of 5 for this system.

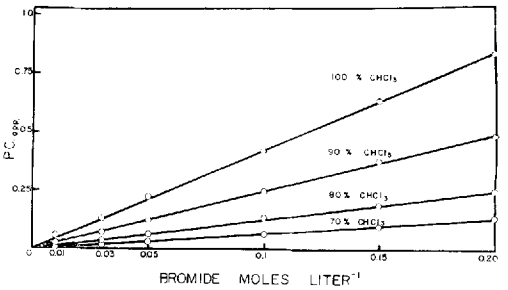

Fig. 1.—The effect of bromide ion on the partitioning of chlorpheniramine at pH 3.5 as a function of chloroform concentration in the organic phase.

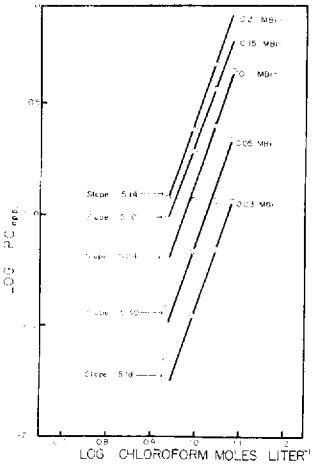

Fig. 2.—The dependency of apparent partition coefficient of chlorpheniramine on the concentration of $CHCl_3$ in the presence of Br at pH 3.5.

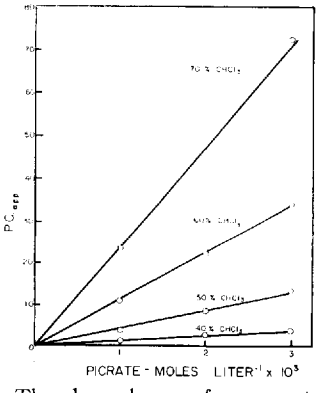

Fig. 3.—The dependency of apparent partition coefficient of chlorpheniramine on picric acid concentration at pH 3.5. The concentration of chlorpheniramine was 5×10^{-5} *M*. The ionic strength was 0.1.

A similar study was carried out using the picrate anion at pH 3.5. The results shown in Fig. 3 are in keeping with those of the bromide interaction, except that the $PC_{app.}$ is substantially greater. The change in the $PC_{app.}$ appears from this and

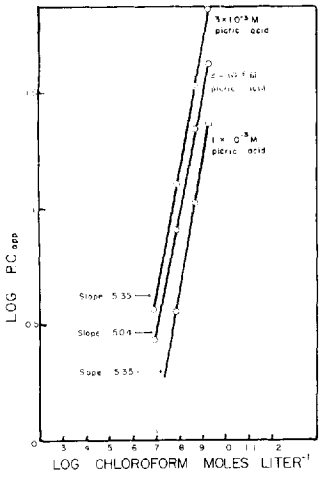

Fig. 4.—The dependency of apparent partition coefficient of chlorpheniramine on the concentration of $CHCl_3$ in the presence of picric acid at pH 3.5.

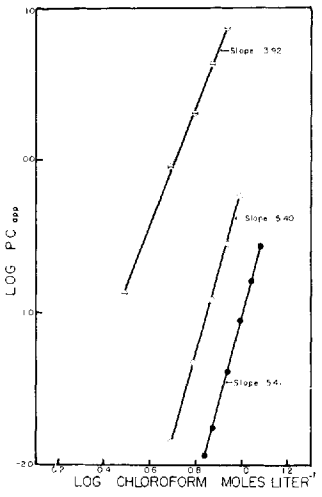

Fig. 5.—The dependency of apparent partition coefficient of chlorpheniramine on $CHCl_3$ concentration in presence of chloride, maleic acid, and trichloroacetic acid at pH 3.5. The concentration of chlorpheniramine was 10^{-3} *M*. Key: ⊖, trichloroacetate, 0.1 *M*; ○, maleate, 0.2 *M*; ●, Cl^-, 0.2 *M*.

TABLE I.—CALCULATED VALUES OF EXTRACTION CONSTANTS FOR CHLORPHENIRAMINE ION PAIRS AT TWO CHLOROFORM CONCENTRATIONS AND DEPENDENCE ON CHLOROFORM CONCENTRATION

$$K_x = \frac{PC_{app.}}{[X^-]} \cdot \left(1 + \frac{[H^+]}{K_a}\right)$$

Anion	1 *M* $CHCl_3$	10 *M* $CHCl_3$	Av. Dependence on $CHCl_3$[a]
Chloride	4.2×10^{-6}	1.9	5.4 = 1
Bromide	1.4×10^{-5}	5.8	5.2 = 1
Maleate	4.4×10^{-5}	15	5.4 = 1
Trichloroacetate	6.7×10^{-2}	550	3.9 = 1
Picrate	7.5×10^{-1}	2.2×10^{4}	5.2 = 1

[a] Slopes of lines such as shown in Figs. 2, 4, and 5.

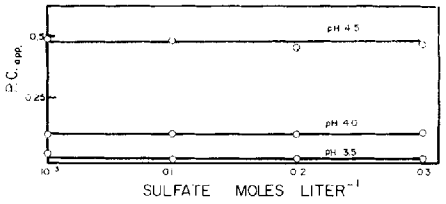

Fig. 6.—The effect of sulfate ion on the partitioning of chlorpheniramine between a pH 3.5 citrate buffer and chloroform.

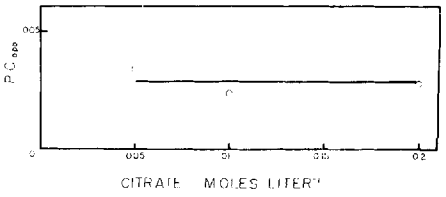

Fig. 7.—The dependency of apparent partition coefficient of chlorpheniramine on citrate buffer concentration as it was extracted by $CHCl_3$ at pH 3.5.

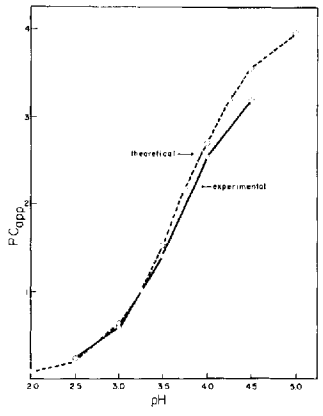

Fig. 8.—pH profile of apparent partition coefficient chlorpheniramine–bromide ion pair. Chlorpheniramine, 10^{-3} *M*; bromide, 0.5 *M*; organic phase, $CHCl_3$; 0.05 *M* citrate buffer.

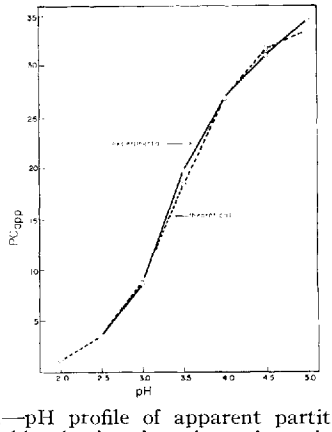

Fig. 9.—pH profile of apparent partition coefficient of chlorpheniramine–picrate ion pair. Chlorpheniramine, 10^{-4} *M*; picrate, 2×10^{-3} *M*; organic phase, 55% $CHCl_3$; in cyclohexane; 0.05 *M* citrate buffer.

other studies to be related to the bulk and hydrophobicity of the anion. In this case, as well as that of the bromide interaction, a plot of log $PC_{app.}$ *versus* $[CHCl_3]$ yielded straight lines as evidenced in Fig. 4 at varying concentrations of picrate anion. From the slopes of the lines it was determined that again 5 molecules of chloroform interacted with each picrate ion pair.

Investigations were also carried out with constant concentrations of chloride, maleate, and trichloroacetate anions under conditions of varying chloroform concentrations. Figure 5 again shows a linear relationship between log $PC_{app.}$ and log $[CHCl_3]$. Table I summarizes the apparent stoichiometry of the interactions of a number of chlorpheniramine–anion ion pairs with chloroform, as well as the extraction constants at 1 and 10 *M* chloroform concentration. Although an expression can be derived for the over-all equilibrium constant as follows:

$$K_e K_c = K_0 = \frac{PC_{app.}}{[X^-][M]^n}\left(1 + \frac{[H^+]}{K_a}\right) \quad \text{(Eq. 3)}$$

where K_0 is the over-all equilibrium constant; K_a is the dissociation constant for $BH^{++} \rightleftharpoons BH^+ + H^+$; in this case 1×10^{-4}, as determined spectrophotometrically.

It is more meaningful to compare the extraction constants at various concentrations of chloroform according to the following equation:

$$K_e = \frac{PC_{app.}}{[X^-]} \cdot \left(1 + \frac{[H^+]}{K_a}\right) \quad \text{(Eq. 4)}$$

Although at this time it is not possible to establish what factors influence the stoichiometry of the complex formed, from this and other work on dextromethorphan, it appears that changes in the anion alter the interaction.

Effect of Polyvalent Anions on the Apparent Partition Coefficient and the Importance of Chlorpheniramine Species in Solution.—As noted earlier, chlorpheniramine exists in aqueous phase in its singly protonated, doubly protonated, or its free base form, the relative concentrations depending on the pH of the system. It was of interest to determine if the sulfate and citrate anions would form ion pairs at different pH values. It was thought that since these anions could exist as doubly charged species they might interact with BH^{++} and form ion pairs.

Figure 6 shows that at a given pH and changing sulfate concentrations the $PC_{app.}$ is essentially independent of $[SO_4^{--}]$ concentration. Below pH 3.0, no chlorpheniramine could be detected in the organic layer. The slight increase in $PC_{app.}$ with pH appears to be extraction of the free base. The effect of citrate anions was determined at pH 3.5 increasing the total citrate concentration from 0.05 mole $L.^{-1}$ to 0.2 mole $L.^{-1}$. The results shown in Fig. 7 show that no ion-pair formation was detected. As sulfate anions show no tendency to form ion pairs, potassium sulfate was used to maintain a constant ionic strength in the studies dealing with bromide, picrate, chloride, maleate, and trichloroacetate anions.

To determine the ion-pair forming ability of the two protonated species of chlorpheniramine, a series of experiments were conducted at constant chlorpheniramine concentrations, constant ionic strength, and varying pH. In one series a constant bromide-ion concentration was maintained and in another a constant picrate. Figures 8 and 9 show similar profiles and would suggest that in both cases the BH^{++} did not form ion pairs, and only BH^+ interacted with the anion to form the extractable species. The theoretical curves in both figures were calculated on this assumption.

Equation 3 can be rearranged to the following form:

$$\frac{1}{PC_{app.}} = \frac{1}{K_0[X^-][M]^n} + \frac{[H^+]}{K_0 K_a [X^-][M]^n} \quad \text{(Eq. 5)}$$

Consequently, a plot of $1/PC_{app.}$ *versus* $[H^+]$ should yield a straight line with a slope of $1/K_0K_a[X^-][M]^n$ and an intercept of $1/K_0[X^-][M]^n$.

Figures 10 and 11 show that both the bromide and picrate data from Figs. 8 and 9 follow the dependency predicted from Eq. 5. The theoretical curve in Figs. 8 and 9 would be calculated from this equation when the appropriate values for n, K_a, and K_0 were utilized.

The K_a for the dissociation of the doubly protonated form was determined spectrophotometrically and found to be 1×10^{-4} at ionic strength of 0.55, but the values obtained from the intercepts and slopes of Figs. 8 and 9 deviated from this value. In the case of the bromide interaction, K_a was found to be 1.84×10^{-4}, and in the picrate system 3.64×10^{-4}.

These differences between the pKa values determined by spectrophotometric determinations and those calculated from Figs. 10 and 11 might possibly be due to interactions resulting in ion-pair formation in the aqueous layer which was not considered in Eq. 1.

From the foregoing and the previous work on dextromethorphan (1), it can be recognized that if chlorpheniramine and dextromethorphan were present in combination in the same system, they would be readily separated by selective ion-pair extraction. At pH 1 in the presence of bromide, dextromethorphan would form ion pairs and could be extracted by the organic phase. Chlorpheniramine being essentially completely in the doubly charged form would not form ion pairs. After separation of dextromethorphan, the pH could be increased to 3.5 and the antihistamine determined.

EXPERIMENTAL

Reagents.—Chlorpheniramine maleate U.S.P.[1] Based upon assay by the U.S.P. nonaqueous titration, it was found to be 99.65% $C_{20}H_{23}ClN_2O_4$.

Chloroform, analytical reagent, was shaken with phosphorus pentoxide and distilled to remove the ethanol. Two per cent (v/v) of *n*-amyl alcohol was added as stabilizer.

All water was glass distilled in the presence of potassium permanganate.

All other reagents were of analytical grade.

Chlorpheniramine Sulfate Stock Solution.—This solution was utilized in the tests determining the apparent partition coefficient in all cases except as described under the maleate system.

Preparation.—Dissolve 3.9 Gm. of chlorpheniramine maleate in 100 ml. of distilled water. Adjust to pH 8–9 and extract with three 50-ml. vol. of

[1] Supplied through the courtesy of the Schering Corp., Bloomfield, N. J.

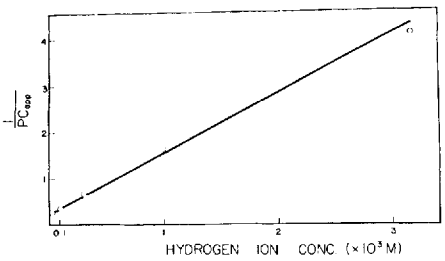

Fig. 10.—Reciprocal apparent partition coefficient of chlorpheniramine in the presence of 0.5 *M* bromide ion as a function of hydrogen-ion concentration.

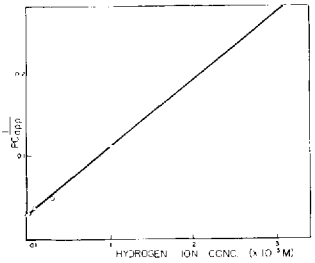

Fig. 11.—Reciprocal apparent partition coefficient of chlorpheniramine in the presence of 2×10^{-3} *M* picrate ion as a function of hydrogen-ion concentration.

cyclohexane. The combined extracts are washed with 50 ml. of distilled water. The cyclohexane is then extracted with three 50-ml. vols. of 0.05 *M* sulfuric acid. The solution is assayed spectrophotometrically and adjusted to a concentration of 5×10^{-2} mole/L. by the addition of distilled water.

Procedure of Partition.—*Procedure A.*—This procedure was employed for the partitioning of chlorpheniramine in the presence of bromide, chloride, maleate, and trichloroacetate anions. Solutions were prepared by dissolving acid or potassium salt of anion in the mixture of chlorpheniramine stock solution and citrate buffer (0.05 *M* citric acid, the pH adjusted by addition of 0.05 *M* potassium hydroxide solution). The concentration of chlorpheniramine in the solution was determined spectrophotometrically at the wavelength of absorption maximum using the calibration curve made from chlorpheniramine stock solution. The wavelengths of absorption maximum at each pH were as follows: pH 2.5, 265 mμ; pH 3.0, 264 mμ; pH 3.5, 263 mμ; pH 4.0, 262 mμ; pH 4.5–5.5, 261 mμ. Twenty milliliters of the aqueous solution was placed in a 60-ml. separator, and an equal volume of the organic phase was added. The mixture was allowed to stand for 1 hr. at 25° to allow the temperature to equilibrate. The sample was then shaken for 30 min. Both phases were separated, and the concentration of chlorpheniramine in the aqueous phase was determined. The concentration in the organic phase was calculated by subtracting the concentration in the aqueous phase from the initial concentration. In order to correct for the concentration of free base extracted into the organic phase, the same procedure was carried out for the blank solution which was prepared from chlorpheniramine, buffer, and containing potassium sulfate to adjust the ionic strength. The apparent partition coefficient was calculated from the following equation:

$$\text{apparent partition coefficient, } PC_{app.} = \frac{\text{total concn. in organic phase} - \text{concn. of free base in organic phase}}{\text{concn. in aqueous phase}}$$

The correction for free base concentration was not made for determining the apparent partition coefficient in the studies of the concentration dependency of sulfate and citrate because of the lack of suitable method. In the experiment with maleate ion, the determination of chlorpheniramine was made in the organic phase using the calibration curve made from chlorpheniramine maleate because the absorption of the anion interfered with the determination in the aqueous phase.

Procedure B.—This procedure was used for the partition of chlorpheniramine in the presence of picric acid. Solutions of chlorpheniramine and of picric acid were prepared, respectively, at the same pH using 0.05 *M* citrate buffer. In a 60-ml. separator 10 ml. of the chlorpheniramine solution and 10 ml. of the picric acid solution were placed, and 20 ml. of organic phase was added to this mixture. After both phases were brought to the temperature equilibrium at 25°, they were shaken for 30 min. Both phases were separated, and a 10-ml. aliquot of the organic phase was placed into a second 60-ml. separator, to which 5 ml. of the picric acid solution and 5 ml. of the buffer were added. The remaining organic phase of the first extraction served for determining the absorbance of the first extraction, A_1. The second separator was treated in the same manner as the first, and the organic phase separated was used for determining the absorbance due to the second extraction, A_2. The absorbance was determined at the wavelength of maximum absorption, 342 mμ, using the pure organic phase as the reference. A blank extraction, without chlorpheniramine, was carried out to correct for the free picric acid which was extracted along with the ion pair. A mixture of 10 ml. of the picric acid solution and 10 ml. of the buffer was shaken with 20 ml. of the organic phase in the same manner as cited above. Absorbances, A_1' and A_2', were determined for the blank extraction, and the apparent partition coefficient was calculated according to the following:

$$PC_{app.} = \frac{A_2 - A_2'}{(A_1 - A_1') - (A_2 - A_2')} u$$

where u is volume ratio of both phases, $V_{aq.}/V_{org.}$ The volume ratio, u, was changed according to the magnitude of the apparent partition coefficient. Chlorpheniramine free base shows no absorption in the wavelengths near 342 mμ.

pKa$_1$ of Chlorpheniramine.—Solutions were prepared from chlorpheniramine stock solution, potassium bromide, and 0.05 *M* citrate buffer at varied pH between 1.6 and 6.4. The concentration of chlorpheniramine was 2×10^{-4} *M* and of bromide was 0.5 *M*. The absorbances were determined at the wavelength of 261 mμ, and pKa was calculated according to the method of Flexner (3).

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Antagonism of Convulsive and Lethal Effects Induced by Propoxyphene

By ROBERT E. FIUT, ALBERT L. PICCHIONI, and LINCOLN CHIN

A toxicological study was performed to demonstrate the effectiveness of several proposed antidotes against the convulsant and lethal effects of propoxyphene hydrochloride. Intravenous administration of levallorphan tartrate, nalorphine hydrochloride, or naloxone hydrochloride at a dose of 1 mg./Kg. significantly increased convulsion threshold and mortality threshold of mice and rats infused with propoxyphene hydrochloride. Treatment with levallorphan tartrate, 1 mg./Kg., plus sodium pentobarbital, 10 mg./Kg., or sodium pentobarbital alone, 10 mg./Kg., prevented propoxyphene-induced convulsions in a large percentage of mice and rats. The combination treatment was effective in elevating mortality threshold in both species, but was no more effective in this respect than a narcotic antagonist alone. Pentobarbital failed to modify mortality threshold. It is recommended that any one of the narcotic antagonists, without pentobarbital, be used to counteract the toxic symptoms of propoxyphene hydrochloride poisoning.

PROPOXYPHENE HYDROCHLORIDE is a widely used analgesic which has been involved in a number of accidental poisonings. The symptoms of massive overdose are referable to the central nervous system and have been reported to include severe depression, hyperactive reflexes, convulsions, respiratory depression, apnea, cyanosis, coma, and death (1–10).

Results of animal studies suggest that the narcotic antagonists are potentially useful for the treatment of propoxyphene toxicito (11–14). In recent years, narcotic antagonists have been used in the treatment of a number of clinical cases of propoxyphene intoxication and appear to be useful in antagonizing the respiratory depression caused by the analgesic (2, 6–8, 10). However, the value of narcotic antagonists against propoxyphene-induced convulsions has been inadequately documented and is controversial. For example, Parker believes that nalorphine may precipitate convulsions when it is used to counteract propoxyphene and cautions against its use as an antidote (5). McCarthy and Keenan (6) reported that nalorphine had no appreciable effect against convulsions in a lethal case of propoxyphene poisoning. On the other hand, in another fatal case, Frasier and coworkers (2) found that the antagonist *N*-allylmorphinan counteracted convulsions caused by the analgesic. Furthermore, a case report by Hara (3) suggests that nalorphine was of value in stopping convulsions displayed by another victim of propoxyphene overdose.

Since there is some uncertainty with respect to the use of narcotic antagonists in propoxyphene poisoning, further investigations to evaluate their effectiveness as specific antidotes are essential. Also, since some investigators have used (2, 8) or suggested the use (1) of central nervous system depressants, *e.g.*, barbiturates, to control convulsions produced by propoxyphene, studies are needed to determine the possible role of depressants in the treatment of poisoning by the analgesic.

The purpose of the present investigation is to determine the relative capacity of three chemically different narcotic antagonists, a barbiturate, and a barbiturate in combination with a narcotic antagonist to control the convulsive and lethal effects induced by propoxyphene in mice and rats. The results obtained constitute the basis of this report.

EXPERIMENTAL

Male CF No. 1 mice, weighing between 25 and 30 Gm., and male Sprague-Dawley rats weighing between 200 and 250 Gm., were randomized according to species into groups of 10. One group from each species was used to test each of the following intravenous treatments: nalorphine hydrochloride, levallorphan tartrate, or naloxone hydrochloride, 1 mg./Kg.; sodium pentobarbital, 10 mg./Kg.; a combination of levallorphan tartrate, 1 mg./Kg.; and sodium pentobarbital, 10 mg./Kg.; or normal saline, 2 ml./Kg. (control group). Three minutes later, propoxyphene hydrochloride, 1.0% for mice and 0.5% for rats, was infused into the animals according to the intravenous infusion technique described by McQuarrie and Fingl (15). The propoxyphene solutions were infused at the rate of 0.005 ml./sec. by means of a constant-infusion apparatus (16) until 2 end points were observed. The first

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This investigation was supported by a grant from Eli Lilly & Co., Indianapolis, Ind.

end point consisted of 3 sec. of persistent clonus, and the second end point consisted of death. Relative effectiveness of the drug treatments was determined by comparing the time required for the onset of convulsions or death in the test group of animals with that of the control groups of animals. The data obtained were evaluated by analysis of covariance (17) and the results expressed as threshold ratios (*i.e.*, test value/control value).

RESULTS

Antagonism of Convulsions Caused by Propoxyphene.—The capacity of the three narcotic antagonists to elevate convulsion threshold in mice and rats is shown in Figs. 1 and 2, respectively. The mean infusion time of propoxyphene for clonus was 55 sec. in control mice and 181 sec. in control rats. The convulsion threshold in mice was increased 60, 41, and 23% ($p < 0.05$), and in rats it was increased 50, 57, and 60% ($p < 0.05$) by levallorphan, nalorphine, and naloxone, respectively. There was no significant difference among the three narcotic antagonists with regard to their relative capacity to elevate convulsion threshold in either mice or rats.

In the case of the pentobarbital-treated animals, 50% of the mice and 70% of the rats displayed no clonus even when propoxyphene was infused to the end point of death. Similarly, when the animals were pretreated with the drug combination levallorphan–pentobarbital, 50% of the mice and 60% of the rats displayed no convulsion.

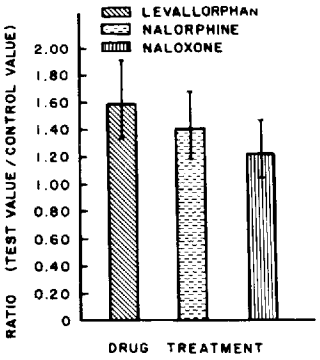

Fig. 1.—The effect of levallorphan tartrate, nalorphine hydrochloride, and naloxone hydrochloride on the convulsion threshold of propoxyphene in mice.

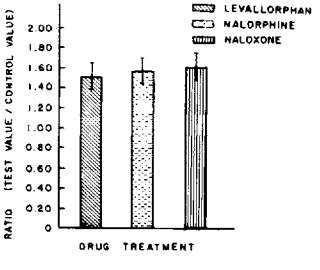

Fig. 2.—The effect of levallorphan tartrate, nalorphine hydrochloride, and naloxone hydrochloride on the convulsion threshold of propoxyphene in rats.

Antagonism of Lethal Effects of Propoxyphene.—The capacity of the various drug treatments to protect mice and rats from the lethal effects of propoxyphene is shown in Figs. 3 and 4, respectively. The mean infusion time for death following intravenous infusion of propoxyphene was 74 sec. in control mice and 372 sec. in control rats. The mortality threshold in mice was increased 60, 50, 59, and 34% ($p < 0.05$), and in rats it was increased 114, 127, 132, and 98% ($p < 0.05$) by levallorphan, nalorphine, naloxone, and the drug combination levallorphan–pentobarbital, respectively. There was no significant difference among these four drug treatments with regard to their relative capacity to elevate mortality threshold in either mice or rats. In contrast to the other drug treatments, pentobarbital, given alone, failed to produce a significant increase in mortality threshold in either animal species.

DISCUSSION

The results of this study show that pretreatment of mice and rats with the narcotic antagonists, nalorphine, levallorphan, or naloxone, markedly increases the amount of propoxyphene required to induce convulsions in these animals, as indicated by an increase in infusion time of the analgesic. Although the experimental design in this investigation required that the antagonists be administered prior to propoxyphene, previous studies in this laboratory have demonstrated that a narcotic antagonist, *e.g.*, nalorphine, arrests convulsions initiated by the analgesic in rats (13). That the narcotic antagonists can counteract established propoxyphene convulsions was also demonstrated by Chapman and Walaszek (12) who administered nalorphine subcutaneously to rats at the onset of the first convulsion and observed a reduction in the duration of convulsion. Even more dramatically, it can be demonstrated in rats that *intravenous* administration of nalorphine after onset of propoxyphene-induced convulsion prevents further episodes of seizure within 90 sec. following injection of the narcotic antagonist (13).

The results of the present study also show that pretreatment with any one of the narcotic antagonists tested markedly increases the amount of propoxyphene required to cause death in mice and rats. In this regard, Chapman and Walaszek (12) reported that pretreatment of rats with nalorphine increased the LD_{50} of the analgesic from 68 to 105 mg./Kg. In addition, these workers showed that the administration of nalorphine, after a toxic dose of propoxyphene which causes 50% mortality in rats, completely abolishes death. Harpel and Mann (14) also reported that nalorphine or levallorphan pretreatment increased the survival rate of mice injected with a lethal dose of propoxyphene.

It is of interest to note that despite the capacity of the drug combination, levallorphan–pentobarbital, to prevent convulsions caused by lethal doses of propoxyphene in a high percentage of mice and rats, the mortality thresholds of these animals were not significantly different from those of animals

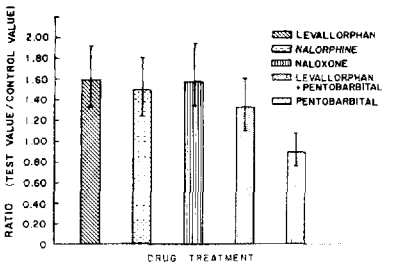

Fig. 3.—The effect of levallorphan tartrate, nalorphine hydrochloride, naloxone hydrochloride, levallorphan tartrate plus sodium pentobarbital, and sodium pentobarbital on mortality threshold of propoxyphene hydrochloride in mice.

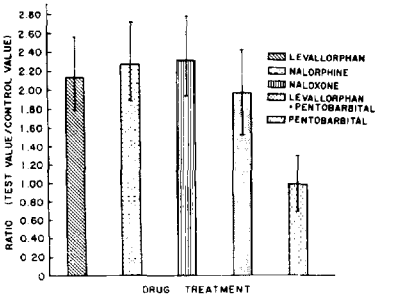

Fig. 4.—The effect of levallorphan tartrate, nalorphine hydrochloride, naloxone hydrochloride, levallorphan tartrate plus sodium pentobarbital, and sodium pentobarbital on mortality threshold of propoxyphene hydrochloride in rats.

pretreated with levallorphan, nalorphine, or naloxone. Indeed, *no* increase in mortality threshold occurred in mice and rats administered pentobarbital alone, a pretreatment procedure which prevented convulsions in a high percentage of the animals given lethal doses of propoxyphene. In view of these findings, it is tempting to speculate that convulsion *per se* may not contribute as much to the lethal effect of propoxyphene as do other factors, such as respiratory depression induced by this drug. If this be the case, then a barbiturate would not reduce mortality, because it does not antagonize the respiratory depression. On the other hand, the results of the present study do not rule out the possibility that convulsion *per se* may contribute to the lethal effect of propoxyphene; the dose of pentobarbital employed could have reduced the convulsive component of toxicity but simultaneously increased the respiratory component such that no significant alteration in propoxyphene-induced lethality was apparent. It is conceivable that there may be an optimal dose of barbiturate which produces an anticonvulsive effect against propoxyphene without causing respiratory depression. However, clinically speaking, it would be difficult as well as potentially hazardous to determine such an optimal dose because of differences in degree of toxicity displayed by victims of propoxyphene poisoning and because respiratory depression resulting from overtitration with a barbiturate would add to postconvulsive and propoxyphene-induced respiratory depression. When a barbiturate is used in conjunction with one of the narcotic antagonists, it is also possible that the respiratory depressant action of the barbiturate, which is not antagonized by these antidotes (18, 19), could reduce the salutary effect of a narcotic antagonist against the lethal action of propoxyphene. Hence, it would appear inadvisable to use a barbiturate alone or in combination with a narcotic antagonist, in the treatment of intoxication caused by propoxyphene. On the other hand, since the narcotic antagonists are known to antagonize the respiratory depressant action of propoxyphene (2, 6–8, 10), and since the present investigation confirms the effectiveness of these drugs in counteracting convulsions and in counteracting the lethal action caused by propoxyphene, it is recommended that one of the narcotic antagonists alone be employed in the treatment of propoxyphene poisoning.

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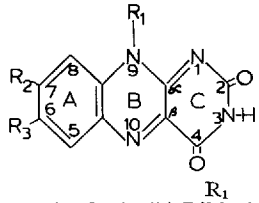
Hydrolytic Behavior of Isoalloxazines Related to Riboflavin I

Identification of an Intermediate and Products in the Base-Catalyzed Degradation of 9-Methylisoalloxazine

By DEODATT A. WADKE and DAVID E. GUTTMAN

Hydrolytic breakdown of 9-methylisoalloxazine was investigated in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide. Spectral and thin-layer chromatographic analysis of the degraded solutions revealed that different products were formed depending on the reaction conditions. Thus, at high pH and in the presence of air, 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid was predominantly formed, while at pH 9.0, 1,2,3,4-tetrahydro-1-methyl-2,3-dioxo-quinoxaline was the main product. At pH 9.0 under anaerobic conditions the predominant products were the keto acid and 1,2-dihydro-1-methyl-2-oxo-quinoxaline. Examination of the degrading solutions by thin-layer chromatography revealed the presence of an intermediate in the reaction pathways. This intermediate was successfully isolated and identified to be 5-(6-methylaminophenylimino) barbituric acid anil.

THE PHOTOLYTIC instability of riboflavin (I) has been well documented. The intermediates and the reaction products have been isolated and identified, and the mechanistic picture is fairly clearly understood (1–4). This mode of degradation in pharmaceutical systems can, of course, be prevented by use of containers which are opaque to deleterious radiation. Riboflavin and other flavins, however, in aqueous solutions can undergo hydrolytic decomposition involving the isoalloxazine nucleus and which occurs with concomitant loss of biological activity. This latter route of degradation is particularly pronounced in alkaline media, and several studies of both qualitative and quantitative nature have been reported. Among the first investigations were those of Kuhn and Rudy (5). They degraded lumiflavin (II) in alkaline medium and were able to isolate urea and 1,2-dihydro-1,6,7-trimethyl-2-keto-3-quinoxaline carboxylic acid from the reaction mixture. Other reports including those of Surrey and Nachod (6) substantiated these findings. The first detailed kinetic study on the hydrolysis of riboflavin has been reported by Farrer and MacEwan (7). They studied the hydrolysis of riboflavin over the pH range 0.5 to 12.0 and observed the reaction to be general acid-base catalyzed. They also followed the progress of the reaction by paper chromatography and noted transient appearance of lumichrome. Their completely degraded solutions showed the presence of the quinoxaline carboxylic acid and an unidentified compound with mauve fluorescence under ultraviolet light. The presence of several unidentified compounds in degraded solutions of riboflavin and other isoalloxazines has been also reported by other workers. Thus, Svobodova (8), who resolved solutions of lumiflavin which were partially degraded under alkaline conditions, was able to see several unidentified spots on chromatographic paper strips. Similar studies were also conducted by a group of workers led by Wada (9), who reported as many as seven unidentified spots. These reports together with some preliminary studies on 3,9-dimethylisoalloxazine (10) gave some indication as to the complexity of the hydrolytic breakdown and emphasized the need for further work. This communication summarizes the results of the preliminary studies on this problem.



	R_1	R_2	R_3
Riboflavin (I)	Ribityl	CH_3	CH_3
Lumiflavin (II)	CH_3	CH_3	CH_3
9-Methylisoalloxazine (III)	CH_3	H	H

9-Methylisoalloxazine (III), rather than riboflavin, was chosen for the purpose of detailed investigation because of its stability to light and because the absence of ribityl side chain precluded certain complexities observed with riboflavin (11). Like riboflavin, 9-methylisoalloxazine is susceptible to the base-catalyzed degradation and is a useful model for studies on the properties of iso-

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alloxazines. The hydrolysis of this compound was investigated under conditions of high pH (0.5 *N* NaOH) and at pH 9.0. In addition, the pH 9 systems were also studied under anaerobic conditions. A chromatographic separation technique was developed to follow the progress of the reaction under different conditions. Identification of the reaction intermediates and the products was accomplished by comparison of their R_f values, fluorescence, and spectral characteristics with those of known compounds.

As it will be seen, spectral and thin-layer chromatographic analysis of solutions of 9-methylisoalloxazine that were degraded under different experimental conditions revealed that different products were formed depending upon the reaction conditions. Thus, in the aerobic systems maintained at high hydroxide-ion concentrations, 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid (IV) was the major product, while at pH 9 and in the presence of air 1,2,3,4-tetrahydro-1-methyl-2, 3-dioxo-quinoxaline (V) was predominantly formed. At the same pH, in the absence of air, the predominant products were the keto carboxylic acid and 1,2-dihydro-1-methyl-2-oxo-quinoxaline (VIII). Evidence was obtained to indicate that the anil of 5-(6-methylaminophenylimino) barbituric acid (VI) and/or 1,2 - dihydro - 1 - methyl - 2 - oxo - quinoxaline-3-carboxyureide (VII) was an intermediate in the reaction under all conditions investigated.

EXPERIMENTAL

Materials.—9-Methylisoalloxazine was obtained as described previously (12).

5-(6-Methylaminophenylimino) Barbituric Acid Anil (VI).—This was synthesized according to the method of Kuhn (13). The anil was purified by dissolving it in water, by adding base, and precipitating the dissolved compound through acidification. The procedure was repeated several times until the precipitated solid was no longer yellowish. Finally, the compound was crystallized from acetone–water to yield silky white needle-like crystals, m.p. 242–244° dec. The anil was titrated potentiometrically under nitrogen atmosphere in DMF, against standard base, and gave a neutral equivalent of 252. Theoretical, based on the molecular formula $C_{11}H_{10}N_4O_3 \cdot {}^1/_2H_2O$ and one titrable hydrogen, was 255.

Anal.—Calcd. for $C_{11}H_{10}N_4O_3 \cdot {}^1/_2H_2O$: C, 51.76; H, 4.31; N, 21.96. Found: C, 52.22; H, 4.77; N, 21.70.

The anil was reported by Kuhn (13) and later by Tishler and co-workers (14) to be yellow in color. It appears that their product was contaminated with the ureide (VII) to which the anil can be easily converted by simple heat or acid treatment. Both Kuhn and Tishler used aqueous acetic acid as their crystallizing solvent and as a result probably obtained a mixture of the anil and the ureide.

1,2-Dihydro-1-methyl-2-keto-3-quinoxaline Carboxylic Acid (IV).—This was synthesized from the anil (VI) in the manner suggested by Kuhn (13). The compound which was obtained as straw-yellow needle-like crystals melted at 176° dec. [King and Clark-Lewis (15) gave m.p. 173–174° dec.] The equivalent weight, determined by nonaqueous titration against methanolic KOH, was 201.6. Theoretical, based on the molecular formula of $C_{10}H_8N_2O_3$ and one titrable hydrogen, was 204.

Anal.—Calcd. for $C_{10}H_8N_2O_3$: C, 58.82; H, 3.92; N, 13.73; O, 23.53. Found: C, 58.97; H, 4.03; N, 13.61; O, 23.53.

1,2-Dihydro-1-methyl-2-oxo-3-quinoxaline Carboxy Ureide (VIII).—This was prepared in the manner suggested by King *et al.* (15), m.p. 246–248° (uncorrected). [Lit. m.p. 248° (15).]

1,2,3,4-Tetrahydro-1-methyl-2,3-dioxo-quinoxaline (V).—This was synthesized according to the procedure described by Miles *et al.* (16). A mixture of equimolar quantities of *N*-methyl-*o*-phenylenediamine hydrochloride and anhydrous oxalic acid was heated at 160° in an oil bath for about 15 min. The melt was cooled and crystallized several times from anhydrous methanol to yield shiny white needle-shaped crystals, m.p. 282–283° (uncorrected).

Anal.—Calcd. for $C_9H_8N_2O_2$: C, 61.36; H, 4.55; N, 15.91. Found: C, 61.22; H, 4.7; N, 15.76.

1,2-Dihydro-1-methyl-2-oxo-quinoxaline (VIII).—This was prepared by sublimation of the keto acid (IV) under reduced pressure at 160° (17). The compound was purified through resublimation, m.p. 119–120° (uncorrected).

Anal.—Calcd. for $C_9H_8N_2O$: C, 67.50; H, 5.00; N, 17.50. Found: C, 67.70; H, 5.08; N, 17.24.

All other chemicals were obtained from commercial sources. The microanalyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn.

Spectral Studies.—Solutions of 9-methylisoalloxazine and of the anil were completely degraded in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide. The spectra of the degraded solutions were obtained on a Beckman DB spectrophotometer.

Thin-Layer Chromatographic Studies.—The progress of the hydrolysis of 9-methylisoalloxazine in pH 9.0 borate buffer and in 0.5 *N* base was followed by thin-layer chromatography. Silica Gel G thin-layer plates (4 × 20 cm.²) of 500 μ thickness were used. Sixty-microliter samples were withdrawn at different time intervals and spotted on the plates. Care was exerted to limit the diameter of the spots to about 5 mm. The chromatograms were developed using $CHCl_3$–acetic acid–pyridine (5:1:1). The solvent front was allowed to run 10 cm. from the point of application of samples. The developed plates were warmed to remove the solvent and examined under long-wave ultraviolet light. The different fluorescent spots were identified by comparison of their R_f values with those of authentic samples.

Isolation and Characterization of the Anil (VI) from Partially Degraded Reaction Mixture.—9-Methylisoalloxazine was degraded in 0.5 *N* sodium hydroxide at 45° for 1.5 hr. This partially degraded solution was rapidly cooled and quickly acidified to pH 5.0. The acidified solution was exhaustively extracted with ether. The ethereal solution was dried over anhydrous sodium sulfate, filtered, and the solvent removed under vacuum. The residue was dissolved in 2 ml. of absolute

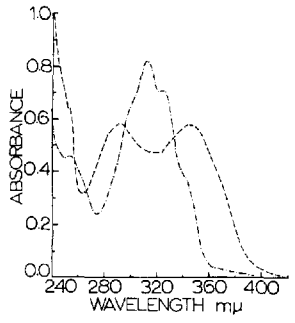


Fig. 1.—Spectra of the solutions of 9 - methylisoalloxazine that were degraded in pH 9.0 borate buffer (-·-·-) and in 0.5 *N* sodium hydroxide (- - - - - -).

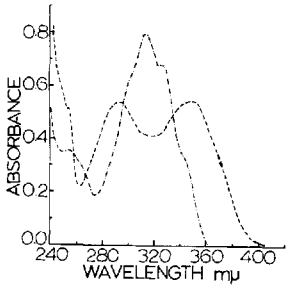


Fig. 2.—Spectrum of the keto acid in 0.5 *N* sodium hydroxide (- - - - - -) and that of the dioxo compound in pH 9.0 borate buffer (-·-·-.)

ethanol and introduced over a silica gel column (10 cm. long and 1 cm. in diameter). Elution was effected with anhydrous ether and the eluate collected in 5-ml. fractions. Earlier studies with synthetic mixtures of the anil (VI), the ureide (VII), and the isoalloxazine (III) showed that such a procedure was effective in separating the anil from the other two compounds. The anil was contained in the first few fractions of eluate.

Anaerobic Studies.—Here the reaction systems containing 9-methylisoalloxazine in pH 9.0 borate buffer were formulated in 10-ml. ampuls. Each ampul was thoroughly flushed with nitrogen and sealed immediately. The sealed ampuls were suspended in a constant-temperature bath adjusted at 45°. The ampuls were opened at different times and the spectrum of each solution was obtained. Concomitant with this, 60-μl. samples were developed on Silica Gel G thin-layer plates using $CHCl_3$–acetic acid–pyridine (5:1:1) solvent system. The developed plates after drying were examined under long-wave ultraviolet light, and the R_f values of different spots were compared with those obtained with known compounds.

RESULTS

Isolation and Identification of Reaction Products.—A preliminary indication of the dependence of the reaction pathways on the hydroxide ion concentrations was obtained by comparison of the spectra of solutions of 9-methylisoalloxazine which were degraded under two different conditions of hydroxide-ion concentration. Figure 1 illustrates such spectra. The striking difference between the spectra clearly indicated that different products were favored depending on the pH of the reaction medium. In Fig. 2 are illustrated the spectra of two possible decomposition products, *i.e.*, the keto acid and the dioxo compound. It can be seen from Figs. 1 and 2 that the spectrum of the isoalloxazine solution degraded at pH 9.0 closely resembled that of the dioxo compound while that of the solution degraded in 0.5 *N* sodium hydroxide corresponded to that of the keto acid. The results of the thin-layer chromatographic studies (see t_∞ samples) summarized in Figs. 3 and 4 revealed that both the keto acid and the dioxo compound were formed under all conditions of pH. However, the relative intensities of the spots corresponding to the two compounds, together with spectral data, demonstrated that the keto acid was the major product at high pH, while at the lower pH, the dioxo compound was mainly formed.

It should be pointed out that the appearance of the dioxo compound as a reaction product was unexpected since the formation of this compound from the parent isoalloxazine must involve both hydrolytic and oxidative reactions. It was the work of Miles *et al.* (16) which presented fluorescence, spectral, and chromatographic properties of a similar compound formed in the bacterial degradation of riboflavin that first drew the authors' attention to the possibility that the dioxo compound was indeed formed.

Thin-Layer Chromatographic Studies.—Fluorescence characteristics under long-wave U.V. light and R_f values for various compounds which could conceivably be formed in the reaction are presented in Table I. It can be seen here and in Fig. 3, which illustrates the progress of the reaction in 0.5 *N* base, that a compound with sky-blue fluorescence and with an R_f approximately 0.38, appeared transiently during the hydrolysis. This R_f value and the fluorescence characteristics agreed with that of a suspected intermediate, *viz.*, the anil compound. The results of a similar study at pH 9.0 are presented in Fig. 4. Only three spots are apparent here, those of the parent compound, the dioxo compound, and a very weak one due to the keto acid.

Isolation and Characterization of the Intermediate from the Reaction Mixture.—A suspected intermediate, the anil, was successfully isolated from partially degraded solution of the isoalloxazine.

Fig. 3.—Chromatograms illustrating the progress of hydrolysis of 9-methylisoalloxazine in 0.5 *N* sodium hydroxide and at 45°. Key: *A*, keto acid; *B*, anil; *C*, isoalloxazine; *D*, dioxo compound.

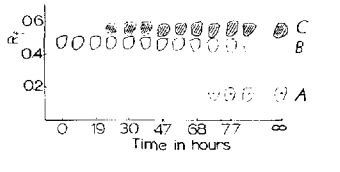


Fig. 4.—Chromatograms illustrating the progress of hydrolysis of 9-methylisoalloxazine in pH 9.0 borate buffer and at 45°. Key: *A*, keto acid; *B*, isoalloxazine; *C*, dioxo compound.

The isolation was achieved through ether extraction of the acidified reaction mixture. Further purification of the isolated material was accomplished through the use of silica gel columns. The spectrum of the isolated compound is shown in Fig. 5 and is identical with that of the pure anil. In addition, the isolated compound exhibited the same fluorescence and chromatographic behaviors as authentic anil. Figure 5 also shows the spectrum of the ureide which could also conceivably be an intermediate in the reaction pathway. It was observed, however, that the ureide in aqueous solution was rapidly and almost quantitatively converted to the anil to form what appeared to be an equilibrium mixture. A typical spectrum of such a mixture in 0.01 *N* HCl is also shown in the figure. The equilibrium was found to be pH dependent, and the anil appeared to be the predominant species in the neutral and alkaline pH range.

Solutions of the anil were formulated in 0.5 *N* NaOH and in pH 9.0 borate buffer and the compound was allowed to degrade. Spectra of the degraded solutions are shown in Fig. 6. These spectra are remarkably similar to those obtained with the parent isoalloxazine.

TABLE I.—R_f VALUES AND THE FLUORESCENCE CHARACTERISTICS OF VARIOUS COMPOUNDS ON SILICA GEL G THIN-LAYER PLATES IN $CHCl_3$–ACETIC ACID–PYRIDINE (5:1:1) SOLVENT SYSTEM

Compd.	Fluorescence Behavior under U.V.	R_f Values
Isoalloxazine (III)	Yellow-green	0.45
Keto acid (IV)	Sky-blue	0.18
Dioxo compd. (V)	Dark blue	0.57
Anil (VI)	Faint sky-blue turning greenish	0.38
Ureide (VII)	Yellow-green	0.78
Quinoxaline compd. (VIII)	Dark blue	0.89
Unidentified compd.	Aquamarine	0.69

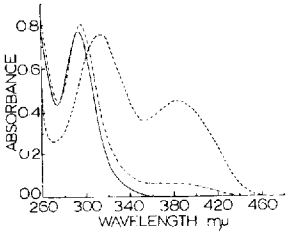


Fig. 5.—Spectra of the anil (————), the ureide (- - - - - - -), and that of the equilibrium mixture in 0.01 *N* HCl (- · - · -).

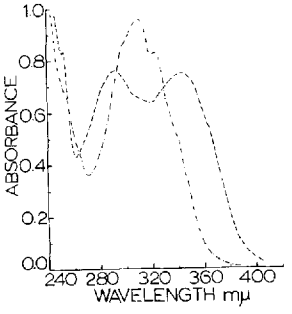


Fig. 6.—Spectra of the solutions of the anil that were degraded in 0.5 *N* sodium hydroxide (- - - - - - -) and in pH 9.0 borate buffer (- · - · -).

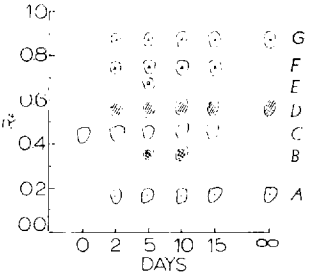
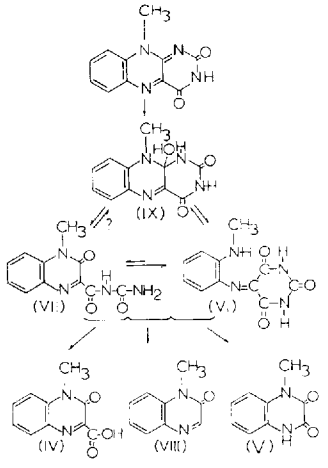


Fig. 7.—Chromatograms illustrating the progress of the anaerobic hydrolysis of 9-methylisoalloxazine in pH 9.0 borate buffer and at 45°. Key: A, keto acid, *B*, anil; *C*, isoalloxazine; *D*, dioxo compound; *E*, unidentified compound; *F*, ureide; *G*, quinoxaline compound.

Anaerobic Studies.—Thin-layer chromatographic results of anaerobic hydrolysis of the isoalloxazine at pH 9.0 are presented in Fig. 7. It can be seen here that the degrading solution of the isoalloxazine under these conditions exhibited spots which corresponded to the keto acid and the quinoxaline compound. In addition, it also showed the anil, the ureide, and an unidentified spot exhibiting aquamarine fluorescence under U.V. light and with an R_f of 0.69.

DISCUSSION

The information which has been collected in this study demonstrated that 9-methylisoalloxazine, in aqueous solution, was transformed into a number of different products, and that the anil compound was an intermediate in such transformations. A proposed scheme for the degradation is presented in Scheme I. The isoalloxazine is seen to be initially



Over-All Reaction Mechanism for the Degradation of 9-Methylisoalloxazine.
Scheme I

converted to the anil compound. Such a transformation can possibly result from hydroxide-ion attack at the α carbon to form a carbinolamine (IX), which in turn can rupture to form either the anil (VI) or the ureide (VIII). Evidence, which will be presented in a future communication, has been ob-

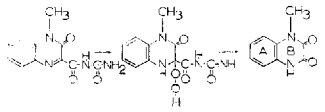
tained to indicate the involvement of such a carbinolamine intermediate. Although the anil compound has definitely been implicated in the reaction scheme, the existence of the ureide is somewhat speculative. As was indicated, however, there is evidence that ureide and anil existed in equilibrium in solution and that the equilibrium favored the anil under the pH conditions which prevailed in this study.

The interesting and somewhat unexpected observation that high pH degradation yielded the keto acid (IV) as the predominant product, while at pH 9.0 the dioxo compound (V) was the main product, can be explained by noting that the anil compound could undergo further reaction by both hydrolytic and oxidative routes and that the relative concentrations of the products would depend on the relative rates of the processes. Thus, at high pH the hydrolytic reaction was much faster than that of oxidation, while at pH 9 the reverse was true. Exclusion of air from the systems at pH 9 markedly decreased the rate of oxidation and made apparent that the hydrolytic reaction which yielded the keto acid was also operant under these conditions. Another reaction pathway which resulted in the formation of the quinoxaline compound (VIII) also became apparent under anaerobic conditions.

The failure to detect the anil in solutions of the isoalloxazine which were degraded at pH 9 in the presence of air can also be explained on the basis of relative reaction rates. It appears that under these conditions, the rate of disappearance of the anil was faster than the rate of its formation from the parent compound. Support of this stems from the fact that solutions of isoalloxazine, which were partially degraded at pH 9 in the absence of air, where the oxidative transformation of the intermediate anil was suppressed, did contain demonstrable concentrations of anil. That the anil was formed as an intermediate under all conditions investigated is also indicated by the spectral studies on completely degraded solutions, which showed that under both conditions of pH the anil yielded the same reaction products as the parent isoalloxazine.

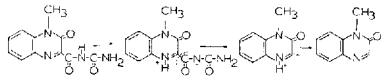
The anaerobic studies with the isoalloxazine also revealed the formation of yet another compound with a weak aquamarine fluorescence under U.V. light and with an R_f value of 0.69 in the solvent system used. This compound has, as yet, not been identified.

Although no definitive evidence was obtained to indicate whether the formation of the final products proceeded through the anil or the ureide, the presence of the intact B ring in the products suggest the latter as the intermediate. Simple hydrolysis would yield the keto acid and urea. It can be speculated that the dioxo compound was formed through a hydroperoxide intermediate such as that depicted in Scheme II, which could undergo homolytic cleavage as shown. Studies on solutions of the keto acid showed that it was stable under the experimental conditions employed and did not undergo any detectable degree of decarboxylation even after prolonged exposure to the reaction conditions. Thus, the quinoxaline compound (VIII) was not derived



A Possible Mechanism for the Formation of Dioxo Compound from the Ureide.
Scheme II

from the keto acid (IV). It could conceivably arise directly from the ureide (VII) which has a weakly acidic imino hydrogen and could be considered to be analogous to a quinaldic carboxylic acid. Such acids are known to decarboxylate readily particularly when unionized (18). A possible mechanism for such a transformation of the ureide is given in Scheme III.



A Possible Mechanism for the Formation of the Quinoxaline Compound from the Ureide.
Scheme III

The formation of an oxidation product analogous to the dioxo compound which was identified in this study, was reported by Stadtman and co-workers (16, 19) to result from bacterial degradation of riboflavin and to involve both hydrolytic and oxidative enzymatic mechanisms. This apparent analogy between chemical and biological systems is interesting and suggests that information on the hydrolytic behavior of isoalloxazines in simple chemical systems might be of significance in gaining a better understanding of certain biochemical reactions involving riboflavin.

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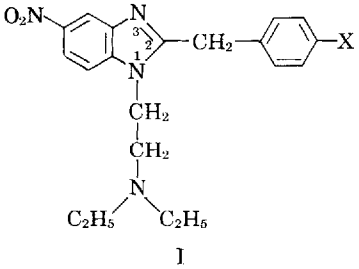
Synthetic Analgesics I

N-(2-Dialkylaminoethyl)-2-phenylacetanilides

By JAMES F. STUBBINS* and TAITO O. SOINE

A series of 24 *N*-(2-dialkylaminoethyl)-2-phenylacetanilides was prepared for testing as potential analgesics. The structure of these compounds can be related to certain nitrobenzimidazole and propionanilide analgesics. The compounds were prepared by treating *N,N*-dialkyl-*N'*-phenyl- or *N,N*-dialkyl-*N'*-(*p*-nitrophenyl)-ethylenediamine hydrochlorides with various phenylacetyl chlorides. The products were obtained as the hydrochloride or perchlorate salts. The previously unknown intermediates—*p*-ethoxyphenylacetyl chloride, *N,N*-dimethyl-*N'*-(*p*-nitrophenyl)-ethylenediamine hydrochloride, and 1-[(*p*-nitroanilino)-ethyl]-piperidine hydrochloride—also are described.

HUNGER *et al.*, in 1957, reported the synthesis of some nitrobenzimidazole derivatives (I) possessing very significant analgesic activity (1). These compounds differ rather radically in structure from other potent analgesics. The degree of activity in this series of compounds is highly dependent upon the *p*-substituent in the benzyl moiety. I*c* and I*d* are 100 and 1000 times as potent as morphine, respectively (2), the latter compound being the most active analgesic known at that time. The unsubstituted (I*a*) or chloro-substituted (I*b*) compounds are only slightly more active than morphine. The nitro group in the 5 position of the benzimidazole ring appears to be an essential feature in this series; if the nitro group is removed or moved to a different position of the ring, activity is markedly reduced or abolished. Another peculiarity of these compounds is that the diethylamino moiety in the basic side chain seems to lead to higher activity than a dimethylamino or heterocyclic amino group.

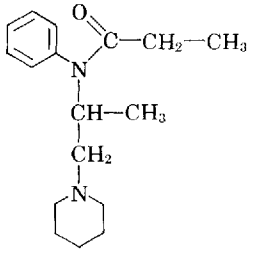


I*a*, X = H
b, X = Cl
c, X = OCH_3
d, X = OC_2H_5

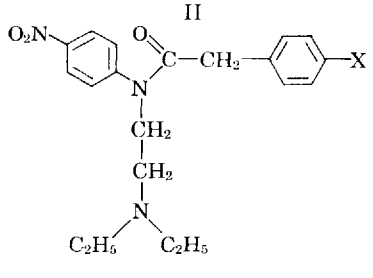
I

Later Wright *et al.* reported that a series of propionanilides showed analgesic properties (3, 4). A typical member of this series is phenampromid (II). These propionanilides resemble the methadone analgesics except that they contain one less phenyl ring and that the usual quaternary carbon atom has been replaced by a tertiary nitrogen atom.

If it is assumed that the carbon-nitrogen double bond of the imidazole ring can simulate a carbonyl group, then the nitrobenzimidazole and propionanilide analgesics may be closely related. Opening of the imidazole ring of I between the benzene ring and the 3 position and replacement of the N^3 nitrogen atom by oxygen leads to compounds of general structure III. This is seen to differ from the propionanilide-type only in the replacement of the propionyl group by the phenylacetyl group and by the added nitro group. Structure III has served as the model for the present series of compounds.



II



III

The structure of III was varied at X to provide compounds for comparison with the active benzimidazoles; thus, X may be a hydrogen, chloro, methoxy, or ethoxy group. In addition, compounds were prepared lacking the nitro group

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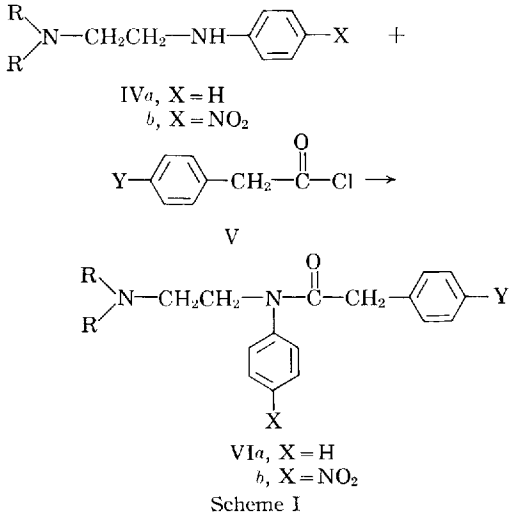
and having dimethylamino or piperidino groups in place of the diethylamino group of the model. It was felt that the special requirements for the nitro and diethylamino groups in the benzimidazole analgesics might be peculiar to that ring system and might be unnecessary in non-benzimidazole analogs.

Thus, all combinations of the variations mentioned yield a set of 24 compounds of general structure (VI). It was hoped that the synthesis and testing of these compounds would establish more structure–activity relationship in this area and that, perhaps, a bridge linking the benzimidazole analgesics to others could be found.

DISCUSSION

In the first attempts to prepare the phenylacetanilides, *N,N*-diethyl-*N'*-phenylethylenediamine (IV*a*, R = C_2H_5) and *N,N*-diethyl-*N'*-(*p*-nitrophenyl)-ethylenediamine (IV*b*, R = C_2H_5) were treated with phenylacetyl chloride in benzene. In both cases large amounts of tar were obtained, and most of the diamine could be recovered. No amide was detected in repeated trials. (Scheme I.)



Scheme I

When phenylacetic anhydride was employed in place of the acid chloride, the desired amides (VI*a* and *b*, R = C_2H_5; Y = H) were obtained in low yield. The products were isolated as the free bases. VI*b* (R = C_2H_5, Y = H) was converted to the hydrochloride salt. All attempts to obtain a salt of VI*a* (R = C_2H_5, Y = H) with various organic and inorganic acids failed to yield a solid until a crystalline perchlorate was prepared according to the method of Caudle *et al.* (5).

The substituted phenylacetic anhydrides required proved to be difficult to obtain in a pure state. Therefore, attempts were made to prepare the amide directly from the acid by use of the "mixed anhydride" methods. Both the sulfonic acid (6) and carbonic acid (7) anhydride methods failed to yield amide. Numerous attempts employing dicyclohexylcarbodiimide (8) as a condensing agent also failed.

When the diamines were treated with the acid chlorides (V), dehydrohalogenation might have occurred in preference to amide formation. To avoid this difficulty, the hydrochloride salt of the diamines (IV) was used in place of the free base. When the various substituted ethylenediamine hydrochlorides were treated with phenylacetyl or *p*-substituted phenylacetyl chlorides in benzene, the phenylacetanilides were obtained as the hydrochloride salts. If the hydrochloride salt would not crystallize, the free base was isolated and converted to the perchlorate salt. All 24 of the desired compounds were obtained by this procedure (Table I). The crude products usually were badly contaminated with the starting material, and purification frequently was difficult. In a few cases the product was soluble in the hot benzene and slowly separated upon cooling, resulting in a much cleaner product.

The structural assignment of the products is supported by the microanalyses and the infrared spectra. All the compounds have a prominent band near 6.0 μ typical of tertiary amides (9). Since this work was started, compound *11* (as the free base) was reported in an Austrian patent (10). It was one of a series of compounds described for use as "narcotic and analgesic drugs," but no pharmacological results were given.

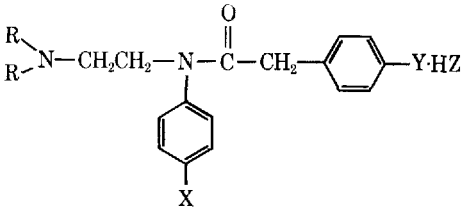
The acid chlorides required were prepared by the method of Elderfield and Meyer (11). *p*-Ethoxyphenylacetyl chloride was the only one not previously known. The intermediate *N,N*-dialkyl-*N'*-phenylethylenediamines were synthesized by condensing dialkylaminoethyl chlorides with aniline according to the method of Stahmann and Cope (12). All the hydrochloride salts have been reported previously (4). However, this method is unsatisfactory for the *N,N*-dialkyl-*N'*-(*p*-nitrophenyl)-ethylenediamines. These were prepared by arylation of *N,N*-dialkylethylenediamines with *p*-nitrochlorobenzene by modification of the method of Mann *et al.* (13). Pyridine was used as solvent and proton acceptor. *N,N*-Diethyl-*N'*-(*p*-nitrophenyl)-ethylenediamine hydrochloride previously has been reported (12).

PHARMACOLOGICAL RESULTS[1]

All of the products were screened initially for possible analgesic activity by the phenyl-*p*-quinone writhing test (14). The compounds were given orally at a dose of 100 mg./Kg. to mice. Compounds *2*, *3*, *5*, *7*, *11*, *14*, *20*, *21*, and *24* (Table I) reduced writhing by at least 50%. These nine compounds were then tested in rats by the D'Amour–Smith procedure (15). At an interperitoneal dose of 25 mg./Kg., only compound *2* increased response time by at least 50%. This is not the compound expected to have the most activity based on comparison with the benzimidazole or propionanilide analgesics. The general lack of activity in the phenylacetanilides is disappointing in view of their close relationship to compounds of significant activity. This would seem to indicate that the benzimidazole ring is an essential feature of that class of analgesics, and that

[1] The authors are indebted to the Pharmacology Department, Lederle Laboratories, Pearl River, N. Y., for the pharmacological results.

TABLE 1.—*N*-(2-DIALKYLAMINOETHYL)-2-PHENYLACETANILIDES



Compd.	R_2N	X	Y	Z	M.p., °C.	Solvent[a]	Anal. C Calcd.	C Found	H Calcd.	H Found	N Calcd.	N Found
1	Dimethylamino	H	H	Cl	211–213	H–B	67.80	67.76	7.27	7.30	8.78	8.69
2	Dimethylamino	H	Cl	Cl	118–183	H–B	61.10	61.14	6.28	6.31	7.93	8.05
3	Dimethylamino	H	OCH_3	Cl	159–161	D	65.40	65.73	7.22	7.02	8.03	8.18
4	Dimethylamino	H	OC_2H_5	Cl	162–163.5	D	66.19	66.01	7.50	7.10	7.72	7.63
5	Dimethylamino	NO_2	H	Cl	185–187	E	59.41	59.36	6.10	6.24	11.55	11.20
6	Dimethylamino	NO_2	Cl	Cl	159–161	D	54.28	54.10	5.32	5.57	10.55	10.68
7	Dimethylamino	NO_2	OCH_3	Cl	203–205	B	57.93	58.24	6.14	6.27	10.67	10.47
8	Dimethylamino	NO_2	OC_2H_5	Cl	163–164	I–B	58.89	58.80	6.43	6.51	10.30	10.28
9	Diethylamino	H	H	ClO_4	106–107	B	58.46	58.44	6.62	6.66	6.82	6.76
10	Diethylamino	H	Cl	ClO_4	106–108	B	53.94	53.93	5.89	5.87	6.29	6.38
11	Diethylamino	H	OCH_3	ClO_4	119–120	B	57.20	56.90	6.63	6.53	6.35	6.38
12	Diethylamino	H	OC_2H_5	ClO_4	80–82	B/F	58.08	57.90	6.87	6.72	6.16	6.11
13	Diethylamino	NO_2	H	Cl	195–196.5	G–B	61.29	61.12	6.69	6.75	10.72	10.40
14	Diethylamino	NO_2	Cl	Cl	179.5–181	D–I	56.34	56.54	5.91	6.05	9.86	10.01
15	Diethylamino	NO_2	OCH_3	Cl	99–101	C/K	59.78	60.04	6.69	6.47	9.96	9.34
16	Diethylamino	NO_2	OC_2H_5	Cl	129–130.5	J/I	60.61	60.34	6.94	7.09	9.64	9.36
17	Piperidino	H	H	Cl	216–218	D–A	70.27	70.21	7.58	7.54	7.81	7.70
18	Piperidino	H	Cl	Cl	149–150.5	D–H	64.12	64.01	6.66	6.77	7.12	7.33
19	Piperidino	H	OCH_3	Cl	137–139	D–H	67.93	68.02	7.52	7.78	7.20	7.50
20	Piperidino	H	OC_2H_5	ClO_4	105–105.7	B/F	59.15	59.38	6.69	6.66	6.00	5.82
21	Piperidino	NO_2	H	Cl	195–197	G–H	62.44	62.76	6.49	6.50	10.41	10.39
22	Piperidino	NO_2	Cl	Cl	194–196	I/G	57.33	57.02	5.75	5.83	9.59	9.59
23	Piperidino	NO_2	OCH_3	Cl	132–134	H/P	60.89	60.58	6.50	6.32	9.69	9.74
24	Piperidino	NO_2	OC_2H_5	Cl	98–100	H	61.66	61.61	6.75	6.99	9.38	9.52

[a] Solvent: A, methanol; B, ethanol; C, acetone; D, methyl ethyl ketone; E, methyl isobutyl ketone; F, diethyl ether; G, tetrahydrofuran; H, ethyl acetate; I, chloroform; J, benzene; K, hexane. A–B, product dissolved in boiling mixture of solvents A and B. A/B, product dissolved in boiling solvent A and reprecipitated by addition of solvent B.

the activity of that group of compounds is unrelated to other morphine-like analgesics. However, the recent suggestion by Portoghese (16) that analgesics may bind to one site in more than one way must be considered. Thus, the benzimidazole and propionanilide analgesics may bind to the same site by different modes. Then a hybrid structure such as the phenylacetanilide may not be well suited for binding by either mode or may bind by still a third mode which does not lead to the analgesic response.

EXPERIMENTAL

All melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are corrected. All analyses were performed by the University of Minnesota Microanalytical Laboratory or the Scandinavian Microanalytical Laboratory, Herler, Denmark.

p - Ethoxyphenylacetyl Chloride.—*p* - Ethoxyphenylacetic acid (50.0 Gm., 0.280 mole) was dissolved in 100 Gm. (0.840 mole) of thionyl chloride. The solution was stirred at room temperature for 1 hr., then heated on a steam bath for 4 hr. After cooling, the reaction mixture was allowed to stand for 16 hr. more at room temperature. The excess thionyl chloride was removed on a rotary evaporator. The residue was then distilled. The yield was 49.8 Gm. of material boiling at 93–97°/0.7 mm.

N,N - Dimethyl - N' - (p - nitrophenyl) - ethylenediamine Hydrochloride.—To 94.6 Gm. (0.600 mole) of *p*-nitrochlorobenzene suspended in 150 ml. of pyridine was added 52.9 Gm. (0.600 mole) of *N,N*-dimethylethylenediamine. The mixture was stirred and heated under reflux for 24 hr. All of the solid dissolved. The solution was cooled, and most of the pyridine was removed by means of a rotary evaporator. The reddish-brown oil that remained had a bright blue fluorescence. Benzene (200 ml.) was added. The mixture was filtered, and the cake was rinsed with benzene. To the filtrate was added benzene saturated with anhydrous hydrogen chloride in small portions with vigorous stirring. The solid was removed by filtration. The filtrate was again treated with hydrogen chloride in benzene to obtain a further small amount of solid. This solid was filtered off, and the filter cakes were combined and air dried. The yield of crude material was 53.9 Gm.

The product was purified by dissolving it in boiling absolute ethanol, filtering, adding ethyl acetate until precipitation began, and then cooling. Repetition of this process finally yielded bright golden flakes free of any brown coloration. After drying overnight at 110°, the product weighed 32.5 Gm. It melted at 180.5–182.5°.

Anal.—Calcd. for $C_{10}H_{16}ClN_3O_2$: C, 48.88; H, 6.56; N, 17.11. Found: C, 48.77; H, 6.67; N, 17.07.

1 - [2 - (p - Nitroanilino) - ethyl] - piperidine Hydrochloride.—1-(2-Aminoethyl)-piperidine (19.2 Gm., 0.150 mole) and *p*-chloronitrobenzene (23.6 Gm., 0.150 mole) were dissolved in 50 ml. of pyridine. The solution was stirred and heated under reflux for 36 hr. The solution was cooled, and most of the pyridine was removed *in vacuo*. The dark oily residue was dissolved in 100 ml. of benzene. Benzene, saturated with hydrogen chloride, was added in small portions with vigorous stirring until

precipitation was complete. The slurry was filtered, and the filter cake was washed with cold benzene. The crude product weighed 23.6 Gm.

The product was recrystallized once from an ethanol–ethyl acetate mixture, then twice from tetrahydrofuran. The pure material consisted of shiny, golden flakes melting at 207–209°. The yield was 12.8 Gm.

Anal.—Calcd. for $C_{13}H_{20}ClN_3O_2$: C, 54.63; H, 7.05; N, 14.71. Found: C, 54.93; H, 7.23; N, 14.41.

N - (2 - Dialkylaminoethyl) - 2 - phenylacetanilides.—From 0.020–0.030 mole of substituted ethylenediamine hydrochloride was suspended in 100–150 ml. of benzene. The appropriate acid chloride was added in 10–25% molar excess. The suspension was stirred and heated under reflux 24–72 hr. The mixture was cooled and filtered. The crude product was recrystallized from the appropriate solvent (Table I).

In a few instances the product was soluble in the benzene even after cooling. In these cases, an equal volume of petroleum ether was added to force the salt out of solution.

If the crude hydrochloride salt was liquid and could not be induced to crystallize, it was dissolved in dilute hydrochloric acid and neutralized with 10% sodium hydroxide solution. The free base was extracted into ether. The ethereal solution was dried, and the ether was removed *in vacuo*. The residual base was converted to the perchlorate salt by the method of Caudle *et al.* (5).

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Synthesis of 4-Substituted-7-methylpyrrolo[2,3-*d*]pyrimidines

By RICHARD H. HAMMER

The reactions of 4-chloro-7-methyl-7H-pyrrolo[2,3-*d*]pyrimidine (IV) with ethanolic ammonia, thiourea, and aqueous sodium sulfhydrate solution to give the 4-amino (V) and 4-thione (VI) analogs are described. Ultraviolet data and pKa values for IV, V, and VI are reported.

TUBERCIDIN (I*a*), a naturally occurring nucleoside in streptomyces species (1, 2), has been assigned the structure 4-amino-7-β-D-ribofuranosyl-7H-pyrrolo[2,3-*d*]pyrimidine (7-deazaadenosine) (3–5). It is an inhibitor of several tumor systems, not cross-resistant to 6-mercaptopurine-resistant line tumor systems (6), and incorporated into both DNA and RNA of mouse fibroblasts and several viruses (7, 8). During structural elucidation studies of tubercidin (I*a*), hydrolysis of I*a* to the aglycone (I*b*) and D-ribose was accomplished by refluxing I*a* in 1–3 *N* HCl for 5 hr. (4). From these data the base ribose bond of I*a* appears to be more resistant to acid hydrolysis than a purine base-ribose bond. Subsequently, resistance of I*a* to enzymatic cleavage by *E. coli* nucleoside phosphorylase was demonstrated while 6-mercaptopurine riboside was observed to be rapidly cleaved (9). Significance of the stability of the pyrrolo[2,3-*d*]pyrimidine base-ribose bond in relation to drug distribution and cancer chemotherapy remains to be explained.

Recent studies by Montgomery and Hewson (10, 11) on the cell culture cytotoxicity of 6-mercaptopurine and 6-mercaptopurine-deaza analogs suggests that deaza structures such as 6-mercapto-7-deazapurine (11–13) are not metabolized by the cells to the ribotide form and consequently are 300–500 times less active than 6-mercaptopurine which is readily converted to the ribotide. This raises the question as to whether antitumor activity for pyrrolo[2,3-*d*]-pyrimidine structures may be dependent on a substituent such as a sugar group, cycloalkyl or alkyl group on the 7-nitrogen (corresponding to the 9-position of purine). This is evident by the fact that tubercidin (I*a*) with a β-D-

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By RICHARD H. HAMMER

The reactions of 4-chloro-7-methyl-7H-pyrrolo[2,3-*d*]pyrimidine (IV) with ethanolic ammonia, thiourea, and aqueous sodium sulfhydrate solution to give the 4-amino (V) and 4-thione (VI) analogs are described. Ultraviolet data and pKa values for IV, V, and VI are reported.

TUBERCIDIN (I*a*), a naturally occurring nucleoside in streptomyces species (1, 2), has been assigned the structure 4-amino-7-β-D-ribofuranosyl-7H-pyrrolo[2,3-*d*]pyrimidine (7-deazaadenosine) (3–5). It is an inhibitor of several tumor systems, not cross-resistant to 6-mercaptopurine-resistant line tumor systems (6), and incorporated into both DNA and RNA of mouse fibroblasts and several viruses (7, 8). During structural elucidation studies of tubercidin (I*a*), hydrolysis of I*a* to the aglycone (I*b*) and D-ribose was accomplished by refluxing I*a* in 1–3 *N* HCl for 5 hr. (4). From these data the base ribose bond of I*a* appears to be more resistant to acid hydrolysis than a purine base-ribose bond. Subsequently, resistance of I*a* to enzymatic cleavage by *E. coli* nucleoside phosphorylase was demonstrated while 6-mercaptopurine riboside was observed to be rapidly cleaved (9). Significance of the stability of the pyrrolo[2,3-*d*]pyrimidine base-ribose bond in relation to drug distribution and cancer chemotherapy remains to be explained.

Recent studies by Montgomery and Hewson (10, 11) on the cell culture cytotoxicity of 6-mercaptopurine and 6-mercaptopurine-deaza analogs suggests that deaza structures such as 6-mercapto-7-deazapurine (11–13) are not metabolized by the cells to the ribotide form and consequently are 300–500 times less active than 6-mercaptopurine which is readily converted to the ribotide. This raises the question as to whether antitumor activity for pyrrolo[2,3-*d*]-pyrimidine structures may be dependent on a substituent such as a sugar group, cycloalkyl or alkyl group on the 7-nitrogen (corresponding to the 9-position of purine). This is evident by the fact that tubercidin (I*a*) with a β-D-

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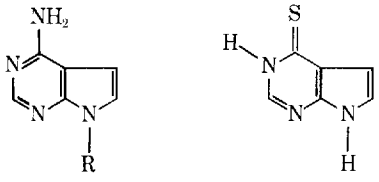
ribose moiety on the 7-position is active while II, which is unsubstituted, is inactive. Pyrrolo [2,3-*d*]pyrimidine structures with a methyl group on the 7-nitrogen and amino or mercapto groups at the 4-position (corresponding to the 6-position of purine) are therefore of biological interest and prompted the described synthesis of 4-amino-7-methyl-7H-pyrrolo[2,3-*d*]pyrimidine (V) and 7 - methyl - 7H - pyrrolo[2,3 - *d*]pyrimidine - 4-(3H)-thione (VI) from 4-chloro-7H-pyrrolo[2,3-*d*] pyrimidine (III).



Ia, R = β-D-Ribose
Ib, R = H

II

EXPERIMENTAL

Melting points were obtained on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Infrared and ultraviolet analyses were recorded on Beckman IR-5 and model DB spectrophotometers. Titrimetric pKa values (14) were obtained on a Sargent titrator, model D, and spectrophotometric pKa values (15) were obtained using buffered solutions (16) verified with standard buffer solutions on a Beckman expanded scale pH meter, model 76. Microanalyses were conducted by Galbraith Laboratories, Inc., Knoxville, Tenn.

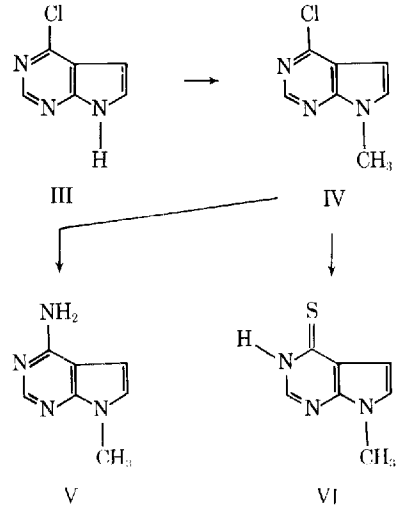
4-Chloro-7-methyl-7H-pyrrolo[2,3-d]pyrimidine (IV) (17).—To a solution of 1.50 Gm. of crude 4-chloro-7H-pyrrolo[2,3-*d*]pyrimidine (III) (12) in 10.0 ml. of dimethylformamide (dried over petroleum ether extracted sodium hydride) cooled to 5° was added 0.492 Gm. of sodium hydride (represents 0.492 Gm. sodium hydride, 50% dispersed in oil, prior to extraction with petroleum ether). The mixture was kept at room temperature until all the hydrogen gas was liberated (4.5 hr.). The reaction mixture was cooled to 5° and 0.60 Gm. of methyl iodide was added. An immediate tan precipitate was observed with agitation in the ice-bath, and after standing for 12 hr., 10.0 ml. of water was added and cooled for an additional 6 hr. Filtration yielded 1.16 Gm. of crude tan product, m.p. 120–122°. The filtrate gave 0.160 Gm. of additional product, m.p. 118–120°. The combined crops were recrystallized from methanol to give light tan crystals (IV). Yield, 1.009 Gm. (62%), m.p. 126–127°. [Reported m.p. 130° (17).] $\nu_{max.}$, cm.$^{-1}$, 3050, 2880 (CH); 1615, 1580, 1530, 1510 (C=C, C=N).

Anal.—Calcd. for $C_7H_6ClN_3$: C, 50.18; H, 3.58; N, 25.08. Found: C, 49.90; H, 3.70; N, 24.84.

4-Amino-7-methyl-7H-pyrrolo[2,3-d]pyrimidine (V).—To 0.20 Gm. of IV in a Parr stainless steel bomb was added 10.0 ml. of ethanolic ammonia solution (prepared by saturating absolute ethanol at 5° with ammonia). The bomb was sealed and maintained at 125–130° with stirring on a combination hot plate-magnetic stirrer. The reported reaction temperature was obtained by simultaneously heating a beaker of mineral oil adjacent to the Parr bomb on the same hot plate. After 10 hr., the mixture was cooled and filtered 5 times to remove decomposition material. The clear brown filtrate was evaporated to dryness yielding a brown crystalline residue which was recrystallized 2 times from hot water to give tan crystals (V). Yield, 0.044 Gm. (25%), m.p. 207–208° dec. $\nu_{max.}$, cm.$^{-1}$, 3280, 3050, 2890 (NH, CH); 1640, 1580, 1550 (NH, C=C, C=N).

Anal.—Calcd. for $C_7H_8N_4$: C, 56.76; H, 5.40; N, 37.82. Found: C, 56.75; H, 5.60; N, 37.61.

7-Methyl-7H-pyrrolo[2,3-d]pyrimidine-4(3H)-thione (VI).—*Method A.*—To a solution of 0.30



Scheme I

TABLE I.—ULTRAVIOLET ANALYSIS

	$\lambda_{max.}$ mμ (ε)			
	pH 1.0[a]	pH 7.0	pH 13.0	pKa
III		222(20,200)[b]		
		271(4,950)		
IV	229(23,050)	225(25,580)	225(24,310)	2.06[c]
	272(4,550)	270(3,980)	271(3,700)	
V[e]	229(21,710)		224(12,930)	5.25[c]
	274(9,270)	271(9,130)	270(8,810)	5.02[d]
VI	266(6,530)	266(6,530)	221(18,150)	9.35[d] (SH)
	318(19,180)	318(19,450)	306(16,450)	

[a] pH 1.0 (0.1 *N* HCl); pH 7.0 (phosphate buffer) (16); pH 13.0 (0.1 *N* NaOH). [b] Ethanol, absolute (13). [c] Spectrophotometric method (15). [d] Titrimetric method (14). [e] Reported (4): $\lambda_{max.}^{0.01\ N\ HCl}$ 230 (—), 275(10,300); $\lambda_{max.}^{0.01\ N\ NaOH}$ 273(9,400).

Gm. of IV in 8.0 ml. of absolute ethanol was added 0.272 Gm. of thiourea. After 5 min. of refluxing the $\lambda_{max.}$ had shifted from 270 to 325 mμ. Refluxing was continued for an additional 3 hr. at which time the $\lambda_{max.}$ was still 325 mμ. Upon evaporation, the crude residue was recrystallized from methanol (48 hr. at room temperature) to give pale yellow needles (VI). Yield, 0.154 Gm. (52%), m.p. 305–307° dec. $\nu_{max.}$, cm.$^{-1}$, 3125, 3010, 2860 (CH); 1575, 1530 (C=C, C=N).

Anal.—Calcd. for $C_7H_7N_3S$: C, 50.90; H, 4.24; N, 25.44. Found: C, 51.08; H, 4.47; N, 25.20.

Method B.—To a solution of 0.20 Gm. of IV in 10.0 ml. of absolute ethanol was added 30.0 ml. of an aqueous sodium sulfhydrate solution (2.0 *N*). The solution was refluxed for 5 hr. at which time the $\lambda_{max.}$ had shifted from 271 to 319 mμ. The yellow solution was cooled and neutralized to a pH of 7 with 10% acetic acid. After cooling overnight the reaction mixture was filtered to give 0.161 Gm. of a pale yellow residue, m.p. 300–302°. Recrystallization from methanol (48 hr. at room temperature) gave pale yellow needles (VI). Yield, 0.097 Gm. (33%), m.p. 307–308° dec. $\nu_{max.}$, cm.$^{-1}$, 3130, 3000 (CH); 1575, 1545, 1530 (C=C, C=N). (Scheme I.)

Anal.—Calcd. for $C_7H_7N_3S$: C, 50.90; H, 4.24; N, 25.44. Found: C, 50.62; H, 4.27; N, 25.21.

RESULTS AND DISCUSSION

The intermediate compound 4-chloro-7H-pyrrolo-[2,3-*d*]pyrimidine (III) was prepared by a 5-step reaction sequence from starting materials of ethylcyanoacetate and bromoacetal (12). Reaction of III with sodium hydride to form the nucleophile and subsequent reaction with methyl iodide gave the 4-chloro-7-methyl-7H-pyrrolo[2,3-*d*]pyrimidine (IV) compound (17). Nucleophilic displacement of the 4-chloro group of IV by reaction with either thiourea (method *A*) or sodium sulfhydrate (method *B*) gave 7-methyl-7H-pyrrolo[2,3-*d*]pyrimidine-4(3H)-thione (VI). Both reactions proceed smoothly at reflux temperatures. Method *A* gave a yield of 52% of VI compared to 33% by method *B*. Infrared and ultraviolet curves of VI from methods *A* or *B* were superimposable and identical in every respect. Optimum conditions for synthesis of the 4-amino congener (V) required heating IV in ethanolic ammonia for 10 hr. at 125–130° in a stainless steel bomb. The reaction was also run at temperatures of 105–110° and 155–160°. The higher temperature produced more extensive decomposition and the lower temperature gave a lower yield than the 125–130° range. Synthesis of the 4-mercapto analogs of pyrrolo[2,3-*d*] pyrimidines can readily be followed by a bathochromic shift in $\lambda_{max.}$ from 265–275 to 315–325 mμ as previously demonstrated during the synthesis of II (13) and as described here (Table I).

Facile substitution of the 6-chloro group of 6-chloropurine and 6-chloro-9-substituted purines by nucleophilic groups has provided useful synthetic pathways leading to a variety of purine nucleosides and purine analogs. Similarly, 4-chloropyrrolo-[2,3-*d*]pyrimidine moieties, without a 7-nitrogen substituent, are useful intermediates which can be reacted with ammonia (18), alkyl amines and thiourea (12), phosphorus pentasulfide (9), sodium sulfhydrate and sodium methoxide (13), and alkyl mercaptans (19) to give the corresponding 4-substituted derivatives. Even though nucleophilic groups can be substituted in place of the 4-chloro group, on the basis of the conditions required for the amination reactions of III, IV, and 6-chloro-purine compounds, the 4-chloro group appears to be more difficult to displace than the corresponding 6-chloro of purine compounds. Lewis *et al.* (20) reported the synthesis of 6-amino-9-(tetrahydro-2-furyl)purine by treating the 6-chloro precursor with methanolic ammonia at room temperature for 48 hr. However, Hitchings *et al.* (18) reported that to replace the 4-chloro group of III with an amino group it was necessary to heat III in a stainless steel bomb at 155–160° for 20 hr., and as reported here, conversion of IV to V required heating IV in ethanolic ammonia for 10 hr. at 125–130°. For the amination reactions, the reaction conditions indicate that the 4-chloro group of pyrrolo[2,3-*d*]pyrimidines is more difficult to displace than the corresponding 6-chloro group of purines.

The pKa values reported in Table I were obtained by titrimetric (14) or spectrophotometric (15) methods. Due to its insolubility in aqueous perchloric acid, the pKa value for IV was obtained spectrophotometrically. Replacement of the 4-chloro group of IV with an amino group (V) gave an expected shift to a higher pKa which would normally occur when an electron-withdrawing group (Cl) is replaced with an electron-donating group (NH_2). Tubercidin (I*a*) has been reported to have a pKa of 5.3 (4). The pKa of the 4-amino-7-methyl analog (V) was found to be 5.02. Providing V has a favorable partition coefficient, it would be expected to produce high intracellular concentrations. The ratio of unionized to ionized species of V at a pH of 7.4 (plasma) would be 240/1 (pKa 5.02) which should enhance its absorption across cellular membranes. The pKa of the SH group of VI was found to be 9.35. The pKa value was not obtained for the pyrrolo nitrogen of VI.

Screening of these compounds for antitumor properties is being conducted by the Cancer Chemotherapy National Service Center and will be reported at a later date.

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Morning Glory Tissue Cultures: Growth and Examination for Indole Alkaloids

By E. JOHN STABA and PAUL LAURSEN*

The seeds and aerial portions of three *Ipomoea violacea* varieties contained significant amounts of indole alkaloids. The roots, callus tissue, and callus medium of these three varieties; the callus tissue and callus medium of *Rivea corymbosa;* and the seeds of three Japanese morning glory varieties contained traces of indole alkaloids.

IT HAS BEEN conclusively established that some *Convolvulaceae* plants contain ergot-type alkaloids. Among them are the seeds of *Argyreia nervosa* (baby Hawaiian wood rose) (1) and *Ipomoea argyrophylla* Vatke (2), *I. coccinea* L. (3), *I. muelleri* Benth. (4), *I. rubro-caerulea* Hook (5) [*I. violacea* (6)]; the seeds (7, 8) and plants (9) of *I. violacea* L. (morning glory, Badoh Negro, *I. tricolor);* and the seeds (7) and plants (10) of *Rivea corymbosa* (L.) Hall. f. (Ololiuqui, wild morning glory).[1]

Certain varieties of *I. violacea* are reported to induce a physiological response similar to lysergic acid diethylamide (11), although a recent study indicates sedation is the principal physiological effect of *I. violacea* and *R. corymbosa* seeds (12). Crude seed extracts of either heavenly blue or pearly gates varieties of *I. violacea* will cause isolated rat uterus muscle to contract (11). Sheep fed dried *I. muelleri* plants for 3 to 6.5 weeks lost weight, became incoordinated, and acquired a rapid panting respiration (4).

The principal objective of this study was to examine three varieties of *I. violacea* and *R. corymbosa* seed callus tissue cultures for indole alkaloids by thin-layer chromatography and spectrophotometry. In addition, the seeds and plants of these three varieties, and the seeds of three varities of Japanese morning glory were examined for indole alkaloids.

MATERIALS AND METHODS

The source of seeds for this study were: *Ipomoea violacea* L. cv. flying saucer (FS), cv. heavenly blue (HB), and cv. pearly gates (PG) (Park Seed Co., Greenwood, S. C.); *Ipomoea nil* (L.) Roth [*Pharbitis nil* Chois, Japanese morning glory (13)] cv. matzukaze, yuki, and chiyo no okina (H. Saier Seedsman, Dimondale, Mich.); and *Rivea corymbosa* (L.) Hall. f. (Prairie Regional Laboratory, Saskatoon, Canada, who purchased them from Atkins Garden and Research Laboratory, Cienfuegos, Cuba). Plants studied were grown from seeds in the Drug Plant Greenhouse, University of Nebraska.

Tissue Cultures.—*Ipomoea* seeds were sterilized in a 5.25% sodium hypochlorite solution (Purex Corp., Ltd., South Gate, Calif.) diluted 1:1 with sterile distilled water for 15 min. in vacuum, and *Rivea* seeds in a 1:3 dilution for 10 min. in vacuum. An unusually strong seed sterilization treatment was required for *Ipomoea* seeds to avoid mold contamination. As the seeds normally germinated within 2 days, they were directly transferred, after thorough rinsing with sterile distilled water, to 1-oz., square glass vials containing 18 ml. of Murashige's and Skoog's tobacco medium (14) with 1.0 p.p.m. 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0% agar.

Extraction Procedure.—Plant, seed, callus tissue and agar medium were extracted for indole alkaloids by a modification of the procedures published by Taber *et al.* (9) and by Abe and Yamatodani (15).

The aerial and root portions were ground in a Wiley mill to a 40-mesh powder; whereas, the seed and dried callus tissue were ground with a mortar and pestle. The plant samples (seed, 2.0 Gm.; aerial, 3.0 Gm.; root, 6.0 Gm.; callus tissue, amount indicated in Table I) were wetted with 10% ammonium hydroxide and allowed to macerate overnight. The ground seeds were first wetted with ethyl ether to facilitate more thorough wetting of the seeds by alkali. The samples were then extracted with ethyl ether in a Soxhlet apparatus for 24 hr.

The agar media were diluted approximately 3:1 with water, made alkaline with 10% ammonium hydroxide, and extracted for 2 to 3 days with ethyl ether in a liquid–liquid extractor.

Each ethereal fraction was then evaporated to dryness in vacuum and the residue dissolved in 10 ml. of 0.2 *N* sulfuric acid. The acidic solution was washed with 10-ml. portions of ethyl ether to remove residues and color. The acidic solution was then neutralized with an excess of sodium bicarbonate and extracted with three 15-ml. portions of chloroform (fraction A). The aqueous solution was then adjusted to pH 6.8 with 0.2 *N* sulfuric acid and again extracted with three 15-ml. portions of chloroform (fraction B). The remaining aqueous layer was filtered and designated fraction C. Fractions A, B, and C were divided into equal aliquots—A_1 and A_2; B_1 and B_2; and C_1 and C_2. Aliquots A_1, B_1,

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[1] Some of these species contain the lysergic acid-type alkaloids ergosine (2); ergometrine (ergonovine), ergine, and isoergine (7); ergometrinine, ergosinine, ergotamine, and lysergic acid (9) and/or the clavine-type alkaloids agroclavine (2); chanoclavine, elymoclavine, and lysergol (7); and penniclavine (9).

TABLE I.—ALKALOID CONTENT OF MORNING GLORY SEEDS, PLANTS, AND TISSUE CULTURES[a]

Plant Variety	Plant Age, Months	Seeds Fraction[b] A	Seeds Fraction[b] B	Aerial Fraction[b] A	Aerial Fraction[b] B	Roots Fraction[b] A	Roots Fraction[b] B	Callus Age, Months[c]	Callus Tissue Dry Wt., Gm.	Callus Fraction[b] A	Callus Fraction[b] B	Agar Medium Agar Wet Wt., Gm.	Agar Medium Fraction[b] A	Agar Medium Fraction[b] B
Heavenly blue	5	0.486	0.007	46.0	0.9	13.3	1.3	15	7.1	0.0	0.0	1259	Trace	0.0
Heavenly blue	4	0.296	0.006	22.6	2.2	...	...	...	...	...	...	...	...	...
Flying saucer	5	0.240	0.039	Trace	0.9	1.3	2.0	15	6.8	0.6	0.6	987	0.0	8.0
Flying saucer	4	...	...	2.6	0.9	...	...	...	...	...	...	...	...	...
Pearly gates	5	0.053	0.003	132.0	2.2	4.0	1.3	15	9.2	0.2	0.4	804	4.0	4.0
Pearly gates	4	...	...	30.0	1.7	...	...	...	...	...	...	...	...	...
Rivea	...	...	...	...	...	...	...	6	18.7	1.0	2.8	490	Trace	0.0
Rivea	...	...	...	...	...	...	...	15	8.2	2.2	1.0	425	0.0	Trace
Matzukaze	...	0.005	0.002	...	...	...	...	...	...	...	...	...	...	...
Yuki	...	0.006	0.005	...	...	...	...	...	...	...	...	...	...	...
Chiyo no okina	...	0.007	0.004	...	...	...	...	...	...	...	...	...	...	...

[a] Alkaloid content for seeds expressed as mg./Gm. of seed material; for aerial, roots, and tissue cultures as mcg./Gm. wet weight. Average of three samples of seeds and aerial portion; and of one sample of roots, callus, and agar medium. [b] Fraction A: alkaline chloroform extract; fraction B: pH 6.8 chloroform extract. [c] Time interval since callus established in culture.

and C_1 were examined for indole alkaloids spectrophotometrically, and A_2, B_2, and C_2 examined for indole alkaloids by thin-layer chromatography.

Quantitative Alkaloid Assay.—Each aliquot was analyzed colorimetrically by the modified *p*-dimethylaminobenzaldehyde (PDAB)–nitrite procedure of Michelon and Kelleher (16). Total alkaloids present were calculated from an ergonovine maleate standard curve which was measured at 590 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer.

Aliquots A_1 and B_1 (in chloroform) were evaporated to dryness in vacuum. The residue of aliquot A_1 was dissolved in 5.0 ml. of 0.2 *N* sulfuric acid, while the residue of aliquot B_1 was dissolved in 10.0 ml. of 5% aqueous tartaric acid. Aliquot C_1 (the filtered aqueous remainder) was analyzed without further purification. However, a bright yellow color in aliquot C_1 prevented its accurate assay, and the data for it are not reported. For each assay 2.0 ml. of extract was added to 2.0 ml. of 0.1% PDAB dissolved in a concentrated sulfuric acid–water solution (1:1, v/v). After 10 min., 0.1 ml. of freshly prepared 0.1% sodium nitrite aqueous solution was added, mixed, and the absorbance recorded. Each assay was repeated three times.

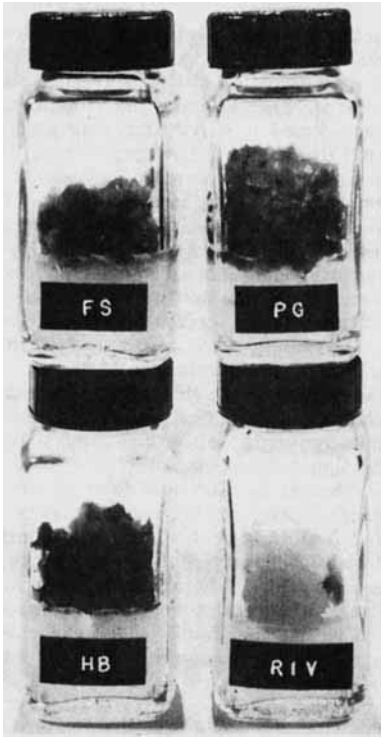

Fig. 1.—Callus tissue cultures after approximately 4 weeks' growth. Key: FS, flying saucer; PG, pearly gates; HB, heavenly blue; RIV, *Rivea corymbosa*.

Qualitative Alkaloid Assay.—Aliquots A_2, B_2, and C_2 were evaporated to dryness in vacuum. The residue of aliquot A_2 was dissolved in 0.5 ml. of 100% ethanol; the residue of aliquot B_2 was dissolved in 0.5 ml. of 95% ethanol; and the residue of aliquot C_2 was dissolved in 1.0 ml. of 95% ethanol. Qualitative comparisons of the alkaloids present in these aliquots were made on thin-layer plates.[2] The plates were spotted with extract amounts ranging from 50 to 200 μl. The solvent system used was a 17:3 mixture of chloroform and methanol (9). Developed plates were sprayed with 1% PDAB in 100% ethanol acidified with concentrated hydrochloric acid, and after 10 min. again sprayed with freshly prepared 0.1% sodium nitrite in 50% ethanol.

RESULTS AND DISCUSSION

Although callus tissue often appeared within 2 weeks on germinated seeds, it required 2 to 3 months before a sufficient size formed for subculture. Once established, callus tissue grew rapidly (Fig. 1) and required subculture approximately every 4 weeks. *Rivea* callus were consistently cream colored, whereas *Ipomoea* callus were considerably darker.

The results of the spectrophotometric assay are shown in Table I, and the chromatographic analysis in Fig. 2. Both analyses reconfirm that fraction A extracts of the seed and aerial portions of *I. violacea* (flying saucer, heavenly blue, and pearly gates) contain alkaloids in the amounts reported by previous investigators (1, 2, 7–9, 11). The authors' results are in agreement with Hofmann (7), but not with Taber *et al.* (9), that alkaloids are present in the plant's roots. Although the three Japanese varieties of *I. nil* seeds assayed contained trace amounts of alkaloids, six domestic varieties are reported not to contain alkaloids (8, 17). Trace amounts of alkaloids remained in most of the alkaline aqueous solutions extracted with chloroform (fraction A). The trace alkaloids were extracted with chloroform

[2] The thin-layer plates were prepared with Adsorbosil-1, Applied Science Lab., Inc., State College, Pa.

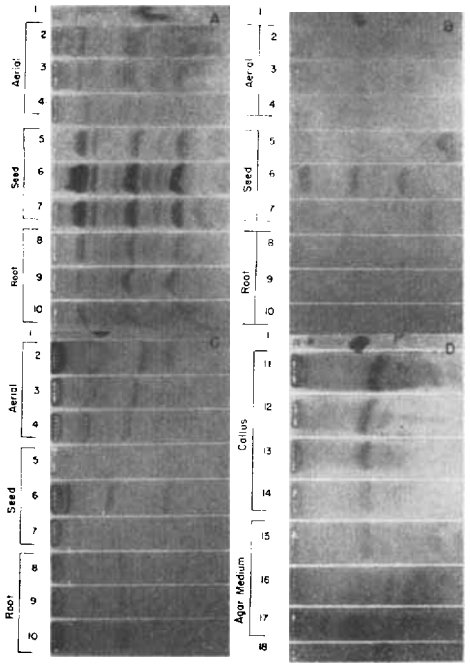

Fig. 2.—Thin-layer chromatograms of morning glory seeds, plants, and tissue cultures. Key: A, plants and seeds: alkaline chloroform extract (fraction A); B, plants and seeds: pH 6.8 chloroform extract (fraction B); C, plants and seeds: pH 6.8 aqueous extract (fraction C); D, tissue culture: alkaline chloroform extract (fraction A). Number code: clymoclavine, 1; pearly gates, 2, 5, 8, 11, 15; heavenly blue, 3, 6, 9, 12, 16; flying saucer, 4, 7, 10, 13, 17; *Rivea corymbosa*, 14; agroclavine, 18. Amounts applied: A, aerial 100 μl.; seed, 50 μl.; root, 150 μl.; B, C, and D, 100 μl. Adsorbent: Adsorbosil-1 (Applied Science Lab., Inc., State College, Pa.). Solvent system: chloroform–methanol (17:3). Spray reagent: PDAB–sodium nitrite.

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FOR CENTURIES the natives of the South Pacific islands have employed the root and rhizome of the kava-kava shrub (*Piper methysticum* Forst.), also known as "ava," "kava," "yangona," and "hoi," to prepare an intoxicating beverage called kava which is consumed at various rituals. The kava beverage, if consumed in sufficient quantity, produces a state of euphoria, followed shortly by muscular relaxation, loss of control of the extremities, and finally a period of dreamless sleep which may last 10 hr. or more. Upon awakening there are apparently no undesirable effects (1–3).

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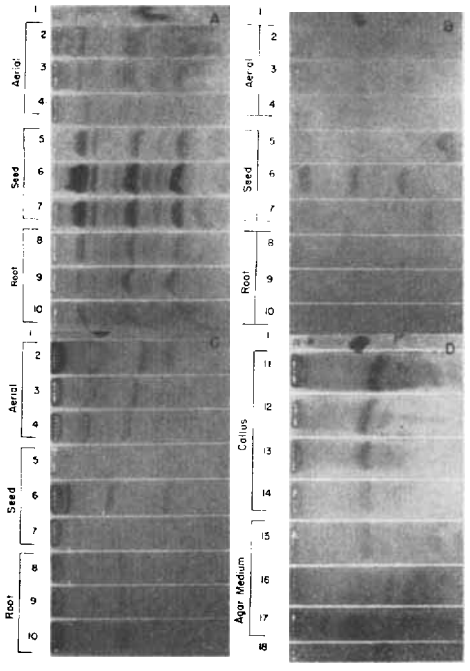
This investigation was supported by grant NB 02733 from the National Institutes of Health, U. S. Public Health Service, Bethesda, Md.



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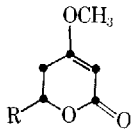
nucleus, only two, dihydrokavain (I) and dihydromethysticin (II), have been shown to produce the characteristic soporific effect (4–6).



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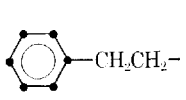
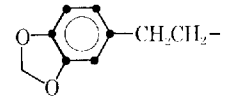
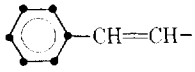


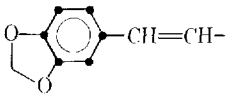
Dihydrokavain (I)



Dihydromethysticin (II)



Kavain (III)



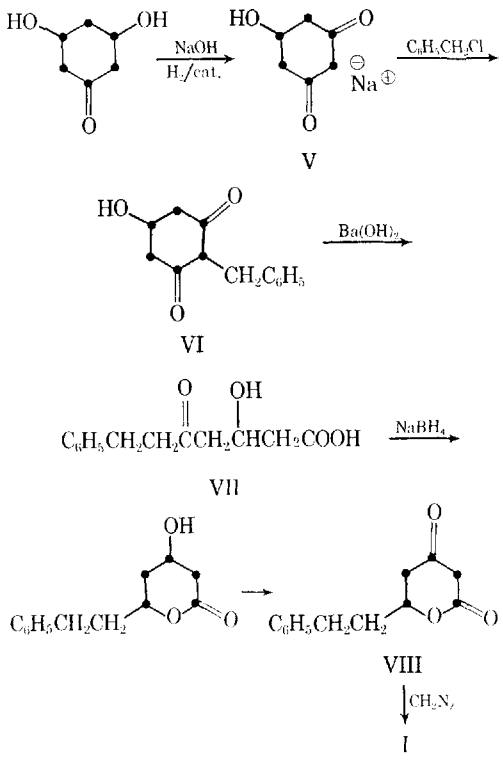
Methysticin (IV)

Dihydrokavain (I) has been synthesized; however, the reaction sequence is lengthy and the yield is low (7). The present source of dihydrokavin (I) and dihydromethysticin (II) is either by isolation from the kava-kava plant or by reduction of kavain (III) and methysticin (IV), respectively.

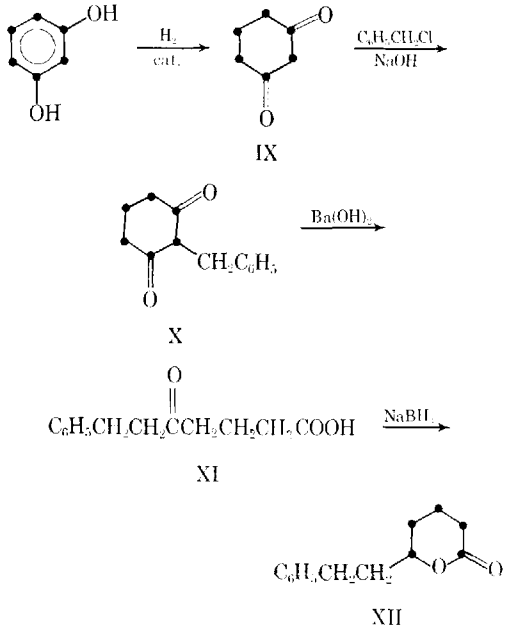
The unique chemistry and pharmacology associated with these dihydropyrones prompted this investigation. It was envisioned that the desired lactones could be prepared by the scheme shown in Scheme I.

Reduction of phloroglucinol followed by alkylation of the monosodium salt (V) would yield the alkylated dione (VI). A reverse Claisen reaction would afford the keto-acid (VII), which in turn could be lactonized and oxidized to the keto-lactone (VIII). Treatment with diazomethane would then afford dihydrokavain (I). Dihydromethysticin (II) and other analogs could be prepared in an analogous manner, using the appropriately substituted benzyl chloride.

Before investigating this approach, it was decided to use resorcinol as a model starting compound (Scheme II). Reduction of resorcinol with Raney nickel catalyst afforded dihydroresorcinol (XI) which was alkylated with benzyl chloride, giving rise to 2-benzylcyclohexane-1,3-dione (X). A reverse Claisen reaction using barium hydroxide as the base, followed by reductive lactonization of the keto-acid (XI) gave rise to the lactone (XII).



Scheme I

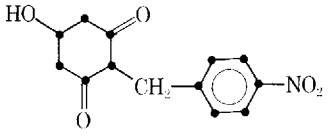


Scheme II

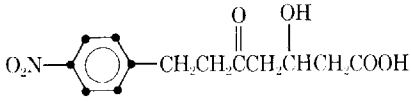
Applying the same sequence of reactions (Scheme I) to phloroglucinol afforded much lower yields of the corresponding compounds. Reduction of phloroglucinol with Raney nickel catalyst yielded monosodiodihydrophloroglucinol

(V) in a 35% yield. When 5% rhodium on alumina was used as the catalyst, the monosodium salt (V) was obtained in 95% yield. Alkylation of the salt (V) with benzyl chloride gave rise to 2-benzyl-5-hydroxycyclohexane-1,3-dione (VI) in 15% yield. Attempts to increase this yield by the use of benzyl iodide, bromide, and tosylate, or by employing various solvents and temperatures were unsuccessful. Formation of the heptanoic acid (VII) from the dione (VI) by means of a reverse Claisen reaction was also unsuccessful. Various temperatures and bases were employed, but the keto-acid was not produced.

The use of a modified procedure of Kornblum (8) in which *p*-nitrobenzyl chloride is used as the alkylating agent, afforded a 30% yield of the nitro dione (XIII). Attempts to open the dione ring (XIII) by means of a reverse Claisen reaction did not afford the keto-acid (XIV).



XIII



XIV

EXPERIMENTAL

Dihydroresorcinol (IX). This material, m.p. 103–104°, was prepared in 90% yield by the procedure of Thompson (9). (Lit. m.p. 103–104°.)

2-Benzylcyclohexane-1,3-dione (X).—This substance, m.p. 184°, was prepared in 70% yield by the procedure of Stetter and Dierichs (10). (Lit. m.p. 184°.)

5-Oxo-7-phenylheptanoic Acid (XI).—This material, m.p. 58°, was prepared in 78% yield by the procedure of Stetter and Dierichs (10). (Lit. m.p. 58°.)

δ-Phenylethyl-δ-valerolacetone (XII).—The procedure for the preparation of this material was patterned after that of Chaikin and Brown (11). Five per cent aqueous sodium hydroxide was added dropwise to 4.3 Gm. (0.019 mole) of 5-oxo-7-phenylheptanoic acid (XI), which was suspended in water, to phenolphthalein end point. This solution was added over a period of 15 min. to 0.23 Gm. of sodium borohydride (0.006 mole) dissolved in 10 ml. of water. The reaction was stirred during the addition and for 1 hr. after the addition was complete. The solution was then acidified to Congo red with 10% hydrochloric acid and stirred for 15 min. at room temperature. The cloudy mixture was then extracted with three 50-ml. portions of ether, the ether dried over anhydrous magnesium sulfate, and evaporated. The resulting oil was distilled under reduced pressure to give 2.8 Gm. (70%) of the lactone, XII, b.p. 140–143° (0.05 mm.); n^{25} 1.5320. The infrared spectrum (liquid film) exhibited strong absorption at 6.75 μ (C=O).

Anal.—Calcd. for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.64; H, 8.38.

Monosodiodihydrophloroglucinol (V).—The procedure for the reduction of phloroglucinol was patterned after a method for the reduction of resorcinol reported by Esch and Schaeffer (12). To a solution of 15 Gm. (0.376 mole) of sodium hydroxide in 100 ml. of water was added 61.0 Gm. (0.376 mole) of phloroglucinol dihydrate and the total volume of the solution was made 200 ml. This solution was placed in a 500-ml. hydrogenation bottle, 4.7 Gm. of 5% rhodium on alumina (Engelhard Industries, Inc.) was added, and the mixture was shaken at room temperature (31°) for 5.5 hr. at an initial pressure of 60 p.s.i. The catalyst was filtered, rinsed with water (2–20 ml.), and the filtrates were combined. After cooling in an ice bath, the filtrate was acidified (pH 6) with 10% hydrochloric acid. The unchanged phloroglucinol was filtered, and water was added to make the total volume of the solution 350 ml. Attempts to obtain the diketone by neutralization of the salt resulted only in polymeric material, so it was necessary to isolate the sodium salt.

Into ten 250-ml. round-bottom flasks was placed 35 ml. of this solution and the water was removed by freeze drying (0.03 mm.) for 18 hr. Fifty milliliters of a solution of 40% ethyl acetate–60% ethyl alcohol was added to each flask, stirred for 30 min., filtered, and the maroon residue was rinsed with a small amount of ethyl acetate–ethyl alcohol mixture. This dark red filtrate was poured with stirring into ethyl acetate (about 1 l.); the sodium salt of dihydrophloroglucinol (V) was filtered rapidly and dried under reduced pressure (0.02 mm.).

Based on the recovered phloroglucinol, the yield of monosodiodihydrophloroglucinol (V) was 98%. This salt is quite hygroscopic and necessitates storage *in vacuo*, $\lambda_{max.}^{EtOH}$ 280 mμ (ε 18,800).

2-Benzyl-5-hydroxycyclohexane-1,3-dione (VI).—The procedure (9) for the preparation of 2-benzylcyclohexane-1,3-dione (X) was modified as follows. In a 100-ml. round-bottom flask fitted with a condenser and magnetic stirrer was placed 7.7 Gm. (0.057 mole) of monosodiodihydrophloroglucinol, 7.0 ml. (0.06 mole) of benzyl chloride, 8.0 ml. of water, and 0.4 Gm. (0.24 mmole) of potassium iodide. The mixture was stirred for 2 hr. in an oil bath (95°), cooled to room temperature, and 5% sodium hydroxide was added until the mixture was distinctly alkaline (pH 9–10). The gummy mixture was extracted with two 50-ml. portions of ether and the ether solutions were discarded. The dark alkaline solution was cooled to 5° in an ice bath and made acidic (pH 2) with 10% hydrochloric acid. A viscous brown oil separated after the flask stood in the refrigerator for several hours. The aqueous solution was decanted and 25 ml. of chloroform was added to the oil. After standing at room temperature for 12 hr., yellow crystals separated. The crystals were dissolved in a small amount of ethyl acetate, heated to boiling, petroleum ether was added to the cloud point, the mixture was allowed to cool to room temperature, and then placed in a

refrigerator for 2–3 hr. The yellow crystals were recrystallized from ethyl acetate–petroleum ether in the same manner, affording 2.0 Gm. (18%) of the alkylated dione (VI), m.p. 161°; $\lambda_{max.}^{EtOH}$ 263 mμ (ε 12,800), $\lambda_{max.}^{EtOH}$ 289 mμ (ε 23,200). The infrared spectrum (Nujol mull) of this material exhibited a broad band at 3.2 μ (bonded OH) and a sharp band at 14.2 μ (mono-substituted benzene).

Anal.—Calcd. for $C_{13}H_{14}O_3$: C, 71.54; H, 6.47. Found: C, 71.79; H, 6.91.

Attempted Preparation of 3-Hydroxy-5-oxo-7-phenylheptanoic Acid (VII).—The procedure (9) for the preparation of 5-oxo-7-phenylheptanoic acid (XI) was followed using 0.486 Gm. (2.23 mmoles) of 2-benzyl-5-hydroxycyclohexane-1,3-dione (VI), 3.2 Gm. (10.7 mmoles) of barium hydroxide octahydrate, and 10 ml. of freshly distilled water. After stirring for 25 hr. at 105–110° the mixture was cooled in an ice bath and 10% hydrochloric acid was added dropwise to a Congo red end point. The cloudy aqueous solution was extracted with three 25-ml. portions of ether, the combined ether solutions dried over anhydrous magnesium sulfate, and the ether was evaporated. A dark viscous oil (350 mg.) was obtained which had a sweet odor but which would not form a 2,4-DNP derivative.

This oil did not solidify and crystallization could not be induced. It was insoluble in 20% sodium bicarbonate, but was soluble in 5% sodium hydroxide. The infrared spectrum (liquid film) exhibited absorption at 3.0 (OH), 5.9 (C=O), and 14.3 μ (monosubstituted benzene). It was not possible to purify this material.

2-(p-Nitrobenzyl)-5-hydroxycyclohexane-1,3-dione (XIII).—A solution of 4.5 Gm. (0.026 mole) of *p*-nitrobenzyl chloride dissolved in 20 ml. of DMF was added to a stirred solution of 3.5 Gm. (0.026 mole) of monosodiodihydrophloroglucinol in 30 ml. of DMF. After stirring 24 hr. at room temperature (25°) the sodium chloride was filtered and the DMF was removed under reduced pressure. Chloroform was added to the residue, heated to boiling, and decanted from the dark insoluble material. This was repeated twice, and the chloroform extracts were combined. The yellow organic solution was heated to boiling, petroleum ether was added to the cloud point, and the mixture was allowed to cool to room temperature; it was then placed in a refrigerator. The crystals were filtered and the filtrate was treated in the same manner twice again. The three crops of crystals were combined and recrystallized twice from ethyl acetate, affording 2 Gm. (30%) of the dione (XIII), m.p. 184–185°; $\lambda_{max.}^{EtOH}$ 265 mμ (ε 37,400), $\lambda_{max.}^{alk.\ EtOH}$ 287 mμ (ε 64,800). The crystalline material gave a negative Beilstein (halogen) test and exhibited strong bands in the infrared spectrum (Nujol mull) at 6.45 and 7.4 μ (aromatic nitro).

Anal.—Calcd. for $C_{13}H_{13}NO_5$: C, 59.32; H, 4.98; N, 5.32. Found: C, 59.80; H, 5.06; N, 5.28.

Attempts to prepare the keto-acid (XIV) from the dione (XIII) by means of a reverse Claisen reaction using barium hydroxide as the base were unsuccessful.[1]

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[1] Melting points were obtained on a calibrated Thomas-Hoover Unimelt and are corrected. Infrared data were recorded on Beckman IR5 and IR8 spectrophotometers. Ultraviolet data were recorded on a Bausch & Lomb 505 spectrophotometer. Microanalyses were conducted by Drs. G. Weiler and F. B. Strauss, Oxford, England.

Anti-Inflammatory and CNS Depressant Properties of 1-(4-Chlorophenyl)-4-{2-[3-(2-pyridyl)acrylyloxy]-ethyl}-piperazine (DA 1529)

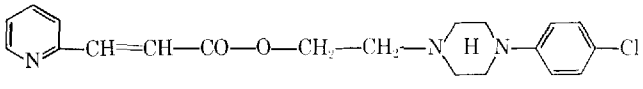
By ERNESTA MARAZZI-UBERTI, CARLA TURBA, and CAMILLO BIANCHI

1-(4-Chlorophenyl)-4-{2-[3-(2-pyridyl)acrylyloxy]ethyl}-piperazine (DA 1529) has been found the most active among 29 alkylpiperazine esters studied. Following oral administration, DA 1529 prevents the edema formation provoked in rats by subplantar injection of carrageenin, formalin, and dextran; it has antinociceptive effect on inflamed (not on normal) foot of rats; it has antipyretic effect in yeast-treated pyrexial rats. DA 1529 is as active or only a little less active than phenylbutazone as anti-inflammatory; it is more active than phenylbutazone as analgesic and antipyretic. The acute toxicity in mice of DA 1529 is similar to that of phenylbutazone. DA 1529 has been found to possess mild CNS depressant properties as evidenced in mice by the results of the barbiturate potentiation test, of the rotarod test, by the reduced spontaneous motility, by analgesia in the hot-plate test, by the hypothermic effect.

AMONG 29 alkylpiperazine esters synthesized (1) and tested for anti-inflammatory, analgesic, and antipyretic activity, the 1-(4-chlorophenyl) - 4 - {2 - [3 - (2 - pyridyl)acrylyloxy]-ethyl}-piperazine (DA 1529) proved to be the more interesting one. Since the completion of the above-mentioned researches, the pharmacological properties of the compound have been explored more fully, and this report is a complete account of the results obtained.

The prevention in rats of the carrageenin-induced edema has been regarded as a reliable method for testing the anti-inflammatory properties of nonsteroid anti-inflammatory drugs (2). Since it has been proved that many compounds unrelated to the anti-inflammatory drugs are very active on this test (3), it was thought advisable to expand the investigation on our drug and look for many other activities in order to draw a clear and correct picture of its pharmacological profile. From this research DA 1529 turned out to be an anti-inflammatory drug with some mild CNS depressant properties.

The compound is a colorless, crystalline material, soluble in water as hydrochloride, and has a molecular weight of 371.86 and the following formula:



METHODS

Male albino mice of 18–22 Gm. and male Sprague-Dawley albino rats of 140–180 Gm. were used in these experiments.

In all the tests, DA 1529 was administered as hydrochloride in aqueous solution at the constant volume of 10 ml./Kg. in rats and 20 ml./Kg. in mice. The control animals received corresponding volumes of saline. The median effective doses (LD_{50} or ED_{50}) were calculated according to Litchfield and Wilcoxon (4) for the quantal responses, and according to Burn (5) for the graded responses. The significance of the differences was estimated using the Student t test as test of significance.

Acute Toxicity.—The acute toxicity tests were performed on mice and rats. The animals in groups of 10 to 20 for each dose tested were treated intraperitoneally or orally and observed for 96 hr. The effects appearing within 3 hr. after treatment were studied according to the scheme suggested by Irwin (6). The doses used ranged from 25 to 200 mg./Kg. for the intraperitoneal route and from 50 to 300 mg./Kg. for the oral route.

Anti-Inflammatory Activity.—The anti-inflammatory activity was estimated in rats following subplantar injection in the left hind paw of formalin (0.1 ml./rat of a 0.75% v/v solution), dextran (0.05 ml./rat of a 6% w/v solution), and carrageenin (0.1 ml. of a 1% w/v suspension in sterile normal saline) (7).

The degree of the edema was expressed as percentage increase of the thickness of the paw after the injection of the edema-provoking agent (measured with a dial thickness gauge) over the thickness of the same paw before injection of the edema-provoking agent. The anti-inflammatory effect was estimated at different time intervals, as specified in the tables, following oral administration of 50 100, and 200 mg./Kg. Phenylbutazone, 200 mg./Kg. orally, was used as reference compound.

Antipyretic Activity.—The antipyretic activity was studied according to the method of Smith and Hambourger (8) in groups of 5 rats for each dose. The animals were housed in a thermostatically controlled room at 23°. The rectal temperatures were measured using a thermocouple applicator before ad-

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ministration of DA 1529 and at hourly intervals for 5 hr., following intraperitoneal administration of 25, 50, and 100 mg./Kg., and oral administration of 50, 100, and 200 mg./Kg. Phenylbutazone, 100 mg./Kg. intraperitoneally and orally, was used as reference compound.

Analgesic Activity on Inflamed Tissue.—The analgesic activity was tested in rats, according to the technique of Randall and Selitto (9). DA 1529 was administered 90 min. after subplantar injection of brewer's yeast (0.1 ml./rat of a 20% suspension in normal saline); 10 to 15 animals were used for each dose. In evaluating the results, the regression line and the corresponding ED_{50} were calculated correlating the logarithm of the dose with the percentage increase of the pain threshold, 90 min. after treatment, of the inflamed paw and of normal paw in treated animals over the corresponding thresholds in control animals. DA 1529 was administered intraperitoneally at the dose of 25, 50, and 100 mg./Kg. and orally at the dose of 50, 100, and 200 mg./Kg. Phenylbutazone administered intraperitoneally, 100 mg./Kg., and orally, 200 mg./Kg., was used as reference compound.

Analgesic Activity—Hot Plate Method.—The analgesic activity was studied in mice, according to the method previously described in detail in another paper (10). DA 1529 was administered intraperitoneally at the dose of 50, 100, and 200 mg./Kg. and orally at the dose of 50, 100, 150, and 200 mg./Kg. to groups of 29 to 40 mice for each dose. Phenylbutazone administered intraperitoneally, 50 mg./Kg., and orally, 200 mg./Kg., was used as reference compound.

Hypothermic Activity.—The activity on the body temperature was evaluated in mice housed in a thermostatically controlled room at 23°. The rectal temperatures were recorded by means of a thermocouple applicator before treatment and 1, 2, 4, and 6 hr. after intraperitoneal administration of 25, 50, and 100 mg./Kg. and oral administration of 12.5, 25, 50, and 100 mg./Kg. of DA 1529. Twenty animals were used for each dose.

Activity on Spontaneous Motility.—The spontaneous motility was studied in mice, using Dews technique (11). DA 1529 was administered intraperitoneally at the dose of 12.5, 25, 50, and 100 mg./Kg. and orally at the dose of 50, 100, and 200 mg./Kg. 15 min. before the test. Six groups of five animals each were used for each dose and route of administration. Corresponding groups of normal mice were used as controls. The results were evaluated by determining the percentage variations of the motility of the treated groups compared with the controls and calculating the corresponding ED_{50} from the activity-log dose line.

Activity of *Hexobarbital* and *Pentobarbital* Sleeping Time.—Hexobarbital and pentobarbital were injected intraperitoneally in mice, at the respective doses of 100 mg./Kg. and 60 mg./Kg., 15 min. after intraperitoneal or oral administration of DA 1529. When hexobarbital was used DA 1529 was adminstered intraperitoneally at 12.5, 25, and 50 mg./Kg. and orally at 12.5, 50, and 100 mg./Kg. When pentobarbital was used DA 1529 was administered intraperitoneally at 25, 50, and 100 mg./Kg. and orally at 12.5, 25, 50, and 100 mg./Kg. The sleeping time was defined as the interval between the loss and the spontaneous return of the righting reflex. Ten to 20 animals were used for each dose and a group of control animals was always prepared in parallel. In evaluating the results the regression line and the corresponding ED_{50} were calculated correlating the logarithm of the dose with the percentage increase of the sleeping time of the treated group over that of the control group.

Anticonvulsant Activity.—The anticonvulsant activity was tested by means of: (*a*) protection from the tonic phase of convulsions due to maximal electroshock (10 ma., alternating current, 0.2 sec.); (*b*) protection from tonic-clonic type convulsions and from death due to lethal doses of pentamethylenetetrazole (125 mg./Kg. i.p.); (*c*) protection from the convulsive and lethal effects of lethal doses of strychnine (3 mg./Kg. i.p.).

The experiments were carried out on unfasted mice. DA 1529 was administered by the intraperitoneal route, 15 min. before the convulsant, to groups of 10 animals each at the dose of 100 mg./Kg.

Activity on the Movement Coordination.—The muscular activity and coordination were studied in mice, using the following methods: (*a*) Boissier's rotarod test (12). The number of mice which lost the capacity to cling to a rotating rod (2.5 cm. diameter, rough surface, 12 r.p.m.) was determined. Before the experiment the mice were selected so as to reject animals falling within 5 min. (*b*) Traction test described by Boissier (12). The mice were suspended by their forepaws from a horizontally stretched wire. The number of animals which lost the capacity of clinging to the wire with at least one hind paw within 5 sec. was determined. (*c*) Inclined screen test according to Randall *et al.* (13). The number of animals incapable of clinging to a fine mesh wire net inclined at approximately 30° to the horizontal was recorded. (*d*) Paralyzing action test according to Berger (14). The loss of the righting reflex for at least 1 min. was taken as test of paralysis.

The tests were performed 2, 5, and 15 min. after intraperitoneal administration and 5, 15, and 30 min. after oral administration of DA 1529. For the rotarod test the doses used were 25, 50, and 100 mg./Kg. intraperitoneally and orally. For the traction test the doses used were 50, 100, and 200 mg./Kg. intraperitoneally and orally. For the inclined screen and righting reflex tests the doses used were 50, 100, and 200 intraperitoneally and 100, 200, and 300 orally.

Action on the Pinna and Corneal Reflexes.—The changes in the pinna and corneal reflexes were determined in mice, according to the method of Witkin *et al.* (15). The tests were performed 2, 5, and 15 min. after intraperitoneal administration and 5, 15, and 30 min. after oral administration of DA 1529. The doses used were 25, 50, and 100 mg./Kg. intraperitoneally and 200, 300, and 350 mg./Kg. orally, 10–15 animals/dose being used.

Activity on the Cardiovascular System and Respiration.—The effect of DA 1529 on the arterial pressure and respiration was studied in male rabbits weighing 2.3–2.5 Kg. in ethyl-urethan narcosis (750 mg./Kg. i.p.). The blood pressure was recorded at the carotid artery using a mercury manometer, and the respiration by means of a Marey tambour connected to a pneumograph fixed to the chest. DA 1529 was administered to the animal

Table I.—Acute Toxicity in Mice and Rats

Animal	Route of Administration	Animals, No.	Doses, No.	LD_{50}, mg./Kg.	Confidence Limits $P = 95\%$
Mouse	i.p.	80	4	323.0	303.3–349.9
Mouse	oral	40	4	529.0	496.7–563.4
Rat	i.p.	50	5	311.0	275.2–351.4
Rat	oral	50	5	478.0	437.4–524.5

Table II.—Activity on Carrageenin-Induced Edema in Rats

Compd.	Dose, mg./Kg. Orally	Animals, No.	Edema, 6 hr. After Administration of the Compd. Degree[a]	Inhibition Compared with Controls, %
Saline	30 ml.	19	27.2 ± 1.40	...
DA 1529	50	20	23.8 ± 1.24	13
DA 1529	100	20	23.0 ± 1.43	16[b]
DA 1529	200	20	18.6 ± 1.29	32[b]
Saline	30 ml.	20	25.1 ± 0.85	...
Phenylbutazone	200	20	12.9 ± 1.32	49[b]

[a] Expressed as percentage increase of the thickness of the inflamed paw compared with the thickness before injection of carrageenin ± S.E. [b] Change significant at 0.02 level.

Table III.—Activity on Formalin-Induced Edema in Rats

Compd.	Dose, mg./Kg. Orally	Animals, No.	Edema, at Intervals After Administration of the Compd.—2 hr., 15 min.— Degree[a]	Inhibition Compared with Controls, %	—4 hr., 15 min.— Degree[a]	Inhibition Compared with Controls, %
Saline	10 ml.	10	69.0 ± 2.36	...	66.9 ± 2.59	...
DA 1529	50	10	62.4 ± 3.24	10	68.6 ± 2.64	0
DA 1529	100	10	55.4 ± 2.94	20[b]	57.0 ± 2.33	15[b]
DA 1529	200	10	43.8 ± 2.07	37[b]	46.8 ± 2.16	30[b]
Saline	10 ml.	20	75.4 ± 2.47	...	78.5 ± 2.51	...
Phenylbutazone	200	20	61.7 ± 2.55	18[b]	63.5 ± 2.55	19[b]

[a] Expressed as percentage increase of the thickness of the inflamed paw compared with the thickness before injection of formalin ± S.E. [b] Change si gnificant at 0.02 level.

Table IV.—Activity on Dextran-Induced Edema in Rats

Compd.	Dose, mg./Kg. Orally	Animals, No.	Edema, 90 min. After Administration of the Compd. Degree[a]	Inhibition Compared with Controls, %
Saline	10 ml.	10	91.1 ± 2.52	...
DA 1529	50	10	94.5 ± 2.11	0
DA 1529	100	10	91.2 ± 3.06	0
DA 1529	200	10	80.0 ± 2.97	12[b]
Saline	10 ml.	10	97.5 ± 2.23	...
Phenylbutazone	200	20	82.0 ± 4.05	16[b]

[a] Expressed as percentage increase of the thickness of the inflamed paw compared with the thickness before injection of dextran ± S.E. [b] Change significant at 0.02 level.

through the marginal ear vein at various doses, maintaining constant the volume and rate of administration (1 ml. in 30 sec.). The coronary vasodilator activity of the compound was determined on isolated rabbit heart prepared according to Langendorff and perfused at constant pressure according to the technique described by Setnikar (16); the amplitude and rate of the heart beats were recorded simultaneously.

Activity on Smooth Muscle.—The action on smooth muscle was studied on segments of guinea-pig ileum suspended in oxygenated Tyrode's solution, in a thermostatically controlled bath kept at 34°. The intestine was stimulated with standard doses of acetylcholine (10^{-7} Gm./ml. of chloride), histamine (10^{-6} Gm./ml. of hydrochloride), nicotine (2×10^{-6} Gm./ml. of bitartrate), and 5 HT (10^{-6} Gm./ml. of creatinine sulfate monohydrate).

RESULTS

Acute Toxicity.—The results of the toxicity tests on DA 1529 are given in Table I. The compound showed slight toxicity when administered by intra-

peritoneal and oral route in either mice or rats. The oral LD_{50}/i.p. LD_{50} ratio was found to be 1.64 for mice and 1.54 for rats. These values are almost identical for the two species and indicate good absorption of the substance under test through the digestive tract. The direct observation in mice performed according to the indications of Irwin (4) revealed that DA 1529 at nonlethal doses (100–200 mg./Kg. i.p. and 100, 200, 300 mg./Kg. orally) caused reduction of the spontaneous motility, curiosity, and pain sensitivity as well as muscular hypotonia, a very slight motor deficit, and reduction of the ipsilateral flexor reflex.

Reduced pain sensitivity was still evident at 50 mg./Kg., for both routes of administration.

Anti-Inflammatory Activity.—Tables II, III, and IV report the data relative to DA 1529's oral inhibiting effect of carrageenin, formalin, and dextran-induced edemas. The results of the experiments performed, reported in Table II, indicate the inhibiting effect of DA 1529 on carrageenin-induced edema; this effect is slightly less intense than that exerted by phenylbutazone. As shown by Table III, DA 1529 demonstrated to be capable of significantly reducing formalin-induced edema, exerting an anti-inflammatory action quantitatively superior to that of phenylbutazone. As for dextran-induced edema, DA 1529 exerted a slight anti-inflammatory action only at the 200 mg./Kg. dose; at this dose the action of DA 1529 was slightly less than that of phenylbutazone.

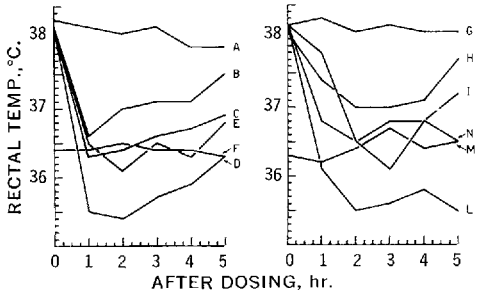

Fig. 1.—Antipyretic activity in rats. Key: A, fevered controls; B, DA 1529, 25 mg./Kg.; C, DA 1529, 50 mg./Kg.; D, DA 1529, 100 mg./Kg.; E, phenylbutazone, 100 mg./Kg.; F, nonfevered controls; G, fevered controls; H, DA 1529, 50 mg./Kg.; I, DA 1529, 100 mg./Kg.; L, DA 1529, 200 mg./Kg.; M, nonfevered controls; N, phenylbutazone, 100 mg./Kg.

Antipyretic Activity.—Figure 1 gives the variations of the rectal temperature. As the graphs show, DA 1529, administered by either the intraperitoneal or oral route, exerted a distinct inhibiting action on experimentally induced pyrexia in rats, the effect still evident, although to a slighter extent, 5 hr. after treatment. The antipyretic effect observed was found to be proportional to the dose administered.

Analgesic Activity on Inflamed Tissue.—Table V summarizes the results of the experiments performed with the gradual pressure method according to Randall and Selitto (19) on the inflamed and controlateral normal paw. The data reported show that DA 1529 exerted a marked effect on the inflamed paw but did not increase the pain threshold in the normal paw.

Under the same experimental conditions phenylbutazone is active only on the inflamed paw. The median effective doses calculated by plotting the percentage increase of the pain threshold in the inflamed paw in function of the logarithm of the dose were found to be 9.5 mg./Kg. for intraperitoneal administration and 40.1 mg./Kg. for oral administration.

Analgesic Activity–Hot-Plate Method.—Table VI summarizes the data relative to the hot-plate analgesic action, and gives the percentage increase of the reaction time (RT) to the heat nociceptive stimulus 1 and 2 hr. after treatment (i.p. and oral) with DA 1529, the total number of mice insensitive to pain during the 2 hr. of the experiment, and the ED_{50} for the two routes of administration. In this test, DA 1529 significantly increased the reaction time to the heat stimulus and provoked an analgesia proportional to the dose administered. The effective dose, producing analgesia in 50% of the animals, was found to be 105 mg./Kg. i.p. and 109 mg./Kg. orally.

Hypothermic Activity.—The results reported in Table VII indicate that after intraperitoneal or oral administration of 100 mg./Kg. of DA 1529, the rectal temperature of mice fell approximately 2°,

Table V.—Analgesic Activity (Randall-Selitto Method) in Rats

Compd.	Dose, mg./Kg.	Animals, No.	Inflamed Paw mm. Hg ± S.E.[a] Pain Threshold	Increase of Pain Threshold, %	ED_{50},[b] mg./Kg.	Normal Paw mm. Hg ± S.E.[a] Pain Threshold	Increase of Pain Threshold, %
Saline	10 ml. i.p.	10	59.2 ± 3.00	...	...	193.6 ± 10.12	...
DA 1529	25 i.p.	10	117.4 ± 8.27	98	9.50	217.2 ± 6.39	12[c]
DA 1529	50 i.p.	10	145.4 ± 16.29	146	...	237.0 ± 6.26	22[c]
DA 1529	100 i.p.	10	161.2 ± 13.15	172	...	199.0 ± 8.33	3[c]
Saline	10 ml. oral	15	53.3 ± 2.67	...	...	168.0 ± 7.59	...
DA 1529	50 oral	15	96.3 ± 9.16	81	40.15	170.5 ± 8.59	1[c]
DA 1529	100 oral	15	129.3 ± 13.95	142	...	203.9 ± 7.92	21[c]
DA 1529	200 oral	15	180.7 ± 14.92	239	...	195.3 ± 9.62	16[c]
Saline	10 ml. i.p.	15	76.0 ± 2.93	...	...	189.7 ± 9.50	...
Phenylbutazone	100 i.p.	15	146.8 ± 9.53	93	...	186.9 ± 5.99	0
Saline	10 ml. oral	15	61.6 ± 2.15	...	...	164.5 ± 5.51	...
Phenylbutazone	200 oral	15	129.2 ± 8.19	109	...	177.1 ± 5.58	8[c]

[a] 90 min. after administration of the compound. [b] Dose provoking a 50% increase in the pain threshold. [c] Difference nonsignificant at 0.05 level.

TABLE VI.—ANALGESIC ACTIVITY (HOT-PLATE METHOD) IN MICE

Compd.	Dose, mg./Kg.	Increase of Reaction Time at Intervals After Administration, % 1 hr.	% 2 hr.	Total No. of Mice Insensitive to Pain No.	%	ED_{50},[a] mg./Kg.	Confidence Limits $P = 95\%$
DA 1529	50 i.p.	124	114	8/40	20	105.0	82.03–134.40
DA 1529	100 i.p.	181	143	22/39	56	...	...
DA 1529	200 i.p.	227	161	28/40	70	...	...
DA 1529	50 oral	96	100	10/39	26	109.0	88.62–134.07
DA 1529	100 oral	131	122	16/39	41	...	...
DA 1529	150 oral	196	165	18/30	60	...	...
DA 1529	200 oral	187	180	21/29	72	...	...
Phenylbutazone	50 i.p.	12	37	0/19	0	...	...
Phenylbutazone	200 oral	47	36	0/10	0	...	...

[a] Dose inducing analgesia in 50% of the animals.

TABLE VII.—ACTIVITY ON BODY TEMPERATURE IN MICE

Compd.	Dose, mg./Kg.	Animals, No.	Body Temp., °C. ± S.E. Before Treatment	After Treatment 1 hr.	2 hr.	4 hr.	6 hr.
Saline	2 ml. i.p.	20	38.0 ± 0.14	37.5 ± 0.15	37.7 ± 0.12	37.2 ± 0.23	37.2 ± 0.19
DA 1529	25.0 i.p.	20	38.7 ± 0.09	38.0 ± 0.15	38.0 ± 0.14	37.5 ± 0.18	37.7 ± 0.13
DA 1529	50.0 i.p.	20	38.1 ± 0.18	37.1 ± 0.25	37.5 ± 0.18	37.5 ± 0.14	37.3 ± 0.14
DA 1529	100.0 i.p.	20	38.4 ± 0.12	34.9 ± 0.39	36.6 ± 0.22	37.6 ± 0.15	37.4 ± 0.17
Saline	2 ml. oral	20	37.9 ± 0.15	38.1 ± 0.13	37.9 ± 0.19	37.5 ± 0.16	37.7 ± 0.13
DA 1529	12.5 oral	20	37.9 ± 0.15	37.7 ± 0.13	37.9 ± 0.11	37.6 ± 0.13	37.3 ± 0.15
DA 1529	25.0 oral	20	37.8 ± 0.17	37.6 ± 0.15	37.6 ± 0.15	37.5 ± 0.15	37.5 ± 0.15
DA 1529	50.0 oral	20	38.3 ± 0.10	37.3 ± 0.27	37.6 ± 0.20	37.5 ± 0.20	37.5 ± 0.15
DA 1529	100.0 oral	20	38.1 ± 0.10	36.8 ± 0.21	37.4 ± 0.19	37.6 ± 0.12	37.3 ± 0.10

TABLE VIII.—ACTIVITY ON SPONTANEOUS MOTILITY, BARBITURATE DEPRESSION, MOVEMENT COORDINATION, AND ON PINNA AND CORNEAL REFLEXES IN MICE

Test	Time After Dosing, min.	Route of Administration	Animals, No.	Doses, No.	ED_{50}, mg./Kg.
Spontaneous motility	15	i.p.	120	4	27.5[a]
	15	os	90	3	94.3[a]
Hexobarbital sleeping time	15	i.p.	30	3	13.39[b]
	15	os	40	4	24.41[b]
Pentobarbital sleeping time	15	i.p.	60	3	35.35[b]
	15	os	70	4	26.74[b]
Rotarod	5	i.p.	30	3	56.0[c]
	15	os	30	3	55.0[c]
Traction	5	i.p.	30	3	88.0[c]
	15	os	30	3	117.0[c]
Inclined screen	5–15	i.p.	30	3	>200[c]
	5–15–30	os	30	3	>300[c]
Righting reflex	5–15	i.p.	30	3	>200[c]
	5–15–30	os	30	3	>300[c]
Pinna reflex	5	i.p.	60	4	98.0[c]
	15	os	30	3	298.0[c]
Corneal reflex	5–15	i.p.	60	4	>200[c]
	5–15–30	os	30	3	>300[c]

[a] Dose provoking a 50% reduction in the spontaneous motility compared with controls. [b] Dose provoking a 50% increase in the sleeping time compared with controls. [c] Dose inducing the effect considered in 50% of the animals.

compared with the controls. The compound exerted only a slight hypothermic effect at 50 mg./Kg. by the oral route. No significant change in the temperature was observed for the other doses tested (25–50 mg./Kg. i.p. and 12.5–25 mg./Kg. orally).

Activity on Spontaneous Motility.—As may be seen from Table VIII, DA 1529 administered intraperitoneally or orally, induced a significant reduction of the spontaneous motility.

Action on Hexobarbital and Pentobarbital Sleeping Time.—On both intraperitoneal and oral administration, DA 1529 markedly potentiated the hypnotic effect of hexobarbital and pentobarbital (Table VIII).

Anticonvulsant Activity.—DA 1529 injected intraperitoneally at the dose of 100 mg./Kg. was found to be inactive on convulsions induced by electroshock, pentamethylenetetrazole, or strychnine.

Action on Movement Coordination and Pinna and Corneal Reflexes.—Table VIII gives the results of the test on muscular activity and coordination and on the pinna and corneal reflexes. Table VIII reveals that the compound administered

intraperitoneally or orally exerted a marked effect on the rotarod and traction tests, at doses far from the toxic levels and from those causing behavioral changes. The effect was well marked 5 min. after intraperitoneal administration and 15 min. after oral administration. Fifteen and 30 min. after dosing the mice were normal. DA 1529 had no action on the inclined screen test and righting reflex. As for the alterations of the superficial reflexes, the pinna reflex was markedly influenced when the product was administered intraperitoneally, and only slightly so on oral administration.

By contrast the corneal reflex was not altered at any of the doses tested.

Activity on the Cardiovascular System and Respiration.—DA 1529, administered intravenously to rabbits at doses of 2, 4, and 8 mg./Kg., lowered blood pressure and increased respiration. These phenomena were only temporary and were all the greater the higher the dose. On isolated rabbit heart, DA 1529 perfused at the concentration of 10 mcg./ml., caused a slight increase in the coronary flow, and a slight reduction in the amplitude of the pulse rate and heart beat.

Action on Smooth Muscle.—On guinea pig ileum *in vitro*, DA 1529 exerted a very slight action against acetylcholine, 5-HT, and nicotine, while the antihistamine activity was still apparent, although slight (17% inhibition of histamine-induced contraction), at the concentration of 10^{-8} Gm./ml.

DISCUSSION

DA 1529 prevents the formation of edemas provoked in rats by subplantar injection of formalin, carrageenin, and dextran. More than as an anti-inflammatory, DA 1529 is active as an analgesic and antipyretic. The antinociceptive effect is very marked on the inflamed foot, insignificant on the normal foot. The antipyretic effect is well marked. Relative to phenylbutazone, DA 1529 is more active as an analgesic and antipyretic, as active or only a little less active as an anti-inflammatory. The acute toxicity of DA 1529 is similar to that of phenylbutazone. The data in Table I relative to DA 1529 have to be compared with data relative to phenylbutazone obtained in this laboratory in comparable experimental conditions (LD_{50} in mg./Kg.): 327 and 680 in mice; 215 and 637 in rats. The first value is relative to the intraperitoneal administration, the second one to the oral administration.

The therapeutic indexes (LD_{50}/ED_{50}) are as favorable for DA 1529 as for phenylbutazone. When the analgesic effect is considered the therapeutic index in rats (Randall-Selitto method) is 33 and 11 after intraperitoneal and, respectively, oral administration; in mice (hot-plate method) it is 3.0 intraperitoneally and 4.8 orally.

The anti-inflammatory, analgesic, and antipyretic effects of DA 1529 are apparent at doses provoking in rats only a mild sedation and not overt neural deficit or derangement of the autonomic nervous system or of the neuromuscular system.

DA 1529 has, however, some CNS depressant properties as the results obtained in mice seem to prove. It is active in the barbiturate potentiation test; in the rotarod test, it reduces the spontaneous motility and the sensitivity to painful stimulation. In addition, it has hypothermic properties. As it is well proved (17) that hypothermia, potentiation of the barbiturate depression, and depression of spontaneous motility are strictly correlated, it may be that the depressant effects observed in these experiments performed at 22–23° have been caused or exaggerated by the hypothermic effect. It may be the same as far as the analgesic effect observed in the hot-plate method is concerned since the thermal sensitivity of the feet of the mice is involved. Drugs modifying rectal or superficial temperature are known to have an effect on the response of mice in the hot-plate method (18–23). The analgesic effect, the superficial temperature of the paw, and the rectal temperature may, therefore, be causally related.

In the authors' opinion, however, the mild hypothermic effect is not fully responsible for the effects observed in their experiments since calming effects were still evident following administration of doses not causing hypothermia. Furthermore, the hypothermic effect of the compound was minimal compared with that caused by reserpine or chlorpromazine since, following oral administration of 50 or 100 mg./Kg., the rectal temperature, while lower than normal, was still in the range of normal values of healthy animals.

From what has been said, it is legitimate to think of DA 1529 as a mild CNS depressant agent. This mild depressant effect does not, however, detract from the potential value of the compound as an anti-inflammatory–analgesic–antipyretic agent since the depressant effect is of very short duration, while the duration of the main effect is considerably longer.

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Single-Tablet Determination of Reserpine at the Microgram Level

Estimation of Reserpine in the Presence of Protoveratrine A and Other Active Drug Components

By WILLIAM J. WEAVER

Mixtures of reserpine and other drugs can be resolved by thin-layer chromatography on silica gel, following extraction from single tablets with chloroform. Quantitation is accomplished by visual inspection, and is accurate to within 10 per cent.

THE PURPOSE of this investigation was to provide a rapid, sensitive, reliable analytical procedure to measure tablet-to-tablet variation of the active ingredients in tablets containing reserpine, protoveratrine A, and hydroflumethiazide.[1] Each tablet contains:

Reserpine	0.125 mg.
Protoveratrine A	0.200
Hydroflumethiazide[2]	50.000

The small amounts of reserpine and protoveratrine A present in single-dosage forms of drugs of this type cannot be determined by conventional analytical procedures because of interferences by other active ingredients which are present in milligram quantities.

Hydroflumethiazide can be determined spectrophotometrically by measuring the ultraviolet absorbance of a filtered methanolic extract of a single pulverized tablet. Reserpine, protoveratrine A, and tablet excipients do not interfere.

The official U.S.P. method (1) for the determination of reserpine involves solvent extraction of the reserpine and subsequent colorimetric determination. The U.S.P. extraction procedure is designed to eliminate degradation products of reserpine. In the procedure described here these purification steps are not required because the degradation compounds do not interfere in the separation and detection.

Protoveratrine A is currently determined in these laboratories by the colorimetric procedure of Craig and Jacobs (2). This procedure requires a reaction time of 16 hr. for samples, standards, and appropriate synthetic tablet blanks. In addition to eliminating these time-consuming operations, the procedure described in this report permits the simultaneous separation and detection of reserpine and protoveratrine A.

Wimer *et al.* (3) have recently reviewed procedures for the separate determination of reserpine and protoveratrine A. Although some of the methods described are extremely sensitive, they are not applicable to formulations which contain certain interfering ingredients. Levine and Fischbach (4, 5) have employed partition and paper chromatography for the separation of the veratrum alkaloids. More recently Montgomery (6) has separated protoveratrines A and B by thin-layer chromatography on cellulose.

This paper describes a thin-layer chromatographic method for the separation of reserpine and/or protoveratrine A from excipients and from other active ingredients present in several commercial tablet dosage forms. Development of the thin-layer plate with the solvent is rapid, requiring only 30 min. The detection reagent, *p*-dimethylaminobenzaldehyde in concentrated sulfuric acid, produces characteristic color reactions with reserpine and protoveratrine A. Graham (7) has investigated the color reactions between the veratrum alkaloids with this and other reagents.

EXPERIMENTAL

Equipment.—Silica Gel G scored-glass Uniplates (20 × 20 cm.) supplied by Analtech, Inc., Wilmington, Del. Reactivate the plate for 30 min. in an oven at 100° immediately before use. Glass developing-

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[1] Marketed as Salutensin tablets by Bristol Laboratories, Division of Bristol-Myers Co., Syracuse, N. Y.

[2] Marketed as Saluron by Bristol Laboratories, Division of Bristol Myers Co., Syracuse, N. Y.

TABLE I.—DETERMINATION OF RESERPINE IN COMMERCIAL SAMPLES

Tablet[a]	Active Ingredients	Label Claim, mg./Tablet	Found, Single Tablet Assay, mg.
A	Reserpine Butabarbital Hydrochlorothiazide	0.1	0.09 to 0.11
B	Reserpine Cryptenamine Methyclothiazide	0.1	0.09 to 0.11
C	Reserpine Trichlormethiazide	0.1	0.09 to 0.11
D	Reserpine Nitroglycerin Pentaerythritol tetranitrate	0.07	0.06 to 0.08
E	Reserpine Protoveratrine A Hydroflumethiazide	0.125 0.200	0.112 to 0.137 0.180 to 0.220
F	Reserpine Pyrrobutamine	0.25	0.225 to 0.275

[a] *A*, Butiserpazide −25 (McNeil); *B*, Diutensen R (Neisler); *C*, Metatensin (Lakeside); *D*, Penite (Carnrick); *E*, Salutensin (Bristol); Sandril ĉ Pyronil (Lilly).

TABLE II.—COMPARISON OF METHODS FOR DETERMINATION OF RESERPINE AND PROTOVERATRINE A IN TABLETS CONTAINING RESERPINE, PROTOVERATRINE A, AND HYDROFLUMETHIAZIDE[1]

Sample	Reserpine, mg./Tablet			Protoveratrine A, mg./Tablet		
	Label	U.S.P.	TLC-Range[a]	Label	Craig & Jacobs (2)	TLC Range[a]
1	0.125	0.122	0.112 to 0.137	0.200	0.197	0.180 to 0.220
2	0.125	0.121	0.112 to 0.137	0.200	0.204	0.180 to 0.220
3	0.125	0.120	0.112 to 0.137	0.200	0.192	0.180 to 0.220
4	0.125	0.124	0.112 to 0.137	0.200	0.194	0.180 to 0.220

[a] Acceptable concentration limits have been established as ±10% of label claim.

tanks (25.5 cm. in diameter, 25.5 cm. deep) lined with solvent-saturated filter paper. Hamilton, No. 701-N, 10-μl. syringe. Fisher 125-ml. chromatographic indicator spray flask.

Solvent.—Prepare 30 vol. % acetone, ACS, in chloroform, ACS, to serve as the developing solvent.

Spray Reagent.—Dissolve 500 mg. of *p*-dimethylaminobenzaldehyde, Eastman, in 50 ml. of concentrated sulfuric acid.

Stock Protoveratrine A Standard.—Weigh 90 mg. (±0.1 mg.) of standard protoveratrine A into a 10-ml. volumetric flask. Dissolve and dilute to volume with chloroform. This solution is stable for 1 week.

Stock Reserpine and Protoveratrine A Standard.—Weigh 11.25 mg. (±0.1 mg.) of reserpine U.S.P. into a 10-ml. low-actinic volumetric flask. Pipet 2.0 ml. of the stock protoveratrine A standard solution into the flask containing the dry reserpine. Dilute to volume with chloroform. Prepare fresh daily.

Working Reserpine and Protoveratrine A Standard. Dilute 2.0 ml. of the stock reserpine and protoveratrine A standard to 10.0 ml. with chloroform in a low-actinic volumetric flask. Prepare just prior to use. This solution contains 1.125 mcg. reserpine and 1.8 mcg. of protoveratrine A/5 μl., and 1.35 mcg. of reserpine, and 2.16 mg. of protoveratrine A/6 μl. These amounts represent reserpine and protoveratrine A corresponding to 90% and 110%, respectively, of the theoretical reserpine and protoveratrine A content in 5 μl. of the final sample concentrate.

Preparation of Sample and Application to TLC Plate.—Pulverize one Salutensin tablet, and transfer quantitatively to a 50-ml. glass-stoppered, low-actinic conical flask. Add 30 ml. of chloroform, and shake 15 min. in a mechanical shaker. Filter the chloroform extract through Whatman No. 30 filter paper into a 50-ml. serum vial protected from direct light with aluminum foil. Wash the flask and filter paper with two consecutive 2-ml. portions of chloroform. Evaporate the chloroform extract to dryness under a stream of nitrogen, then dissolve the residue in the vial with 0.50 ml. of chloroform. Stopper the vial, and swirl to ensure complete solution of the active ingredients.

Employing a 10-μl. syringe, spot consecutively on a thin-layer plate about 10 mm. apart and 20 mm. from the bottom edge of the plate: 5.0 μl. of the working standard, 5.0 μl. of the sample concentrate, and 6.0 μl. of the working standard. Maintain the diameter of the spot at 3 to 5 mm. Allow the spots to air-dry. Develop the plate with the solvent to a distance of 10 cm. from the origin.

Detection of Spots.—Remove the plates after solvent development, and allow to air-dry. Holding the sprayer 30 cm. from the plate, spray the plates lightly with the spray reagent until green spots become visible. Place the plates in an oven at 100° for 5 to 8 min. Remove the plates from the oven, and observe the positions of the spots. Protoveratrine A is indicated by an elongated, purple spot, R_f = 0.1 to 0.2. Reserpine appears as a greenish-black spot, R_f = 0.3 to 0.35.

Quantitation.—Estimate the concentrations of reserpine and protoveratrine A in the sample by comparing the sizes and intensities of the reserpine and protoveratrine A sample zones with those

obtained from the standards. Distinct differences can be noted between the standards at the 90% and 110% concentrations.

RESULTS

The method has been applied to six commercial dosage forms containing reserpine and other active ingredients. The results are shown in Table I. (The active ingredients, rauwolfia serpentina and protoveratrines A and B,[3] were also separated on a thin-layer plate. Reserpine and the protoveratrines were not quantitated against standard reserpine and protoveratrine mixtures.) The commercial tablets were obtained on the open market in bottles of 50 or 100, and single tablets of each were used for assay. None of the active ingredients or excipients present in the tablets interfered in the determination of reserpine.

Table II shows a comparison of results obtained from the determination of reserpine and protoveratrine A in tablets containing reserpine, protoveratrine A, and hydroflumethiazide[1] by the proposed single tablet method *versus* the U.S.P. method (1) for reserpine and the protoveratrine A procedure of Craig and Jacobs (2), using multiple-tablet extracts.

DISCUSSION

Sensitivity.—From 2 to 6 μl. of the working standard solution was spotted on a plate. The plate was developed and sprayed with the reagent. The lower limits of reserpine and protoveratrine A that can be detected are 0.9 and 1.44 mcg., respectively.

Interferences.—Reserpine was hydrolyzed in an alkaline medium according to the method of Neuss (8). The products, reserpic acid and trimethoxybenzoic acid, were dissolved in chloroform at a concentration equivalent to that of the working standard solution and chromatographed in the usual fashion. Reserpic acid and trimethoxybenzoic acid do not react with the reagent. When the plate is exposed to ultraviolet light, faint fluorescent spots are observed at the origin.

When the standard stock solution of reserpine and protoveratrine A is exposed to bright light for approximately 4 hr., diluted, and chromatographed, fluorescent spots are observed at the origin and at an R_f of 0.5 to 0.58. These spots are not found on a developed plate when freshly prepared, light-protected standard solutions are chromatographed. Excipients and active components found in the seven formulations reported above do not interfere.

[3] Marketed as Rauprote tablets by The Vale Chemical Co., Inc., Allentown, Pa.

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Determination of Hydrocortisone and Hydrocortisone Acetate in Antibiotic Mastitis Preparation

By ALFRED BRACEY, LEON GARRETT, and PETER J. WEISS

A modified column partition chromatographic method has been developed for the determination of hydrocortisone and hydrocortisone acetate in antibiotic mastitis preparations. A sodium bicarbonate trap under a methyl alcohol–water stationary phase in Celite is used with two mobile phases. First, the interfering oil is eluted with methylene chloride–isooctane, then the steroid to be assayed by the blue tetrazolium method is eluted with methylene chloride. This procedure shows a marked improvement over the U.S.P. XVII hydrocortisone ointment method in the removal of interferences and results in satisfactory assays for the two steroids in mastitis formulations. Recoveries of the steroids added to blank mastitis preparations ranged from 90.3 to 100.9 per cent. The average percentage of recovery for hydrocortisone and hydrocortisone acetate was 96.9 and 98.2, respectively.

CORTICOSTEROIDS have been incorporated in a large number of drug preparations, primarily for their anti-inflammatory activity. Two of the most common, hydrocortisone and hydrocortisone acetate, are often used in ointments and oils for the treatment of mastitis in dairy animals. These mastitis preparations frequently contain procaine penicillin G and a vegetable oil, *e.g.*, peanut oil, and may contain, in addition, one or more of the following: dihydrostreptomycin sulfate, neomycin sulfate, polymyxin B sulfate, sulfamerazine, sulfamethazine, sulfathiazole, sulfanilamide, methylpara-

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A modified column partition chromatographic method has been developed for the determination of hydrocortisone and hydrocortisone acetate in antibiotic mastitis preparations. A sodium bicarbonate trap under a methyl alcohol–water stationary phase in Celite is used with two mobile phases. First, the interfering oil is eluted with methylene chloride–isooctane, then the steroid to be assayed by the blue tetrazolium method is eluted with methylene chloride. This procedure shows a marked improvement over the U.S.P. XVII hydrocortisone ointment method in the removal of interferences and results in satisfactory assays for the two steroids in mastitis formulations. Recoveries of the steroids added to blank mastitis preparations ranged from 90.3 to 100.9 per cent. The average percentage of recovery for hydrocortisone and hydrocortisone acetate was 96.9 and 98.2, respectively.

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ben, propylparaben, papain, chlorobutanol, cobalt sulfate, and polyvinylpyrrolidone. Aluminum monostearate or colloidal silica is frequently used as a gelling agent.

Of the several chemical methods widely used for the assay of steroids (1–4), the blue tetrazolium method, official in U.S.P. XVII and N.F. XII, with minor modifications was chosen for this study.

The U.S.P. XVII method for hydrocortisone ointment proved unsatisfactory for mastitis preparations. Assays for hydrocortisone revealed positive interferences (ranging from less than 1 to 39% and varying with the manufacturer) when blanks were used which contained, in all cases, procaine penicillin in an oil base together with some of the above-mentioned adjuncts. The main interfering substances are procaine penicillin G and the oil.

A Celite chromatographic column with methyl alcohol–water as the stationary phase and methylene chloride–isooctane as the mobile phase is useful for removing fatty alcohols while retaining the steroid; the steroid can then be eluted with methylene chloride (5). This method is applicable to ointments containing steroids and is especially effective for removing the interfering substances contained in lanolin.

A modification of this method is presented for the determination of hydrocortisone and hydrocortisone acetate in antibiotic mastitis preparations. It consists of the following: (*a*) inserting a sodium bicarbonate trap under the methyl alcohol–water stationary phase to prevent procaine penicillin from interfering; (*b*) changing the concentration of methyl alcohol–water (1:1) stationary phase from 1 ml./Gm. of Celite to 0.5 ml./Gm. to improve recovery; (*c*) changing the isooctane–methylene chloride mobile phase to accommodate the polarity range of the two steroids; (*d*) packing the column with Celite support mixture in dry form rather than as a suspension in isooctane–methylene chloride mobile phase, and placing the sample on the column in a Celite mixture rather than in an isooctane–methylene chloride suspension. The Celite mixture technique proved more satisfactory in the authors' experience. Details of the modified assay procedure are as follows.

ASSAY PROCEDURE

Apparatus.—Chromatographic column, 15 mm. i.d., and 450 mm. long with Teflon stopcock.

Reagents.—Celite 545, methyl alcohol, isooctane, methylene chloride, glacial acetic acid, tetramethyl ammonium hydroxide (10% in water), blue tetrazolium (Dajac Laboratories), ethyl alcohol, sodium bicarbonate. All reagents are reagent grade.

Preparation of Column.—Use either glass wool or a fritted disk as the column support. Add 1 ml. of 8% sodium bicarbonate aqueous solution to 2 Gm. of Celite in a small beaker, mix well, and transfer to the column in three portions, gently packing each portion with a tamping rod made of glass or stainless steel. Add 3 ml. of methyl alcohol–water (1 + 1) to 6 Gm. of Celite, mix well, and transfer to the top of the bicarbonate column in several portions, packing gently after each portion.

Preparation of Sample.—Accurately measure or weigh a sample of mastitis preparation containing 2 mg. of steroid into a 150-ml. beaker containing 1 Gm. of Celite and mix thoroughly. Transfer this mixture to the column. Dry wash the beaker with 0.5 Gm. of Celite and add to the column.

Preparation of Standard.—Place into a 150-ml. beaker an aliquot equivalent to 2 mg. of the appropriate steroid reference standard dissolved in ethyl alcohol and evaporate to dryness on a steam bath. Dissolve the residue in 1 ml. of methyl alcohol, add 1 ml. of water, and mix well into 2 Gm. of Celite. Transfer to a column and treat standard and sample alike. This procedural standard gave assay values between 98 and 100% of a direct standard.

Elution of Column.—Add portions of methylene chloride–isooctane (1 + 9) to a sample beaker and transfer to the column. Collect 100 ml. of eluate with a flow rate of 1 drop/sec. Discard the eluate and use the same container to add methylene chloride to the column. Collect 100 ml. of eluate in a volumetric flask at the same rate. This methylene chloride fraction contains the steroid.

Spectrophotometric Assay.—Evaporate a 10.0-ml. aliquot of the methylene chloride fraction in a 50-ml. glass-stoppered conical flask to dryness on a steam bath under a gentle stream of air. Add 20.0 ml. of ethyl alcohol to the flask, swirl to complete solution, and add 2.0 ml. of blue tetrazolium solution (400 mg. in 100 ml. of ethyl alcohol) and 2.0 ml. of diluted tetramethyl ammonium hydroxide (1 ml. to 10 ml. with ethyl alcohol). After 45 min., stop the reaction by adding 1.0 ml. of glacial acetic acid. The color is then stable for 24 hr. Determine the absorbance of the solution against a reagent blank at 520 mμ on a suitable spectrophotometer, and compare with the standard treated in like fashion.

RESULTS AND DISCUSSION

Development of the Assay Procedure.—Initially the column was made with 1 ml. of methyl alcohol–water (1 + 1) per gram of Celite. Incomplete (80–85%) recoveries of steroid were obtained. A change to 0.5 ml. of stationary phase per gram of Celite improved the recoveries to over 95%. The initial mobile phase for removing the oil was a methylene chloride–isooctane mixture (2 + 8). Recovery experiments showed that this solvent was satisfactory for the hydrocortisone alcohol but not for hydrocortisone acetate because of some loss in the initial elution. A change to methylene chloride–isooctane (1 + 9) proved satisfactory, not only in removing the oil but also in yielding satisfactory recoveries of both steroids. In addition to the above steroids, satisfactory recoveries were obtained with prednisolone, prednisolone acetate, and dexamethasone alcohol during the investigational phase of this work. Some interfering substances

TABLE I.—INTERFERENCE IN DETERMINATION OF STEROIDS IN BLANK FORMULATIONS OF MASTITIS PREPARATIONS CONTAINING NO STEROID[a]

	Apparent Steroid Content, mg./Gm.				
	U.S.P. XVII Method for Hydrocortisone Ointment		New Method		
Mfr.	Anal. 1	Anal. 2	Anal. 1	Anal. 2	Anal. 3
1	0.29	0.63	0.10	0.00	0.01
2	0.44	0.78	0.17	0.10	0.10
3	0.53	0.59	0.10	0.04	...
4	0.17	0.35	0.07	0.06	0.02
5	0.01	0.00	0.07	0.01	...
6	0.25	0.29	0.16	0.05	0.07

[a] Usual concentration in commercial samples: 2–2.5 mg./Gm.

TABLE II.—RECOVERY OF STEROID ADDED TO BLANK FORMULATIONS

		Recovery, %				Recovery, %	
Mfr.	Hydrocortisone Added, mg.	Anal. 1	Anal. 2	Anal. 3	Hydrocortisone Acetate Added, mg.	Anal. 1	Anal. 2
1	2.00	98.1	97.2	...	2.02	98.0	98.5
	2.00	...	...	98.4	...	...	...
2	2.00	98.0	96.9	...	2.02	100.0	100.5
3	2.03	100.0	99.3	...	2.02	98.5	99.5
4	2.03	...	94.5	...	2.02	95.0	100.9
	2.00	...	...	94.5	...	...	...
5	2.03	97.7	98.1	...	2.02	96.5	95.0
	2.00	...	...	90.3	...	...	...

TABLE III.—DETERMINATION OF STEROIDS IN COMMERCIAL PREPARATIONS

Mfr.	Steroid	Label Claim, mg./Syringe[a]	Results	% of Label Claim
1	Hydrocortisone acetate	20	22.4	112.0
1	Hydrocortisone acetate	20	21.1	105.5
3	Hydrocortisone acetate	20	20.1	100.5
4	Hydrocortisone acetate	20	21.9	109.5
5	Hydrocortisone acetate	20	18.5	92.6
6, 1	Hydrocortisone	20	18.3	91.5
2	Hydrocortisone	20	18.4	92.2
3	Hydrocortisone	20	22.0	110.0
7	Prednisolone acetate	4	3.71	92.8

[a] Syringes tested ranged from 6 to 12 Gm. total content.

from the preparations being tested still passed through the column. On the assumption that the interference was caused by the procaine penicillin G and/or its degradation products, a sodium bicarbonate trap was placed on the bottom of the column. This removed almost all of the interference. A stronger alkaline trap would undoubtedly be satisfactory in holding back the interference but would encourage the degradation of the steroid. A comprehensive study of interference by antibiotics and other pharmaceutical ingredients in the various assay methods (2–4) is in progress.

Hydrocortisone and hydrocortisone acetate carried through this column procedure were examined by thin-layer chromatography (6). No degradation was observed.

Preliminary experiments indicate that this chromatographic system is also applicable to a large number of aqueous preparations, ointments, lotions, and creams containing antibiotics and steroids.

Application of the Assay Procedure.—The interferences caused by blank mastitis preparations, which contained all ingredients except the steroids (obtained through the courtesy of six manufacturers), when tested by the U.S.P. XVII method for hydrocortisone ointment and by the new procedure, are shown in Table I.

As shown in Table II, the recoveries of steroids added to blank mastitis ointments range from 90.3 to 100.0% for hydrocortisone and 95.0 to 100.9% for hydrocortisone acetate. These recovery data were based on direct standards rather than procedural standards.

Table III shows that the hydrocortisone and hydrocortisone acetate content of nine commercial preparations assayed by the new procedure ranged from 91.5 to 112% of label claim.

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Dye–Polyethylene Glycol 6000 Interactions in Filmcoating Solutions and Their Effect on Color Uniformity

By D. G. BHATIA*, T. D. SOKOLOSKI†, and V. N. BHATIA

The objective of this study was to evaluate the problems of uneven coloration in tablets filmcoated with solutions utilizing polyethylene glycol (PEG 6000) and cellulose acetate phthalate (CAP) in a solvent system consisting of 65 per cent acetone and 35 per cent absolute alcohol. It was hypothesized that to obtain uniform color the dye needed to be brought into solution due to interaction between it and one of the other ingredients in the solution. Attention was focused on CAP, PEG 6000, and titanium dioxide. The procedures included the following steps: (*a*) study of the interaction between dyes, and PEG 6000; (*b*) study of interaction between dyes and CAP; (*c*) testing of the hypothesis stated above in filmcoating operations; (*d*) study of the function of titanium dioxide in filmcoating solutions. The results show that the interaction of PEG 6000 with the solid dye results in the solubilization of the latter and this is significant in producing uniform color. No interaction was observed between CAP and the dyes. Titanium dioxide was found to act purely mechanically in preventing the aggregation of dye particles.

ONE TYPE of filmcoating solution that is frequently used utilizes a combination of cellulose acetate phthalate (CAP) and polyethylene glycol 6000 (PEG 6000) as the film former in a solvent system consisting of acetone (65%) and absolute alcohol (35%). Such a filmcoating solution was described by Gross and Endicott in 1960 (1). While the formula for such a solution consists of a variety of auxiliary agents such as flavoring agents, sweeteners, etc., the basic ingredients are as follows:

CAP	6%
PEG 6000	14%
Dye	0.05%
Titanium dioxide	2%
Solvent system (acetone 65%, absolute alcohol 35%)	*q.s.* to 100%

The dyes used in this formula are essentially insoluble in the solvent system, and it has been noted that their action is often unpredictable with respect to the uniformity of color deposition. In certain concentrations some of the dyes produce coatings with a mottled appearance, particularly when the filmcoating solution is not rendered opaque by the titanium dioxide.

The objective of this investigation was to study the cause of the lack of uniformity in the color deposition. It was hypothesized that to obtain a uniform color coverage the dye must be brought into solution. This could be achieved only by interaction between the dye and some ingredient in the filmcoating formulation causing the formation of a soluble complex.

Since it was observed that titanium dioxide played some role in obscuring the mottled appearance of the filmcoated tablets, its function in a filmcoating formulation was also investigated.

The procedure adopted in this investigation consisted of the following steps: (*a*) study of any interaction between several dyes and PEG 6000; (*b*) study of any interaction between several dyes and CAP; (*c*) testing of the hypothesis presented above by the use of the interaction data of two of the dyes in actual filmcoating operations; (*d*) study of the function of titanium dioxide in a filmcoating formulation.

EXPERIMENTAL PROCEDURE[1] AND RESULTS

The materials used in this study included the following: dyes (provided by Abbott Laboratories): FD&C Blue No. 1 (brilliant blue), FD&C Yellow No. 5 (tartrazine), FD&C Red No. 1 (Ponceau 3R), FD&C Red No. 2 (amaranth red), FD&C Red No. 4 (Ponceau SX); CAP (Eastman Kodak Co.); PEG 6000 (Union Carbide Chemicals Co.); and titanium dioxide (Fisher Scientific Co.).

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[1] All spectrophotometric analyses were conducted on a Beckman DB recording spectrophotometer.

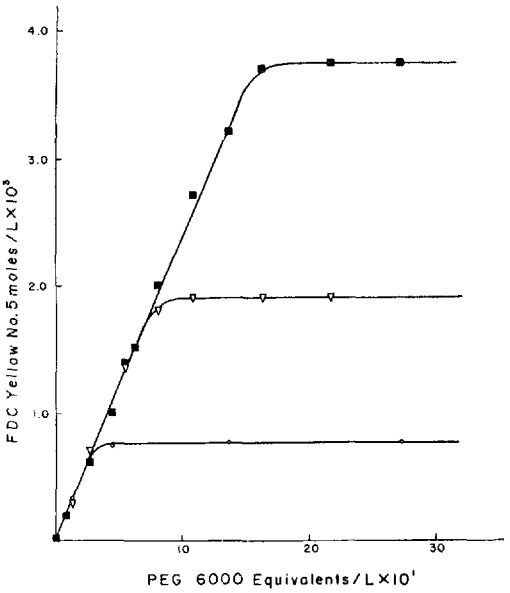

Fig. 1.—Interaction between dye FD&C Yellow No. 5 and PEG 6000. Key: ■, 50 mg.; ▽, 25 mg.; O, 10 mg.

Interaction Studies Between PEG 6000 and Dyes

In this study the following dyes were used: FD&C Yellow No. 5 (mol. wt. = 534.39), FD&C Blue No. 1 (mol. wt. = 792), FD&C Red No. 2 (mol. wt. = 604.49), FD&C Red No. 1 (mol. wt. = 494.46), and FD&C Red No. 4 (mol. wt. = 480).

General Procedure.—All interaction studies between PEG 6000 and dyes were carried out by the phase solubility method (2). A certain amount[2] of the dye under consideration was placed in each of a series of 100-ml. glass-stoppered volumetric flasks. Several stock solutions of PEG 6000 in the solvent system were made. The concentrations of the stock solution were varied, depending on the requirements of a particular experiment. Different volumes of PEG 6000 stock solutions representing different amounts of PEG 6000 were added to each flask. Then, with the aid of a buret, sufficient amounts of the solvent system were added to make up the volume to 25 ml. in each flask. Solutions were shaken in a mechanical shaker at a constant temperature of 25° for 24 hr. to establish dynamic equilibrium. Solutions were then filtered using Whatman No. 2 filter paper, analyzed spectrophotometrically, and the concentrations of the dye were determined from the Beer's law plots.

Three sets of studies using 10 mg., 25 mg., and 50 mg. of dye, respectively, were carried out with FD&C Yellow No. 5 and FD&C Reds No. 1, No. 2, and No. 4. In the case of FD&C Blue No. 1 the amounts of the dye used in the three studies were 200 mg., 250 mg., and 350 mg.

The results of the interaction studies between FD&C Yellow No. 5 and PEG 6000 are shown in Fig. 1. (The interaction between FD&C Blue No. 1 and PEG 6000 gave a similar plot and hence is not illustrated.)

The results of the interaction studies between FD&C Red No. 2 and PEG 6000 are shown in Fig. 2. (The interactions between FD&C Reds No. 1 and No. 4 and PEG 6000 gave similar plots and hence are not illustrated.)

Interaction Studies Between CAP and Dyes

No interaction was noted between CAP and any of the dyes tested.

Filmcoating Studies.—Two of the dyes were selected for this study: FD&C Yellow No. 5 and FD&C Red No. 2. Their selection was based on the fact that they showed two different types of interaction.

Preparation of the Test Tablets.—The test tablets containing equal amounts of sodium bicarbonate and lactose were prepared. Starch paste was used as the granulating agent and magnesium stearate (0.75%) as the lubricant. The tablets were compressed on a Stokes B-2 machine using $^{7}/_{16}$-in. standard concave punches to a pressure of 8–10 Strong-Cobb units. The weight of each tablet was 650 mg. (±5%). The average thickness of the tablets was 5.10 mm.

Preparation of the Filmcoating Solution.—The basic filmcoating formula used in all these studies was:

CAP	6%
PEG 6000	14%
Acetone 65% } q.s.	100%
Absolute ethanol 35% }	

The filmcoating solutions used with the various dyes are presented in Table I (FD&C Yellow No. 5) and Table II (FD&C Red No. 2) and were the modification of the above basic formula.

The test formulations presented above were designed to be of the following two main types.

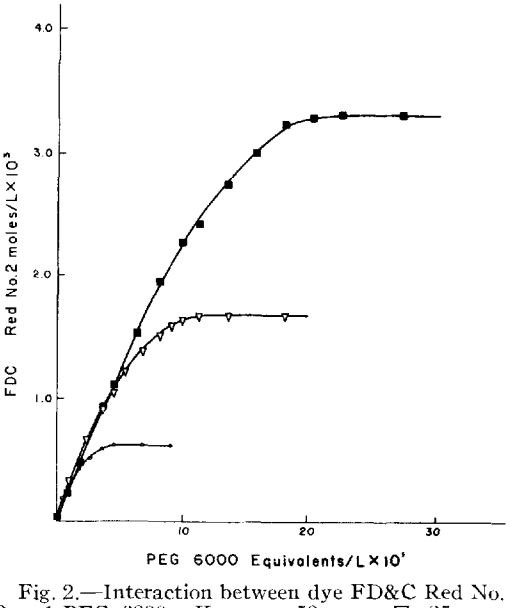

Fig. 2.—Interaction between dye FD&C Red No. 2 and PEG 6000. Key: ■, 50 mg.; ▽, 25 mg.; O, 10 mg.

[2] The amounts used for each dye were chosen on the basis of (*a*) solubility of the dye and (*b*) knowledge of the amount of the dye generally used in the filmcoating formulations.

TABLE I.—COMPOSITION OF TEST FILMCOATING FORMULATIONS USING FD&C YELLOW NO. 5 AND THE OCCURRENCE OF MOTTLING ON THE TABLETS COATED BY THEM

	Formulation No.			
	YI	YII	YIII	YIV
Dye	0.875 Gm.	7 Gm.	0.875 Gm.	7 Gm.
CAP	30 Gm.	30 Gm.	30 Gm.	30 Gm.
PEG 6000	70 Gm.	70 Gm.	70 Gm.	70 Gm.
Titanium dioxide	...	...	10 Gm.	10 Gm.
Acetone, 65% / Absolute ethanol, 35% (q.s.)	500 ml.	500 ml.	500 ml.	500 ml.
Occurrence of mottling	No	Yes	No (Final color lighter than YI)	No (Final color lighter than YII)

TABLE II.—COMPOSITION OF TEST FILMCOATING FORMULATIONS USING FD&C RED NO. 2 AND THE OCCURRENCE OF MOTTLING ON THE TABLETS COATED BY THEM

	Formulation No.			
	RI	RII	RIII	RIV
Dye	0.675 Gm.	5.4 Gm.	0.675 Gm.	5.4 Gm.
CAP	30 Gm.	30 Gm.	30 Gm.	30 Gm.
PEG 6000	70 Gm.	70 Gm.	70 Gm.	70 Gm.
Titanium dioxide	...	...	10 Gm.	10 Gm.
Acetone, 65% / Absolute ethanol, 35% (q.s.)	500 ml.	500 ml.	500 ml.	500 ml.
Occurrence of mottling	No	Yes	No (Final color lighter than RI)	No (Final color lighter than RII)

(*a*) Those containing 4 times (a large excess) the amount of dye that could be solubilized by PEG 6000; *i.e.*, formulation No. YII, YIV, RII, and RIV. (*b*) Those containing half the amount of dye that could be solubilized by PEG 6000; *i.e.*, formulation No. YI, YIII, RI, and RIII.

The determination of the amount of dye used in each formulation was based on the dye–PEG 6000 interaction studies. A typical calculation is shown in the illustration for FD&C Yellow No. 5 (Fig. 1).

On an average, 0.91 equivalents[3] of PEG 6000 (40 Gm.) would completely solubilize 0.0018 equivalents of the dye (1 Gm.). Therefore, 140 Gm. (the amount used per liter of a typical filmcoating formulation) of PEG 6000 would solubilize 3.5 Gm. of the dye.

The two dye concentrations used were $3.5 \times 4 = 14$ Gm./L. (excess dye in suspension) and $3.5/2 = 1.75$ Gm./L. (dye completely solubilized).

In formulations YI, YII, RI, and RII the method of preparation was carried out in the following steps. (*a*) The requisite amount of dye was milled for 24 hr. with 200 ml. of solvent system in a ball mill. (*b*) This mixture was transferred to a bottle containing a solution of 30 Gm. of CAP and 70 Gm. of PEG 6000 in 200 ml. of the solvent system. (*c*) The volume was made up to 500 ml. with the solvent system.

In formulations YIII, YIV, RIII, and RIV, the method of preparation was carried out in the following steps. (*a*) The requisite amounts of dye and titanium dioxide were milled for 24 hr. with 200 ml. of solvent system in a ball mill. (*b*) This mixture was transferred to a bottle containing a solution of 30 Gm. of CAP and 70 Gm. of PEG 6000 in 200 ml. of the solvent system. (*c*) The volume was made up to 500 ml. with the solvent system. All the solutions were kept in a water bath at 55–60°.

[3] Equivalents of PEG 6000 were calculated on the basis of oxyethylene linkages (44 Gm. per equivalent).

Filmcoating Procedure

The filmcoating was carried out using a pear-shaped copper pan of 16 in. maximum diameter with a panload of 5 lb. Four tape-baffles were used to improve the rolling character of the tablets.

Initially a mixture of 50 ml. of the filmcoating solution diluted with 50 ml. of the solvent system was used. This diluted solution was poured in a series of 5 to 10-ml. portions in a thin stream over the tumbling tablets and was added as quickly as possible, making sure that the tablets were perfectly dry between each addition. Then 75 ml. of filmcoating solution (undiluted) was added in a series of 2 to 5-ml. portions. Then the tablets were removed and the pan was washed and dried. The pan was recharged and another 100 ml. of the filmcoating solution (undiluted) was added in a series of 2 to 5-ml. portions. Then the coated tablets were dried and samples were taken.

The results obtained using the various formulations are also shown in Table I (FD&C Yellow No. 5) and Table II (FD&C Red No. 2). It can be seen that mottling occurred only in the tablet coats which contained an excess of the dye and did not contain titanium dioxide. It was also noted that the final color of the tablets coated with formulations containing titanium dioxide was lighter than that of the tablets on which a corresponding formulation without titanium dioxide was used.

Study of the Function of Titanium Dioxide in the Filmcoating Formulation

The following two approaches were used in this study to determine if the presence of titanium dioxide altered the interaction phenomenon between dye and PEG 6000. This study was carried out using FD&C Yellow No. 5 and FD&C Red No. 2.

A.—A constant amount (excess) of the dye under consideration was placed in a series of 100-ml. glass-stoppered volumetric flasks. Amounts of titanium dioxide varying from 0 to 750 mg., in 125-mg. increments, were also placed in the series of flasks. Twenty-five milliliters of the 14% PEG 6000 solution was added to each flask. The solutions were shaken in a mechanical shaker at a constant temperature of 25° for 24 hr. to establish dynamic equilibrium. Solutions were then filtered using Whatman No. 2 filter paper and analyzed spectrophotometrically.

B.—A constant amount (excess) of the dye under consideration was placed in a series of 100-ml. volumetric flasks. Twenty-five milliliters of 14% PEG 6000 solution was added to each flask. The solutions were shaken in a mechanical shaker at a constant temperature of 25° for 24 hr. to establish dynamic equilibrium. Amounts of titanium dioxide varying from 0 to 750 mg., in 125-mg. increments were added to the series of solutions and again the solutions were shaken for another 24 hr. at a constant temperature of 25°. The solutions were then filtered using Whatman No. 2 filter paper and analyzed spectrophotometrically.

The results obtained using the above two approaches were identical to those obtained from experiments conducted under similar conditions without the addition of titanium dioxide.

DISCUSSION

Interaction Studies Between PEG 6000 and Dyes

Interaction Studies Between Dye FD&C Yellow No. 5 and PEG 6000.—The type of interaction between FD&C Yellow No. 5 and PEG 6000 may be explained on the basis of an adsorption phenomenon. It is postulated that there exists an equilibrium between the solid dye, PEG 6000 in solution, and the adsorbed species. Then the adsorbed species goes into solution. The possible scheme of interaction can be represented as:

$$Y + P \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} YP_s \xrightarrow{k_2} YP_l$$

where

Y = FD&C Yellow No. 5,
P = PEG 6000,
YP_s = adsorbed species (solute P adsorbed on solid Y),
YP_l = adsorbed species in solution.

Various approaches were tried to substantiate the existence of the adsorption phenomena mentioned above. These include the use of Freundlich's isotherm (3) and elemental and infrared spectral analysis of the residues from different regions of the interaction curves.

The use of the isotherm could not be applied in the case of FD&C Yellow No. 5 because a method of direct determination of the weight of the solute adsorbed on the dye surface or indirect determination from analyzing the solution at a particular instant for PEG 6000 content was too cumbersome and hence could not be established.

The results obtained from the two analyses did not prove to be definitive and, hence, these experiments neither proved nor disproved the existence of the adsorption phenomenon.

It is observed from Fig. 1 that as the concentration of PEG 6000 is increased, the amount of dye being constant, the curve levels off at some concentration of PEG 6000. Since such a plateau would occur when all the dye molecules are coated by PEG 6000 molecules resulting in the solubilization of the dye, the amount of PEG 6000 (expressed in terms of equivalents of oxyethylene linkages) required to solubilize a mole of dye could be obtained from Fig. 1. For example, in Fig. 1 (10 mg. dye) 4.0×10^{-1} equivalents per liter of PEG 6000 (the point where the curve starts leveling off) are required to solubilize 0.76×10^{-3} moles/L. of dye. Thus,

$$\frac{\text{PEG in equivalents/L.}}{\text{dye in moles/L.}} = \frac{4.0 \times 10^{-1}}{0.76 \times 10^{-3}} = 5.26 \times 10^{2}$$

Similarly, the ratios of 5.26×10^2 and 5.20×10^2 are obtained from 25 mg. dye and 50 mg. dye plots in Fig. 1, respectively. Thus, the average ratio,

$$\frac{\text{PEG in equivalents/L.}}{\text{dye in moles/L.}} = 5.24 \times 10^{2}$$

That is, on an average, 524 equivalents of oxyethylene linkages are required to solubilize 1 mole of FD&C Yellow No. 5.

Interaction Studies Between Dye FD&C Blue No. 1 and PEG 6000.—The type of interaction between FD&C Blue No. 1 and PEG 6000 observed was similar to that observed between FD&C Yellow No. 5 and PEG 6000, and could be explained on a similar basis.

It was found that on an average, 51 equivalents of oxyethylene linkages are required to solubilize 1 mole of FD&C Blue No. 1.

Interaction Studies Between Dye FD&C Red No. 2 and PEG 6000.—The type of interaction between FD&C Red No. 2 and PEG 6000 could also be explained on the basis of adsorption phenomenon and may be represented schematically as:

$$A + P \overset{1}{\rightleftharpoons} AP_s$$

$$AP_s + nP \overset{2}{\rightleftharpoons} AP_{ns} \rightarrow AP_{nl}$$

where

A = FD&C Red No. 2,
P = PEG 6000,
AP_s = primary adsorbed species (solute P adsorbed on the solid A),
AP_{ns} = secondary adsorbed species,
AP_{nl} = secondary adsorbed species in solution.

FD&C Red No. 2 differs from FD&C Yellow No. 5 and FD&C Blue No. 1 in that a shift in the phase solubility curve to the right is observed (Fig. 2) as the initial amount of the dye is increased. Because of this observation the interaction of FD&C Red No. 2 with PEG 6000 was studied in greater detail as shown in Fig. 3. This shift was seen to occur in all the studies shown in this figure. Such a shift indicates that as the dye concentration is

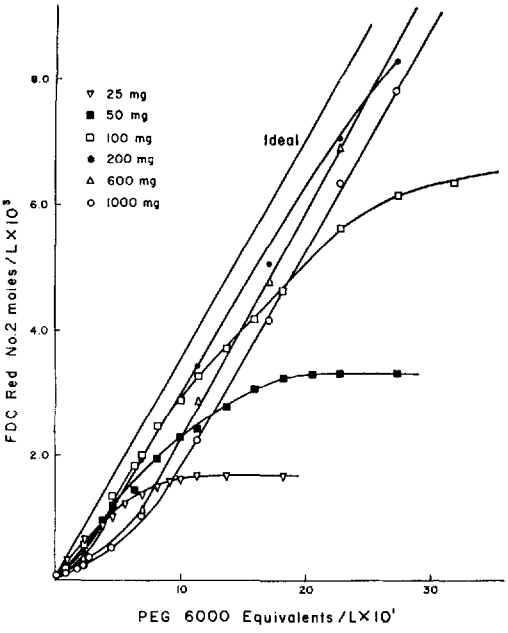

Fig. 3.—Interaction between dye FD&C Red No. 2 and PEG 6000.

increased under conditions in which the PEG 6000 concentration is kept constant, less dye actually enters into solution. This phenomenon could be explained on the basis of the relative insolubility of the primary adsorbed species AP_s (step *1* in the interaction scheme). A greater initial amount of the dye would adsorb more PEG 6000 from its solution forming the insoluble species in relatively larger amounts. Thus a smaller amount of PEG 6000 would be available to form the secondary adsorbed species AP_{ns} (step *2* in the interaction scheme) which leads to solubilization (step *3* in the interaction scheme).

At a lower initial amount of the dye, since less PEG 6000 is required for primary adsorption, more PEG 6000 would be available for secondary adsorption, and, hence, the higher solubility of the dye is observed. It is assumed here that AP_s is practically insoluble and the concentration of dye read at the absorption maximum at any instant is directly proportional to the amount of AP_{nl} in solution.

To substantiate the hypothesis that an adsorption phenomenon (adsorption of solute PEG 6000 from solution onto the solid dye) from solution occurs, the empirical isotherm suggested by Freundlich (3) mentioned earlier may be applied here.

An ideal line, *i.e.*, the line representing no shift due to adsorption phenomenon, is drawn (Fig. 3) passing through the initial point (point representing the saturation solubility of the dye in the absence of PEG 6000) and parallel to the interaction lines in the higher concentration region of PEG 6000.

To apply the Freundlich isotherm, the weight of PEG 6000 adsorbed on the dye is first determined. An illustration to calculate the values of x is as follows:

$$x = x_t - x_i$$

where

x = weight of PEG 6000 (in equivalents per liter) adsorbed,
x_t = weight of PEG 6000 (in equivalents per liter) taken,
x_i = weight of PEG 6000 (in equivalents per liter) required to solubilize the same amount of dye if no shift in the curves occurred.

Referring to Fig. 3 (1000 mg. dye curve which represents the dye in a concentration of 1 Gm./25 ml. or 40 Gm./L.), when $x_t = 0.5$, $x_i = 0.15$, then $x = 0.35$.

Since the plot of log x/m against log C (Fig. 4) yields a straight line, the experimental results conform to the Freundlich expression indicating the existence of adsorption phenomenon.

In the lower concentration region of PEG 6000 and when the amount of dye present is large, the primary adsorption phenomenon would be predominant. However, as the concentration of PEG 6000 in solution is increased, the secondary adsorption and solubilization phenomenon would come into predominance. Approaching the plateau region of the curves, the solubilization process would be predominant in nature.

In case of FD&C Red No. 2 also, the residues from ten different regions of the interaction curves after dynamic equilibrium was established were collected and dried. Here also no definite conclusions could be drawn either from elemental (C, H, N) analysis or infrared spectra (using KBr pellet method) of the residues. The above analyses did not prove or disprove the existence of the adsorption phenomenon. The ratio

$$\frac{\text{PEG in equivalents/L.}}{\text{dye in moles/L.}}$$

for FD&C Red No. 2 is calculated in the same manner as shown with FD&C Yellow No. 5. On an average 721 equivalents of oxyethylene linkages are required to solubilize 1 mole of FD&C Red No. 2.

Interaction Studies Between Dye FD&C Red No. 1 and PEG 6000 and FD&C Red No. 4 and PEG 6000.—These interactions were similar to that ob-

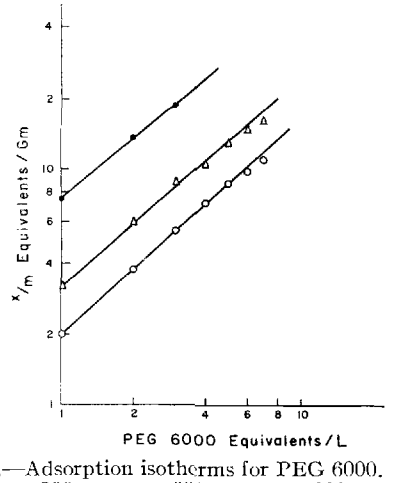

Fig. 4.—Adsorption isotherms for PEG 6000. Key: ●, 200 mg.; △, 600 mg.; ○, 1000 mg.

served between FD&C Red No. 2 and PEG 6000, and can be explained similarly on the basis of adsorption phenomenon. On an average, 700 equivalents of oxyethylene linkages are required to solubilize 1 mole of FD&C Red No. 1, and 450 equivalents to solubilize 1 mole of FD&C Red No. 4.

Filmcoating Studies.—The relative uniformity of color produced by formulations YI (FD&C Yellow No. 5) and RI (FD&C Red No. 2) contrasted with the highly mottled appearance produced by formulations YII (FD&C Yellow No. 5) and RII (FD&C Red No. 2) substantiates the hypothesis presented earlier that to achieve uniform color coverage the dye must be brought into solution. However, the results shown by formulations YIV (FD&C Yellow No. 5) and RIV (FD&C Red No. 2) do not substantiate this hypothesis. Although here the amount of dye is in excess (same as in YII and RII, respectively), the presence of titanium dioxide helps to prevent the expected mottled appearance of the tablets.

Study of the Function of Titanium Dioxide in the Filmcoating Formulation.—The results obtained in the study designed to evaluate the function of titanium dioxide in the filmcoating formulation showed no change in the adsorption curve patterns obtained for PEG 6000–dye interactions. Hence, it is concluded that titanium dioxide does not act as an adsorbent for the dye or PEG 6000 and thus does not aid in preventing mottling by an adsorption process. Furthermore, the studies show that the titanium dioxide does not act in any way to affect the interactions between the dyes and PEG 6000. However, the results in the filmcoating studies show that even when the dye was not completely solubilized by PEG 6000, the mottling was considerably reduced by the addition of titanium dioxide to the filmcoating formula. Therefore, it is concluded that the presence of titanium dioxide promotes color uniformity simply due to "mechanical effect" of its bulk; that is, the larger number of titanium dioxide particles present tends to mechanically prevent the aggregation of the dye particles.

CONCLUSIONS

The results of the present investigation have demonstrated: (*a*) PEG 6000 interacts with the dyes tested (FD&C Red No. 1, 2, and 4; FD&C Yellow No. 5; and FD&C Blue No. 1). The interaction (adsorption of PEG 6000 onto the solid dye) results in solubilization of the dyes which are almost insoluble in the solvent system used (65% acetone and 35% absolute ethanol). (*b*) No interaction is observed between CAP and the dyes tested. (*c*) Presence of titanium dioxide does not alter or affect the adsorption of PEG 6000 onto dyes.

Thus, in a typical basic filmcoating formulation containing dye, PEG 6000, CAP, and titanium dioxide in the nonaqueous solvent system, the interaction between PEG 6000 and dyes plays a significant role in affecting the uniformity of color. In order to assure maximum color uniformity, the amount of dye in a filmcoating formulation should not exceed the amount capable of being brought into solution by PEG 6000. The purely mechanical effect of titanium dioxide in preventing the aggregation of dye particles also plays a significant role in achieving color uniformity.

The information provided by this study gives some insight into methods that may be employed in formulating filmcoating solutions on the basis of scientific data rather than by the use of purely empirical techniques.

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Experiences in Development of Directly Compressible Tablets Containing Potassium Chloride

By JACK LAZARUS and LEON LACHMAN

The influence of particle size distribution, particle shape, apparent bulk density, moisture content, additives, and punch shape on the directly compressible characteristics of potassium chloride were investigated. The relative weight and drug variability of hydrochlorothiazide–potassium chloride tablets prepared by direct compression were compared with those prepared by customary wet granulating techniques.

ACCORDING TO the literature (1, 2) it should be possible to directly compress crystals belonging to the cubic system into conventional flat-faced or biconvex tablets. Potassium chloride crystals fall under this classification and can normally be directly compressed into such tablets. However, when the tablet shape is altered, not all batches of U.S.P. potassium chloride crystals obtained from different suppliers could be directly

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The authors express their appreciation to Mr. James B. Smith for his analytical assistance.

served between FD&C Red No. 2 and PEG 6000, and can be explained similarly on the basis of adsorption phenomenon. On an average, 700 equivalents of oxyethylene linkages are required to solubilize 1 mole of FD&C Red No. 1, and 450 equivalents to solubilize 1 mole of FD&C Red No. 4.

Filmcoating Studies.—The relative uniformity of color produced by formulations YI (FD&C Yellow No. 5) and RI (FD&C Red No. 2) contrasted with the highly mottled appearance produced by formulations YII (FD&C Yellow No. 5) and RII (FD&C Red No. 2) substantiates the hypothesis presented earlier that to achieve uniform color coverage the dye must be brought into solution. However, the results shown by formulations YIV (FD&C Yellow No. 5) and RIV (FD&C Red No. 2) do not substantiate this hypothesis. Although here the amount of dye is in excess (same as in YII and RII, respectively), the presence of titanium dioxide helps to prevent the expected mottled appearance of the tablets.

Study of the Function of Titanium Dioxide in the Filmcoating Formulation.—The results obtained in the study designed to evaluate the function of titanium dioxide in the filmcoating formulation showed no change in the adsorption curve patterns obtained for PEG 6000–dye interactions. Hence, it is concluded that titanium dioxide does not act as an adsorbent for the dye or PEG 6000 and thus does not aid in preventing mottling by an adsorption process. Furthermore, the studies show that the titanium dioxide does not act in any way to affect the interactions between the dyes and PEG 6000. However, the results in the filmcoating studies show that even when the dye was not completely solubilized by PEG 6000, the mottling was considerably reduced by the addition of titanium dioxide to the filmcoating formula. Therefore, it is concluded that the presence of titanium dioxide promotes color uniformity simply due to "mechanical effect" of its bulk; that is, the larger number of titanium dioxide particles present tends to mechanically prevent the aggregation of the dye particles.

CONCLUSIONS

The results of the present investigation have demonstrated: (*a*) PEG 6000 interacts with the dyes tested (FD&C Red No. 1, 2, and 4; FD&C Yellow No. 5; and FD&C Blue No. 1). The interaction (adsorption of PEG 6000 onto the solid dye) results in solubilization of the dyes which are almost insoluble in the solvent system used (65% acetone and 35% absolute ethanol). (*b*) No interaction is observed between CAP and the dyes tested. (*c*) Presence of titanium dioxide does not alter or affect the adsorption of PEG 6000 onto dyes.

Thus, in a typical basic filmcoating formulation containing dye, PEG 6000, CAP, and titanium dioxide in the nonaqueous solvent system, the interaction between PEG 6000 and dyes plays a significant role in affecting the uniformity of color. In order to assure maximum color uniformity, the amount of dye in a filmcoating formulation should not exceed the amount capable of being brought into solution by PEG 6000. The purely mechanical effect of titanium dioxide in preventing the aggregation of dye particles also plays a significant role in achieving color uniformity.

The information provided by this study gives some insight into methods that may be employed in formulating filmcoating solutions on the basis of scientific data rather than by the use of purely empirical techniques.

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Experiences in Development of Directly Compressible Tablets Containing Potassium Chloride

By JACK LAZARUS and LEON LACHMAN

The influence of particle size distribution, particle shape, apparent bulk density, moisture content, additives, and punch shape on the directly compressible characteristics of potassium chloride were investigated. The relative weight and drug variability of hydrochlorothiazide–potassium chloride tablets prepared by direct compression were compared with those prepared by customary wet granulating techniques.

ACCORDING TO the literature (1, 2) it should be possible to directly compress crystals belonging to the cubic system into conventional flat-faced or biconvex tablets. Potassium chloride crystals fall under this classification and can normally be directly compressed into such tablets. However, when the tablet shape is altered, not all batches of U.S.P. potassium chloride crystals obtained from different suppliers could be directly

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compressed to produce tablets with satisfactory physical properties. The term "directly compressed" as used in this text refers to the compression of a mixture of materials which have been subjected to thorough mixing prior to compression in order to obtain a uniform distribution of components of the mixture. By this process, treatments, such as drying the wet granulation, rescreening, and lubricating are not necessary.

Several physical factors appear to influence the direct compression of potassium chloride crystals into modified ball-shaped tablets. This report will relate findings on the influence of particle size distribution, particle shape, apparent bulk density, moisture, drug, and lubricant on the compaction characteristics of potassium chloride crystals.

EXPERIMENTAL

Formulations.—The formula used for screening the direct compressibility of potassium chloride crystals was:

	1000 Tablets
Potassium chloride crystals	1000 Gm.
Stearic acid, powdered	5

Pass the potassium chloride crystals and the stearic acid through a No. 30 mesh stainless steel screen and mix well.

Wet Granulation Formula	1000 Tablets
Potassium chloride crystals	1000 Gm.
Hydrochlorothiazide crystals	50
Gelatin	10
Stearic acid, powder	5

Pass the potassium chloride and hydrochlorothiazide through a No. 30 mesh stainless steel screen and mix well. Dissolve the gelatin in a suitable quantity of deionized water and granulate. Screen the granulation to break up the lumps, dry on trays in a circulating air oven, rescreen, and lubricate.

Direct Compression Formula	1000 Tablets
Potassium chloride crystals	1000 Gm.
Hydrochlorothiazide crystals	50
Polyethylene glycol 6000, powdered	5
Deionized water	1.0 ml.
Colloidal silica	5 Gm.
Stearic acid	5
Talc	10

Pass the potassium chloride, hydrochlorothiazide, and the polyethylene glycol 6000 through a No. 30 mesh stainless steel screen. Mix well and then spray in the water by means of an atomizer or a pressure-actuated spray nozzle. Mix well, then add the colloidal silica, stearic acid, and talc. Mix until blended. The granulation is now ready for compression.

Compaction.—A four-station, single-rotary Colton 204 tablet press, using $^{15}/_{32}$ in. modified ball stainless steel punches, was used to screen the direct compressibility of the different batches of potassium chloride crystals. The batch of crystals which compressed satisfactorily was then formulated with hydrochlorothiazide. The mixtures which produced satisfactory tablets on the Colton 204 were scaled up for compression on a 27-station, double-rotary tablet press. The production-size batches were sufficient to make 240,000 tablets and were compressed on a Stokes 541 double-rotary press, operating at a speed that produced 2000 tablets/min.

The four-station, single-rotary press was used rather than a single-punch machine for the screening studies in order to more closely approximate the compression force distribution encountered when the tablets are compressed in production on a larger rotary press. Furthermore, in a single-punch machine, the lower punch is stationary and only the upper punch exerts the pressure at the time of maximum compression. In a rotary press, both the upper and lower tablet punches approach one another simultaneously up to the point of maximum pressure. Consequently, the strains induced in tablets produced on a single-punch machine would be different than those obtained from a rotary press.

For this investigation, modified ball-shaped tablets were chosen since such tablets provide a minimum volume for a heavy tablet and a narrow edge which facilitates the application of a coating onto the tablets. A tablet compressed with standard concave punches or extra deep concave punches would have a wider edge which is not so easily coated. Figure 1 shows a composite photo of these different shaped tablets.

Friabilator.—A modified Roche friabilator (3) was employed to determine the resistance of the tablet to breaking. Both a plastic and stainless steel frame were used initially for tumbling the tablets. The plastic frame was selected for use after the initial evaluations since it was found that each frame gave comparable results. Twenty tablets were placed in the friabilator which was then set in motion and timed. The test was terminated when one or more tablets were broken. Four minutes was the minimum acceptable time based on experience gained in spray coating of these tablets.

Particle Size Distribution.—Two hundred grams of potassium chloride crystals were sized on 8-in. diameter stainless steel screens (U. S. sieve series) using a Rotap sieve shaker operating for 20 min. The per cent of the crystals retained on each screen was then determined.

Apparent Bulk Density.—A weighed sample of crystals was placed into a graduated cylinder and tamped a given number of times from a uniform height. The volume of the crystals in the cylinder was used to estimate the apparent bulk density.

Moisture Determination.—The potassium chloride crystals were placed into weighing bottles fitted with ground-glass covers and weighed on a Mettler semimicro analytical balance. The bottles were previously exposed to 175° for 20 hr. The samples of crystals were placed in an oven set at 105 ± 1° for 2 hr. to remove surface moisture. The bottles were then stoppered, placed into a desiccator, and weighed when at room temperature. After weighing, the bottles were transferred to the 175 ± 1° for 20 hr., after which they were stoppered, cooled in a desiccator until at room temperature, and reweighed.

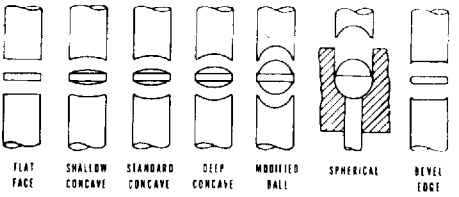

Fig. 1.—Concave punches used in tablet manufacturing.

TABLE I.—MAXIMUM SAFE PRESSURE IN TONS ON TABLET MACHINE PUNCHES[a]

Punch Diam. Fraction	Area of Punch Face, sq. in.		Max. Safe Pressure, Tons—Flatface or Std. Concave Punches	Max. Safe Pressure, Tons—Bevel-Edge Modified Ball or Carbide Tipped Punches
$^{12}/_{32}$	.3750	.1105	5.6	2.2
$^{13}/_{32}$	.4063	.1295	6.6	2.6
$^{14}/_{32}$	.4375	.1506	7.7	3.1
$^{15}/_{32}$	.4688	.1726	8.8	3.5

[a] F. J. Stokes Corp., Philadelphia, Pa.

Microscopic Examination.—The deformation of crystals following compression into modified ball-shaped tablets was evaluated microscopically. The potassium chloride crystals were lubricated with stearic acid, mixed with a small quantity of activated charcoal, and compressed. The tablets were then cut in half and examined under low power magnification.

Sampling Procedure for the Determination of Inter-Unit Tablet Variability.—Six individual batches of the directly compressible formulation of potassium chloride with hydrochlorothiazide, sufficient to make 240,000 tablets per batch, were prepared and individually processed. Three batches were selected for statistical analysis of the inter-unit variability in tablet weight, drug content, and per cent of drug. Four samples of approximately 100 tablets each were taken systematically at 30-min. intervals from each side of the double-rotary tablet press during the compression operation. The initial sample was taken 30 min. after starting compression. During this period, the production personnel made the routine weight adjustments on the machine which may not have been the same in number or in time for the three batches that were analyzed, nor were they the same for each side of the tablet press. Ten tablets were randomly selected from each sample, individually weighed, and each was placed into a coded bottle, which identified the tablet as to batch, time of sampling, side of machine from which sample was taken, and weight of tablet. Four tablets were randomly selected from each set of 10 tablets for analysis of drug content per tablet. A table of random numbers was employed to facilitate random selection.

RESULTS AND DISCUSSION

The failure of some crystals to compact favorably when compressed into modified ball-shaped tablets may be due to the amount of pressure which can safely be applied to the tablets and to the distributions of the forces in the tablet itself. Table I shows the maximum safe pressure which may be applied with different shaped tablet punches. It will be noted that less than half the amount of pressure can be applied safely when using modified ball punches than when using flat-faced or standard concave punches.

Seth (2) stated that deeply biconvex tablets show a greater tendency toward capping because the applied pressure is not uniformly distributed throughout the tablet granulation in the die cavity during compression. As a result, the more deeply biconvex-shaped tablets are, in general, relatively weaker in strength than flat-faced or conventional biconvex tablets. Because of the convexity of the tablet, the pressures reaching the center of the tablet at its maximum diameter would be expected to be a minimum.

Modified ball-shaped tablets which showed poor friability were split in half and examined under low-

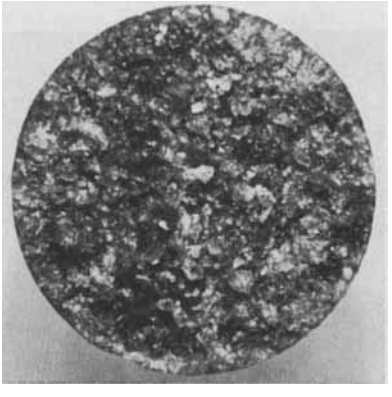

Fig. 2.—Section of compressed tablet of potassium chloride with stearic acid. Carbon has been added to help visualize the crystal boundaries.

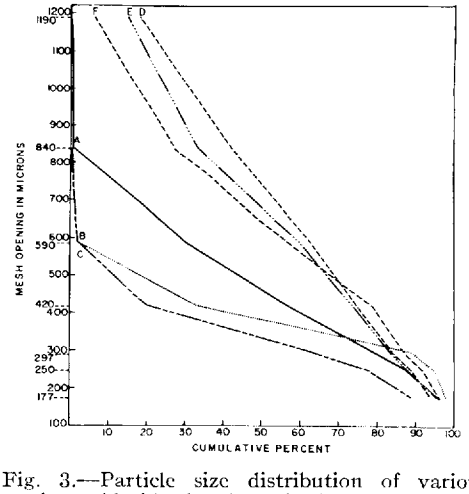

Fig. 3.—Particle size distribution of various potassium chloride batches sized on U. S. sieve series screens.

Fig. 4.- Potassium chloride crystals, batch D.

Fig. 5.—Potassium chloride crystals, batch E.

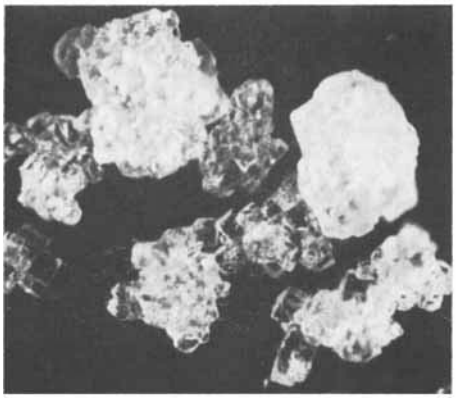

Fig. 6.—Potassium chloride crystals, batch F.

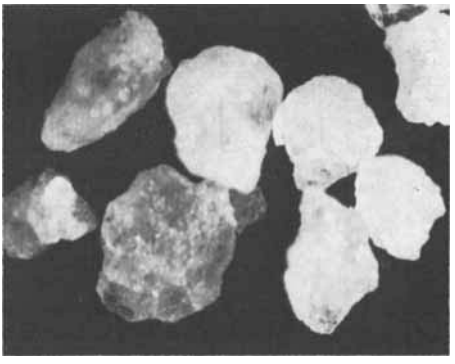

Fig. 7.—Potassium chloride crystals, batch A.

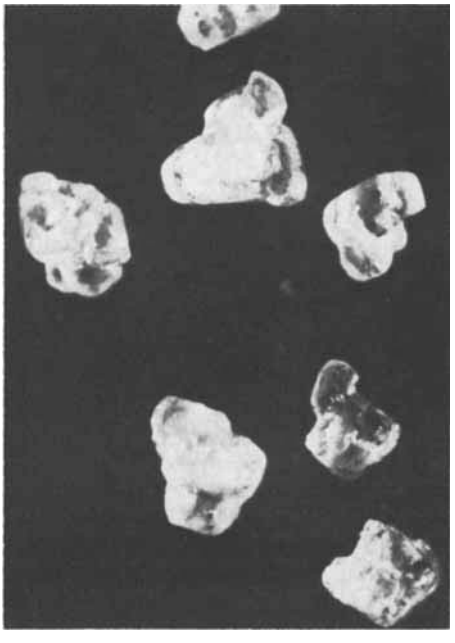

Fig. 8.—Potassium chloride crystals, batch B.

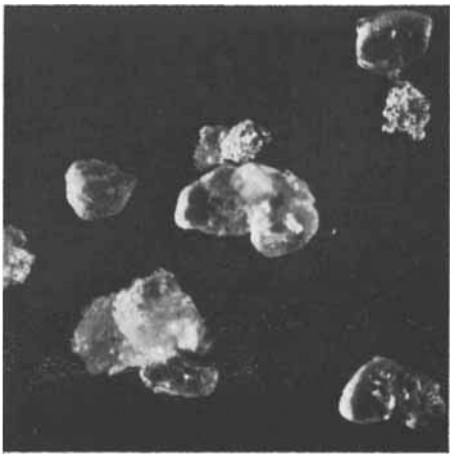

Fig. 9.—Potassium chloride crystals, batch C.

power magnification as shown in Fig. 2. Batch B crystals were used to make the tablet shown in the figure. The crystals at the edges of the tablet, *i.e.*, those crystals in close proximity to the punch face, showed the greatest modification in shape due to the pressure exerted. The crystals in the center of the tablet showed little or no modification from their original shape, indicating that the pressures exerted on the granulation bed were not uniform, a finding which was not unexpected due to the curvature of the punch faces.

Particle Size Distribution.—The particle size distribution of the several batches of potassium chloride crystals is presented in Fig. 3, and it can be seen that there exists a wide range of sizes in the batches. The directly compressible crystals were at least 650 μ or larger at the fiftieth percentile, while the largest crystals at this percentile for those batches that did not exhibit directly compressible properties were no more than 450 μ in size. Approximately 75% of the crystals from the directly compressible batches were 420 μ or larger, while approximately 75% of the nondirectly compressible crystals were 297 μ or larger.

Particle Shape and Appearance.—Examination of the crystals under low-power magnification revealed differences in the appearance and shape of the crystals. The directly compressible crystals (Figs. 4, 5, and 6), batches D, E, and F, respectively, were irregular in shape, frequently having straight jagged edges, often transparent, and appearing to be built up in the form of clusters as a result of growing together in irregular clumps. Other crystals were cubic units built-up in almost pyramid fashion by what appears to be fusion of adjacent crystals. The nondirectly compressible crystals (Figs. 7, 8, and 9), batches A, B, and C, respectively, frequently had rounded surfaces and appeared to lack distinct crystal faces. In addition, these crystals were usually translucent and occasionally opaque in appearance.

Apparent Bulk Density.—The apparent bulk density of the crystals corresponded fairly closely to the particle size distribution of the crystals as shown in Table II. The bulk density represents the packing tendency of the crystals and is a function of the crystal size distribution and the irregularities of the crystal shape. The larger crystals and the highly irregular crystals provide fewer contact points between crystals and create greater void spaces; and if these voids are not filled in by the smaller crystals in the distribution, a low apparent bulk density value will be obtained. It was observed that those crystals which had an apparent bulk density above 1.0 could not be directly compressed.

Moisture Content of Crystals.—When sodium chloride and potassium chloride are crystallized from solution, some water of crystallization may be occluded in the crystals. Smith and co-workers (4) dried sodium chloride and potassium chloride crystals at 140° and then heated them at 550° for 1 hr. A loss in moisture was recorded at the latter temperature. A further loss in occluded moisture was observed when the crystals were heated for 20 min. at 900° which is more than 100° above the fusion point of the crystals. For example, they reported a loss of 0.156% water at 550° and an additional loss of 0.064% water at 900° for potassium chloride crystals prepared by slow evaporation on a hot plate followed by drying to remove surface moisture. In addition, they found that the amount of water occluded varied with the method of precipitating the salts.

The various batches of potassium chloride in the present study were evaluated for the possible presence of occluded moisture, first by drying the crystals at 105° and then at 175° for 20 hr. The 175° temperature was selected because of convenience, no special precautions were necessary to guard against loss by decrepitation, and significant results were apparently attained at this temperature. The data obtained from these drying studies are summarized in Table III. Samples A, B, and C represent crystals which could not be directly compressed or could they be compressed when wet granulated. Samples D, E, and F were directly compressible.

The loss of moisture at 175° was, as one would expect, higher than at 105° which is the usual temperature for removing surface moisture. The loss at 175° which is represented by the difference between total loss of 175° and the observed loss at 105° was generally higher for the directly compressible crystals than for the others.

To determine whether the per cent of occluded water within the crystals varied with the particle size of the crystals, a similar study was performed on different size crystals from several batches, and the results are presented in Table IV. The data in the table indicate that there were generally greater losses in moisture from the larger size crystals than from the smaller crystals. This moisture is not apparently surface moisture because if it were, the small crystals, which have a larger surface area, would be expected to exhibit a greater loss on heating.

The higher percentage of moisture in the larger crystals may be one of the factors that contribute to the compressibility of these crystals. Although these moisture values are small in magnitude, they appear to be of significance when used as a measure

TABLE II.—SUMMARY OF DATA ON VARIOUS POTASSIUM CHLORIDE BATCHES

Sample	Particle Size, mμ		% Loss at 175°	Bulk Density	Hardness	Friability
	50% Up To	50% From				
A	450	450–850	0.01	1.06	7	<2.5 min.
B	360	360–580	0.02	1.10	7	<3
C	320	320–580	0.04	1.14	6	<3
D	730	730–1200	0.05	0.86	13	>10
E	670	670–1200	0.05	0.88	14	>10
F	650	650–1200	0.08	0.92	12	>10

TABLE III.—LOSS OF MOISTURE IN PER CENT AT 105 AND 175° IN VARIOUS SAMPLES OF KCl CRYSTALS[a]

Sample	Loss at 105°	Total Loss at 175°	Difference
A	0.02	0.03	0.01
B	0.01	0.03	0.02
C	0.08	0.12	0.04
D	0.01	0.06	0.05
E	0.01	0.06	0.05
F	0.02	0.10	0.08

[a] Each value represents an average of five determinations. Sample weight ranged from 0.9000 to 4.5000 Gm.

TABLE IV.—MOISTURE LOSS AT 175° OF DIFFERENT BATCHES OF SIZED CRYSTALS OF KCl

	Crystal Size Retained on Screen—		
Sample	No. 20 Mesh, %	No. 30 Mesh, %	No. 60 Mesh, %
A	0.01	0.01	0.00
B	...	0.04	0.01
C	...	0.02	0.01
D	0.09	0.06	0.01
E	0.04	0.03	0.01
F	0.07	0.04	0.02

TABLE V.—EFFECT OF LUBRICANTS ON THE PHYSICAL PROPERTIES OF POTASSIUM CHLORIDE TABLETS

Lubricant	Concn., %	Hardness[a]	Friabilator, min.	Thickness, mm.
KCl + stearic acid	0.5	12	>10	7.9
KCl + magnesium stearate	0.5	5	< 1	7.9
KCl + calcium stearate	0.5	6	< 1	7.9

[a] Hardness was measured on a Strong-Cobb hardness tester, modified so that it was actuated by air pressure rather than the manually operated lever.

TABLE VI.—TYPICAL TABLET PROPERTIES FOLLOWING THE ADDITION OF HYDROCHLOROTHIAZIDE

Formula	1 Tablet, mg.	1 Tablet, mg.
Potassium chloride	1000	1000
Stearic acid	5	5
Hydrochlorothiazide	...	50
Tablet Properties		
Tablet diameter	15/32 in.	15/32 in.
Tablet thickness	7.9 mm.	8.0 mm.
Tablet shape	Modified ball	Modified ball
Hardness	12	9
Friabilator	>10 min.	3.0 min.

of water of occlusion and are in the same order of magnitude as reported by Smith *et al.* (4).

It is reasonable to assume that the loss in weight observed after heating the crystals represents moisture since the crystals are obtained by evaporation from water. Examination of the crystals, before and after heating, under low-power magnification, preferably with the stereomicroscope, will reveal an apparent increase in the number of air bubbles or cavities following the heat treatment. The air bubbles will be more obvious with crystals which exhibit a greater moisture loss at the higher temperature.

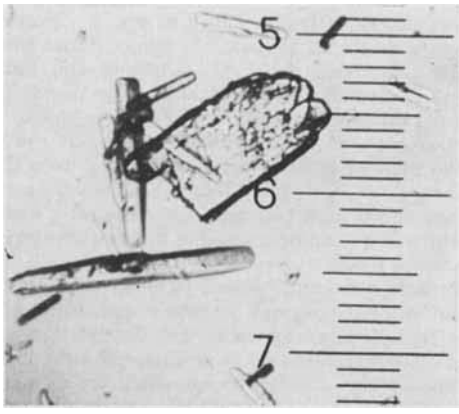

Fig. 10.—Hydrochlorothiazide crystals.

Although batch A crystals had a larger average particle size than B or C and more closely approximated in particle size range the directly compressible crystals, they did not directly compress. This can be attributed to the fact that batch A crystals were compacted on a Chilsonator[1] to attain a larger average particle, and the amount of occluded moisture was low as seen from the data in Table III. The photomicrograph of these crystals (Fig. 7) shows them to be rounded and somewhat opaque with few natural transparent aggregates, unlike the crystals formed by evaporation of solvent and not further subjected to any mechanical compaction process. Since moisture is apparently an important factor in compaction, the crystals should not be subjected to any treatment which will cause excessive loss of moisture.

Lubricant Effect.—The addition of different lubricants was studied in conjunction with the compressibility of the potassium chloride crystals. Stearic acid, magnesium stearate, and calcium stearate were each added in varying amounts to potassium chloride crystals. The magnesium and calcium stearates produced good lubrication, but decreased the bonding characteristics of the salt excessively as seen from the data presented in Table V.

Drug Effect.—The addition of hydrochlorothiazide to the mixture containing potassium chloride and stearic acid reduced tablet hardness and the resistance of the tablets to splitting in the friabilator as evidenced by the data in Table VI. The good bonding properties of the directly compressible potassium chloride crystals were adversely affected as evidenced by the data in this table. The hydrochlorothiazide crystals were essentially needle-like in shape with an occasional flat-platelet and which could be readily fractured and is shown in Fig. 10.

Directly Compressible Formulas.—To overcome the adverse effect of hydrochlorothiazide on the

[1] Manufactured by the Fitzpatrick Co., Chicago, Ill.

TABLE VII.—COMPARISON OF TWO PRODUCTION METHODS

Lots	Tablet Wt. Mean ± S. D.	Tablet Wt. Coeff. of Var., %	Drug Content, mg. Mean ± S. D.	Drug Content, mg. Coeff. of Var., %
Direct Compression				
D	1.0752 ± .0050	.47	48.59 ± .60	1.23
F	1.0704 ± .0052	.49	48.28 ± 1.65	3.42
H	1.0739 ± .0044	.41	47.66 ± .93	1.95
Wet Granulation				
K_4	1.0647 ± .0066	.62	48.67 ± 1.68	3.46
K_6	1.0664 ± .0063	.59	48.60 ± 1.33	2.74
K_8	1.0649 ± .0068	.64	49.00 ± 1.24	2.53

directly compressible properties of potassium chloride, additives were incorporated in the formulation. The rationale for the use of the various raw materials in the formulation will now be presented.

The polyethylene glycol 6000 powder was used as a water-soluble binder, and the small amount of moisture which is atomized into the mixture most likely activates its binding properties. Since the potassium chloride crystal is well balanced ionically, it does not have ideal surfaces for attracting poorly soluble additives, such as a hydrochlorothiazide. The polyethylene glycol 6000 may act as a bridge to provide the necessary bonding between these two crystals.

The moisture may also act to leak-off electrostatic charges induced by mixing the dry powders in the presence of hydrochlorothiazide. The surface of the potassium chloride crystals may be altered by the addition of moisture through surface dissolution of the salt followed by recrystallization on drying.

The colloidal silica improved the flow of the mixture by its adsorbent properties, particularly when the distribution of the small quantity of moisture added was not absolutely homogeneous. In the presence of the water, the colloidal silica may modify the surface of the potassium chloride by forming a surface film.

In order to overcome picking of the tablets at the monogram, it was found necessary to add talc. Such picking did not occur on nonmonogrammed, smooth-faced punches.

Inter-Unit Tablet Variation.—The directly compressible method (method *2*) required less manufacturing and material handling time than the wet granulation procedure (method *1*). At least 12 material handling steps are involved in method *1* as compared to four for method *2*. The manufacturing time for method *1* is approximately 3.5 manhours, not including the delay for drying time. There is no delay for drying in method *2*, and the manufacturing time is about 1.25 manhours.

The purpose of the statistical analysis was to determine the variation in inter-unit tablet weight and drug content existing in the tablets prepared by method *2* and to compare these results with similar data reported for method *1* (5).

The mean, standard deviation, and coefficient of variation for tablet weight and drug content were estimated for each lot. The contribution to heterogeneity introduced by sampling from two sides of the compressing machine and the different times of sampling were disregarded. The data are presented in Table VII.

The tablets from method *1* show a tendency for larger inter-unit tablet weight variability than do the tablets from method *2* as judged from the values for the coefficients of variation. A stronger tendency for larger variability of drug content exists also in method *1*. In view of these results, one can conclude that the directly compressible formulation is at least as good, if not better, than the wet granulated formula.

SUMMARY

Several factors were found to influence the direct compression of potassium chloride crystals into nonconventional shaped tablets. These were particle size distribution, crystal shape, apparent bulk density, moisture, additives, and punch shape.

A larger particle size distribution was observed for the potassium chloride crystals which could be directly compressed into modified ball-shaped tablets. These crystals had many straight edges, were generally cubic or oblong in shape, or formed clusters from these shapes. Rounded surfaces and smaller particle size distribution were characteristic of the potassium chloride crystals which could not be directly compressed into modified ball-shaped tablets. The larger irregular crystals had an expected lower apparent bulk density and exhibited more occluded moisture. As lubricants, calcium and magnesium stearate tended to produce weaker tablets than stearic acid. Hydrochlorothiazide had a similar effect as the stearates. The development of a directly compressible formulation for hydrochlorothiazide-potassium chloride tablets is described. The inter-unit tablet weight and drug content variability for the tablets prepared from the directly compressible formulation and those prepared by the wet granulation technique were found to be comparable.

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Compressibility of Pharmaceutical Solids I

Instrumentation Employed and Preliminary Results Obtained

By JACOB VARSANO and LEON LACHMAN

A description is presented of an Instron physical testing instrument which was adapted to permit its utilization in the evaluation of the compressing characteristics of drug particles and granules. Measurements were performed on a bed of a readily and a poorly compressible solid, as well as granules of these solids made with several commonly used pharmaceutical binding agents. It was found that potassium chloride crystals and granules deformed at lower compression loads than those of potassium citrate. The influence of crystal and granule size and granulating agents on these measurements was found to be relatively small when compared with the inherent properties of each salt. The relationship between the logarithm of the load and the deformation showed a change of slope depending on load range. An attempt was made to obtain a parameter of compressibility having practical implications and leading to a better theoretical understanding of the mechanism of bonding of solids under pressure.

DURING THE past 15 years a considerable amount of research has been performed to elucidate the phenomena and mechanisms involved in the compression of pharmaceutical solids and their granulations.

Higuchi and associates (1–5), using an instrumented single-punch tablet press, were able to demonstrate that a definite relationship existed between compression force and various physical characteristics of the produced tablets, among which were hardness, density, porosity, and specific surface area.

The mechanisms of compaction of powders into pellets and the transmission of the compression forces throughout the powder bed were investigated by Train (6). He also studied the physical changes occurring in the powder undergoing compression by measuring the change of relative volume with respect to load.

Shotton and Ganderton (7) evaluated the relationships between compaction forces and crushing strength, voidage, and ejection forces. The structural changes of granules occurring at different compression levels and the mechanism of bonding were also investigated by these authors (8, 9).

Several pharmaceutical solids were studied for their stress relaxation behavior under constant strain by Shlanta and Milosovich (10). The investigators found a correlation between the relaxation properties of these materials and their bonding characteristics.

Seth and Munzel (11) postulated that particles bind among each other by the formation of surface irregularities during compression. Rumpf (12) investigated the effect of adsorbed water as a factor in the bonding of particles undergoing compression.

In recent years increasing attention has been given to the influences of crystal lattice strength on the bonding characteristics of several pharmaceutical materials. Correlations between crystal hardness and bonding have been demonstrated by Windheuser *et al.* (13). A thorough investigation into the elastic and plastic deformations of some pharmaceutical solids under compression was reported by Höfer and Gstirner (14).

The cohesion between solid surfaces depends on forces of atomic attraction which demonstrate themselves at distances slightly greater than normal interatomic spacing. There exists a direct proportionality between bonding and the contact area of the solid surfaces on which these forces play a role. Solids with a greater tendency for plastic flow will attain a greater contact area when subjected to a given load and, therefore, a higher degree of bonding would result (15). Consequently, the plasticity of the crystal lattice would be a major factor contributing to the bonding of pharmaceutical solids, regardless of the other mechanisms involved.

Although previous reports have considered the importance of this aspect, there is still a need for a systematic evaluation as to the effect of relative crystal and granule hardness on the compression bonding of particulate solids. In light of this, a study was designed to perform hardness measurements on a bed of crystals and granules and determine the effect that particle size and granulating additives have on these measurements. Data accruing from such a study should contribute to the development of

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crystal and granule hardness specifications of materials used in tableting, their relative grading in respect to hardness, and permit an estimation of the compressibility characteristics of new drugs before formulation work is initiated.

This report describes the instrumentation and techniques employed toward achieving the aforementioned objectives and presents the preliminary data obtained on two pharmaceutical solids, potassium chloride and potassium citrate. Previous experience obtained with these salts during formulation work indicated that satisfactory tablets could be obtained by direct compression with potassium chloride but not with potassium citrate. These results led to the selection of potassium chloride and potassium citrate for this study as representative of a readily and a poorly compressible solid.

EXPERIMENTAL

Instrumentation.—The Instron Universal testing instrument TM-M, table model[1] shown in Fig. 1 was utilized for this study. This equipment permits an evaluation of the stress-strain characteristics of materials under selected loads applied at varying rates and patterns. The major considerations for selecting the instrument were its accuracy, versatility, operational convenience, and relative compactness. These qualities make it a valuable tool for both investigational and routine measurements.

A brief description of the operational principles of this instrument follows. The vertically moving crosshead (A) is driven by a synchronous system exerting a compression force at constant rates independent of load. A set of interchangeable gears provides a selection of crosshead speeds. The load cell (B) is a bonded wire strain gauge system excited by a stabilized oscillator and is temperature compensated. The load cell output is amplified and fed to the potentiometer-type strip chart recorder (C), driven synchronously with the crosshead at variable speed ratios. The actuation of the crosshead and its direction of motion is controlled from a panel (D) either manually or automatically by preset conditions.

To allow the measurements of deformation occurring in particulate solid beds under varying stress levels, the special fixture shown in Fig. 2 was designed for the instrument. It consisted of two punches and a die resembling a single-punch tablet press. The upper punch (A) was attached to the moving crosshead. The lower punch (B) was seated on a flange (C) which was fastened by set screws to the load sensitive table of a compression load cell (D). A threaded bushing (E) containing the lower punch provided a means for regulating its height. The die (F) was placed on a metal plate (G) which slides vertically along a pair of sturdy steel rods (H) attached to the base of the instrument. The surface of the rods had inscribed scales in 1-mm. increments to allow the positioning of the die plate at desired heights which in turn is

Fig. 1.—Instron universal testing instrument equipped with compression fixture. Key: A, crosshead; B, load cell; C, recorder; D, control panel; E, fixture.

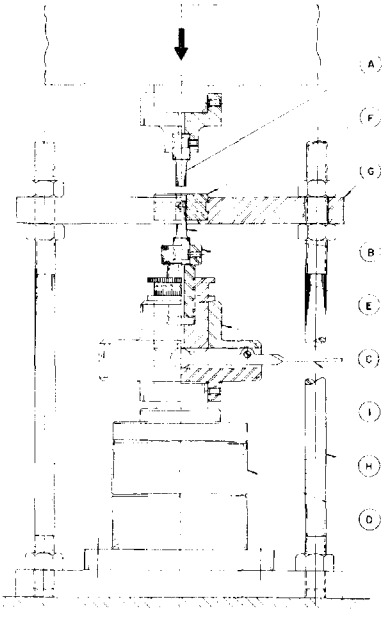

Fig. 2.—Fixture for compressing particulate solids. Key: A, upper punch; F, die; G, metal plate; B, lower punch; E, threaded bushing; C, flange holding lower punch; I, lifting lever; H, steel rods; D, compression cell.

held in place by set screws. A lever (I) served to extrude the compressed compacts from the die by lifting the bushing containing the lower punch. The flat-faced punches employed in this study were of $^{11}/_{32}$-in. diameter. The die and punches were chromium plated to reduce sticking and friction.

The instrument equipped with the fixture described above permitted an evaluation of the deformation properties of solids undergoing compression from the relationship of upper punch travel and the compression force exerted on the lower punch.

Materials Used.—Potassium chloride (Schuylkill

[1] Instron Corp., Canton, Mass.

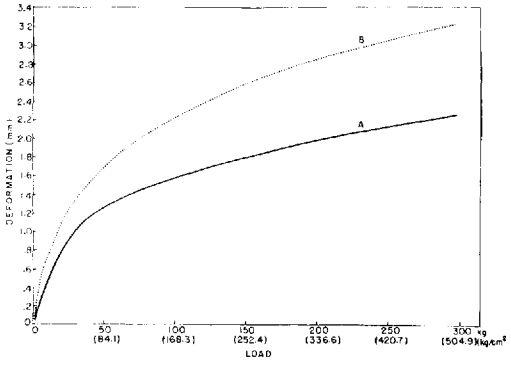

Fig. 3.—Deformation of crystals at varying loads. Key: A, potassium citrate crystals; B, potassium chloride crystals.

Chemical Co.); potassium citrate (Chas. Pfizer and Co., Inc.); cornstarch U.S.P.; acacia U.S.P.; and polyvinylpyrolidone (PVP) (Antara Chemicals).

Procedure.—The potassium chloride and potassium citrate were milled on a Fitzpatrick machine, and granulations of these salts were prepared with water, cornstarch paste 20% w/v, acacia solution 20% w/v, and PVP solution 20% w/v. For each granulation system the ratio of binder to salt was kept at 1.4%. The granulations and the untreated crystalline solids were screened on U. S. standard sieves to obtain the following size fractions:

	Sieve Classification, Mesh
Potassium chloride, crystalline	40–60, 60–80, 80–100, 100–120
Potassium citrate, crystalline	40–60, 60–80, 80–100, 100–120
Granulations	18–20, 20–40, 40–60, 60–80, 80–100

The screened fractions and unscreened material were dried in a 65°-oven until the Karl Fischer test indicated no measurable amount of water.

Compression.—The die wall was dusted with magnesium stearate, and a 300-mg. sample was accurately weighed and fed into the die cavity through a specially designed glass funnel. In order to insure uniform packing of the particles in the die, the funnel was suspended on a vibrating arm operating at a constant frequency.

The instrument was operated on automatic load cycling at a downward crosshead motion of 1 cm./min. until a 300-Kg. (504.9 Kg./cm.^{2}) load was exerted on the lower punch. When this maximum load was reached, the instrument automatically reversed the direction of the crosshead at a speed of 50 cm./min. The ratio of the downward crosshead speed to that of the recorder chart was 1:50. For each screened fraction, five measurements were made, and their mean values were used in subsequent calculations. Corrections were made for the amount of deflection due to the instrument and the fixture.

RESULTS AND DISCUSSION

Figure 3 presents a typical load deformation relationship as replotted from the recorder. It

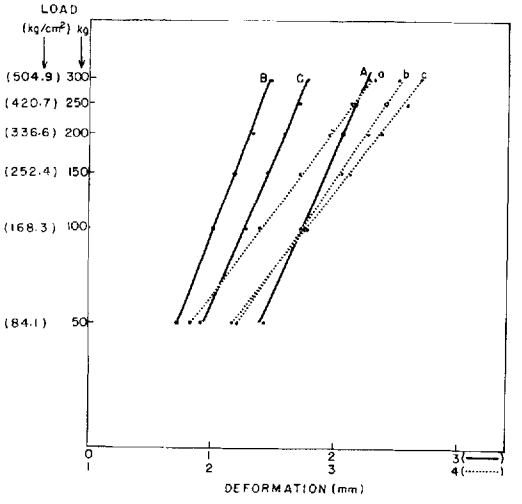

Fig. 4.—Compression curves of potassium citrate and potassium chloride crystals at varying particle sizes. Key: (potassium chloride) a, on 100; b, on 60; c, not screened; (potassium citrate) A, on 100; B, on 60; C, not screened.

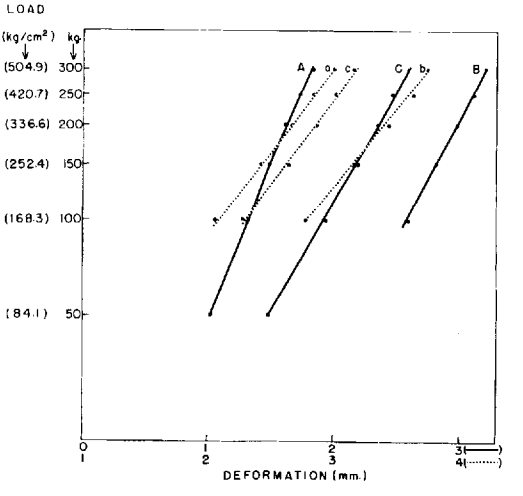

Fig. 5.—Compression curves of potassium citrate (on 80) and potassium chloride (on 80) granulated with several agents. Key: (potassium citrate) A, crystalline; B, starch paste granulation; C, acacia granulation; (potassium chloride) a, crystalline; b, starch paste granulation; c, acacia granulation.

TABLE I.—(Δ LOG LOAD/Δ DEFORMATION) OF POTASSIUM CITRATE AND POTASSIUM CHLORIDE AT VARYING PARTICLE SIZES

	Crystals	
Sieve Size	Potassium Citrate	Potassium Chloride
On 40	.96	.51
On 60	.94	.64
On 80	.90	.50
On 100	.85	.50
On 120	.94	.58
Not screened	.87	.52

TABLE II.—INFLUENCE OF GRANULATING ADDITIVES ON SLOPES (Δ LOG LOAD/Δ DEFORMATION) OF POTASSIUM CITRATE

Sieve Size	Crystals	Granulated with			
		Water	Starch Paste	Acacia	PVP
On 20	...	.87	.85	.85	.84
On 40	.96	.89	.84	.85	.90
On 60	.94	.88	.82	.80	.82
On 80	.90	.94	.79	.74	.75
On 100	.85	.86	.81	.76	.92
On 120	.94	...	...	...	...
Not screened	.87	.87	.85	.76	.89

TABLE III.—INFLUENCE OF GRANULATING ADDITIVES ON SLOPES (Δ LOG LOAD/Δ DEFORMATION) OF POTASSIUM CHLORIDE

Sieve Size	Crystals	Granulated with			
		Water	Starch Paste	Acacia	PVP
On 20	...	.54	.54	.46	.47
On 40	.51	.50	.48	.46	.46
On 60	.64	.54	.49	.40	.54
On 80	.50	.54	.41	.56	.55
On 100	.50	.54	.52	.49	.56
On 120	.58	...	...	...	...
Not screened	.52	.56	.51	.50	.54

TABLE IV.—WORK EXPENDED TO COMPRESS CRYSTALS AND GRANULATIONS OF POTASSIUM CITRATE AND POTASSIUM CHLORIDE AT VARYING PARTICLE SIZES

Sieve Size	Crystals		Starch Paste Granulation		Acacia Granulation		PVP Granulation		Water Granulation	
	K Citr.	KCl	K Citr.	KCl	K Citr.	KCl	K Citr.	KCl	K Citr.	KCl
On 20	...	...	179	300	169	283	171	280	165	254
On 40	145	230	172	281	165	283	164	270	166	265
On 60	138	212	174	277	174	309	174	254	164	250
On 80	153	242	180	261	187	230	182	248	160	243
On 100	152	245	179	252	178	259	164	241	167	242
On 120	151	272	...	...	...	...	...	...	...	...
Not screened	161	246	168	262	172	255	162	252	161	254

shows that the deformation of potassium chloride was greater than that obtained with potassium citrate.

Plotting the logarithm of the load against deformation produced a linear relationship over a load range from approximately 50 to 300 Kg. for both potassium citrate and potassium chloride as shown in Figs. 4 and 5. Because of particle reorganization occurring at the load range below 50 Kg., deviation from linearity takes place in the above relationship. The slopes of the plots in Figs. 4 and 5 (Δ log load/Δ deformation), are an index of the relative ease with which the solid bed undergoes deformation.

The compression slopes of the plots in Fig. 4 are summarized in Table I. It is evident from the data in this table that within each salt, the slope values are for the most part of the same magnitude, independent of particle size. However, a comparison of the slopes of the two salts reveals a significant difference between them.

The effect of granulating additives at a constant particle size on the slopes is demonstrated by the plots in Fig. 5. The slopes of such plots are presented in Tables II and III. It can be seen that the slopes of each salt are essentially unaffected by granule size and granulating agent at concentrations normally used in tablet technology.

The values for work expended in compressing the

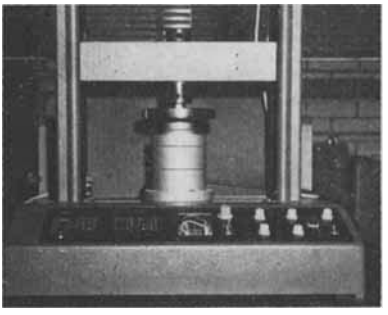

Fig. 6.—The instrument as utilized for measuring the crushing strength of compacts.

TABLE V.—OVER-ALL MEAN CRUSHING STRENGTH OF COMPACTS COMPRESSED FROM CRYSTALS AND GRANULATIONS OF POTASSIUM CITRATE AND POTASSIUM CHLORIDE

	K Citr., Kg.	KCl, Kg.
Crystals	6.15	10.94
Water granulation	5.90	10.06
Starch paste granulation	3.78	9.93
PVP granulation	2.99	9.80
Acacia granulation	10.15	13.69

materials under study over the load range from 50 to 300 Kg. were computed from the areas under the load displacement curves and are presented in arbitrary units in Table IV. The data clearly indicate that in all instances the work done in compressing potassium chloride is greater than that for potassium citrate. This is an indication that potassium chloride has undergone greater deformation at this load level than potassium citrate.

Crushing strength measurements on the compacts obtained in this study were performed on the instrument as illustrated by the photograph shown in Fig. 6. Table V presents the over-all means of these values for each granulation. It is evident from these data that greater apparent bonding was achieved with potassium chloride than with potassium citrate.

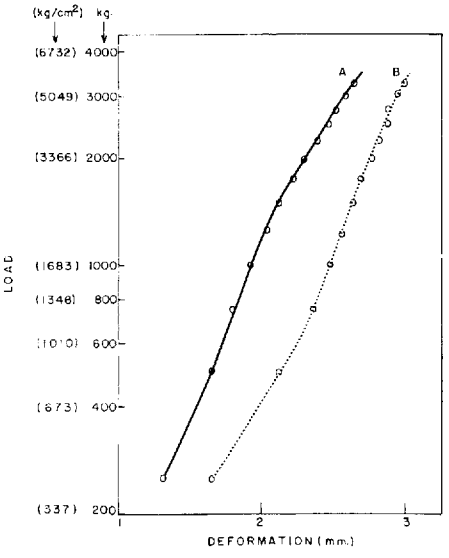

Fig. 7.—Compression curves of potassium citrate (on 80) and potassium chloride (on 80) at high loads. Key: A, potassium citrate; B, potassium chloride.

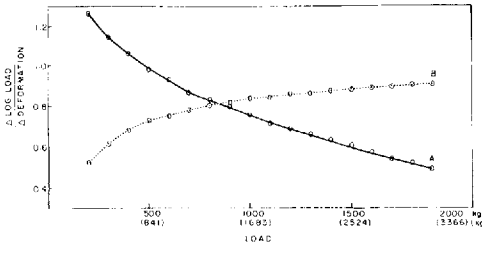

Fig. 8.—Compression slopes of 80-mesh crystals at different loads. Key: A, potassium citrate; B, potassium chloride.

In order to determine whether the slope relationship for the two substances found at lower loads would remain valid at higher loads, a floor model Instron capable of exerting a maximum load of 5000 Kg. was utilized. The data obtained were plotted in Fig. 7, and it is apparent from the nonlinearity of the plots of log load *versus* deformation that the straight line relationship found at the lower loads does not hold true over a wide load range. Because of the curvature of the plots, it is not readily possible to obtain the compression slopes of the two substances. It was felt that if the compression curves were approximated as straight lines, valuable information on the mechanism of compression would be obscured. Instead, the equation of the best fitted line was calculated on a computer by the method of least squares, using the quadratic model $\log y = A_1 + A_2X + A_3X^2$. The slope at each load level of the fitted line was then computed by differentiating the model: $d(\log y)/dx = A_2 + 2A_3X$. This analysis permitted the determination of the compression slopes at any load level. The computer program also recorded the residual sum of squares and the standard deviation. The latter represented the geometric mean of the variations about the fitted line and included both the experimental error and the inadequacy of the model.

The slopes computed in this manner were plotted against load as shown in Fig. 8. This graphical presentation clearly illustrates the influence of varying loads on the compression slopes. From the plot of potassium chloride, it can be seen that the compression slope increases and approaches a plateau with increase in load. This relationship would indicate that the major deformation of the crystal bed took place at the lower load range. As the load increases beyond this range, a greater portion of it is being transmitted through the bed onto the load cell with little further compaction being accomplished. In contrast, for potassium citrate, the major deformation is occurring at the higher load range as evidenced by the considerable decrease in compression slope as the load increases.

The changes occurring in compression slopes at different load ranges provide valuable information about the compression characteristics of a material and permit the selection of the most appropriate load level to obtain optimal compaction. It would be expected that the grading of the relative compressibility of materials could be accomplished by determining the load range at which most active compaction is taking place.

The work expended in compressing potassium chloride and potassium citrate in the load range up

Table VI.—Cumulative Work Expended to Compress 80-Mesh Potassium Citrate and Potassium Chloride Crystals from 50 Kg. to Load Levels Given in Table

	Cumulative Work at Loads in Kg.								
	500	1000	1500	2000	2500	3000	3500	4000	4500
Potassium citrate	281	557	862	1187	1528	1882	2246	2620	3002
Potassium chloride	356	613	870	1129	1388	1647	1906	2166	2425

to 4500 Kg. is given in Table VI. It can be seen from the data in this table that more work is expended on the potassium chloride crystals up to 1500 Kg., while for potassium citrate more work is expended from 1500 to 4500 Kg. This would indicate that the potassium chloride crystals are undergoing greater deformation and compaction at the lower load range while the reverse is true for potassium citrate.

SUMMARY AND CONCLUSION

A description has been presented of a modified Instron physical testing instrument which can be used to obtain a quantitative measure of the compressibility of pharmaceutical solids. Preliminary information is given for potassium chloride and potassium citrate crystals and granulations of these salts which can be summarized as follows.

1. Potassium chloride was found to deform at lower compression loads than potassium citrate.

2. Modifying the two salts by granulating with materials commonly used in tablet technology indicated that the inherent compression properties of the salts predominated. The granulating agents employed were water, acacia, starch paste, and polyvinylpyrrolidone.

3. A linear relationship was found for log load *versus* deformation for both salts at load levels up to 300 Kg. However, at higher loads, this linear relationship did not hold true.

4. In order to permit a measure of the relative compressibility of the materials at high load levels, a computer program was prepared to determine the quadratic equation best fitting the experimental data which were subsequently used to obtain the compression slopes at each load by calculating the derivative.

5. The relationship between compression slope and load can be used to determine the load range at which maximum deformation of the solid bed is taking place.

6. The work expended at different loads can also be used to determine the load range of maximum deformation.

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Powder Flow Studies I

Instrumentation and Applications

By GERALD GOLD, RONALD N. DUVALL, and BLAZE T. PALERMO

Several methods have been used to evaluate the flow properties of pharmaceutical formulations. Although these methods give reproducible results, they often measure different factors. Consequently, it is difficult to interpret the data, and it is debatable whether correlation with actual flow of material is possible. A new approach to the measurement of powder flow is presented and involves measurement of the weight of powder per unit time flowing through a hopper orifice. The instrument, a recording powder flowometer, consists of a hopper, strain gauge balance, and recorder. The flow rate can be calculated from the recorder tracing, and in addition, the tracing serves to characterize the flow qualities of a formulation. Various sized hopper orifices with or without vibration can be used. By incorporation of an ionostat into the instrument, the static charge may also be measured concurrently with the flow rate.

FLOW PROPERTIES of pharmaceutical formulations are extremely important to the industrial pharmacist. Increasing complex manufacturing techniques and modern dosage forms require a more thorough and basic understanding of the science and technology of small particles. In tablet and capsule manufacturing, considerable effort is directed toward obtaining and improving free flowing powders and granulations. Recent compendia standards and law enforce-

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The authors thank Mr. T. M. Anderson and associates for designing the instrument and Mr. J. G. Slater for mathematical assistance.

ment activity relative to intertablet dosage variation have further emphasized the problem. Research in this area has been hindered by the lack of suitable instrumentation, and, until fairly recently, flow properties were often evaluated empirically.

Methods currently in use in an attempt to evaluate powder flow include measurements of the angle of repose or angle of spatula and timed delivery from an orifice. The angle of repose is a measure of the static coefficient of interparticular friction, and is the maximum angle possible between the surface of a pile and the horizontal plane. The angle of spatula is also a measure of interparticular friction. In this method, a spatula is inserted parallel to the bottom of the container and then lifted straight up and out of the material, thereby creating an angle to the horizontal (1). Timed delivery through an orifice has also been used to evaluate flow of materials, with either the volume or weight of powder flowing through the orifice in a specified time period measured (2). Munzel (3) also utilized this principle with a modified Emix powder dispenser. Although, separately, these three methods give reproducible comparative results, they have no specific relation to each other, and are not necessarily influenced by the same factors.

Powder flow problems encountered in this laboratory could not be satisfactorily resolved with the various methods described above. Consequently, an instrumented approach to the problem has been developed and appears to be more closely related to actual flow of materials under production conditions. A recorder tracing of the weight of the powder flowing through a hopper *versus* time is obtained. The tracing serves to illustrate graphically variations or fluctuations in flow patterns. The flow rate through a given hopper orifice with or without vibration is easily calculated from the tracing and, in addition, an ionostat can be used to measure the static charge concurrently with the flow rate. This report describes the recording powder flowometer which should lend itself to basic studies of the flow of powdered or granular materials. An example is presented to illustrate the usefulness of the instrument in identifying the ingredients in a complex formulation which adversely influence flow and in confirming an improved flow of the reformulated material.

EXPERIMENTAL

Materials.—Commercially available materials of either U.S.P., N.F., or pharmaceutical grade were used. The following materials were powders: anhydrous dicalcium phosphate, magnesium stearate, niacinamide, riboflavin, and thiamine. The following were crystalline: ascorbic acid, aspirin, anhydrous citric acid, sodium ascorbate, and tartaric acid. The remaining materials were processed granules.

Description of the Recording Powder Flowometer.—The instrument consisted basically of a strain gauge balance and recorder along with various hoppers. The hoppers were stainless steel, conical in shape, and measured 20 cm. top diameter by 30 cm. in length with orifice diameters of 8.0, 10.0, and 15.0 mm. In order to study poorly flowing materials, a vibrolator[1] was attached to the 10.0-mm. hopper and connected by means of rubber tubing through a pressure regulator[2] to a laboratory air jet. The pressure regulator can be set at any air pressure depending on the amount of vibration desired. In those experiments requiring vibration, a setting of 10 lb./sq. in. was used. The strain gauge balance is shown schematically in Fig. 1. The receiving platform (B) was positioned 11 cm. directly beneath the hopper orifice. This platform was supported by bar (C), the upper of two brass bars mounted horizontally to the wood support. The lower horizontal bar (D), fastened at one end, bends when weight is placed on the platform. Two strain gauges[3] were fastened to this bar. Within the enclosed area (A) were the resistors, resistor controls, and the circuit switch. The electrical diagram indicating the bridge circuit, voltage regulator, and strip chart recorder is also shown in Fig. 1. Voltage regulation was accomplished with a transistorized power supply model 1020[4] set at 8 v. The Brown electronik[5] recorder has a 0–2-mv. range for 25.4 cm. full scale and a chart speed of 0.33 cm./sec.

Calibration of the Instrument.—Due to the change in resistance of the deformed strain gauges when powder flows through the hopper onto the platform, a voltage difference results in the two arms of the bridge circuit. The magnitude of this change in potential, indicated by the recorder, was found to be directly proportional to the weight on the strain gauge balance. The recorder response was calibrated by adding weights to the platform and noting the millivolt response. The linear relationship between recorder response and weight is shown in Fig. 2. The weight of material that has flowed through the hopper at any given time could be ascertained from the calibration curve. In this study, however, an equation based on the method of least squares was used to relate recorder response to weight in grams.

Measurement of Static Charge.—Static charge and flow rate can be measured concurrently if an ionostat described in a previous communication from this laboratory (4) is used and the platform of the strain gauge balance replaces the helipath stand.

RESULTS AND DISCUSSION

Typical results obtained with various materials having different flow rates are illustrated in Fig. 3.

[1] Model UCV-6, Martin Engineering Co., Neponset, Ill.
[2] C. A. Norgren Co., Littleton, Colo.
[3] Series SR4, type C-5, Baldwin-Lima-Hamilton Electronics, Waltham, Mass.
[4] Electronic Instrument Co., Inc., Flushing, N. Y.
[5] Honeywell, Inc., Philadelphia, Pa.

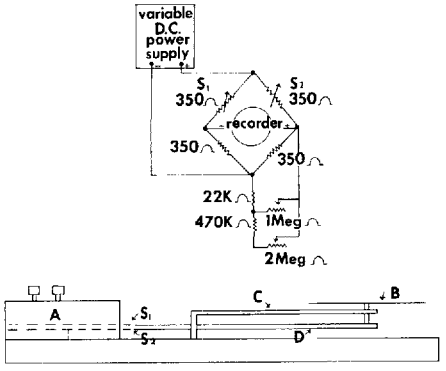

Fig. 1.—Strain gauge balance and electrical diagram.

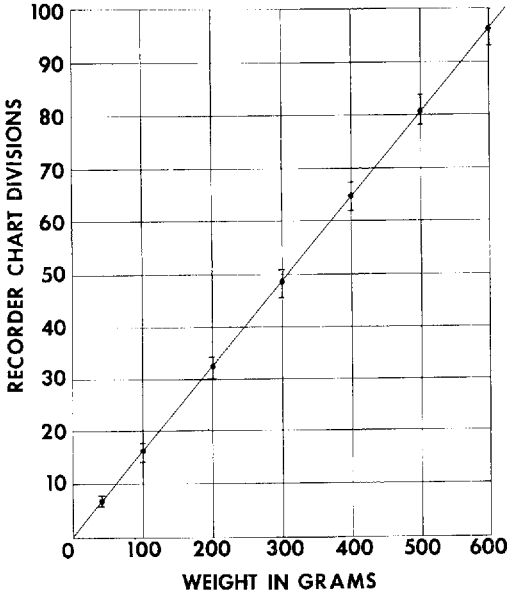

Fig. 2.—Calibration curve relating weight in grams to recorder response. The vertical bracketed lines represent 95% confidence limits.

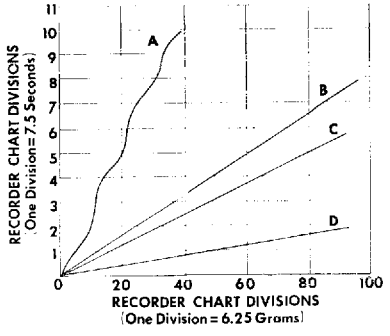

Fig. 3.—Flowometer recording of selected materials. Key: A, dicalcium phosphate anhydrous powder; B, calcium sulfate, dihydrate granulation; C, 20-mesh aspirin crystals; D, 18–25 mesh glass beads.

TABLE I.—FLOW RATE[a] AND CORRESPONDING STANDARD DEVIATION[b] OF SELECTED MATERIALS

Material	Flow Rate, Gm./sec.	S.D.
Glass beads, 18–25 mesh	42.57	0.20
$CaSO_4$ granulation	8.42	0.48
Aspirin crystals	11.17	0.70
Anhydrous $CaHPO_4$[c]	2.72	0.37

[a] 10-mm. diameter hopper orifice. [b] Based on nine determinations. [c] Using vibrator at 10 lb./sq. in.

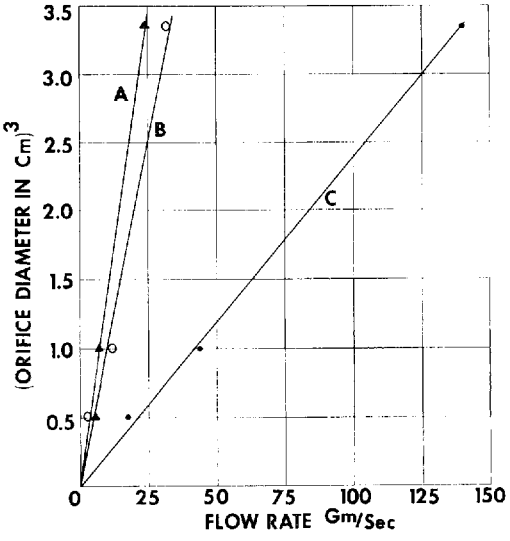

Fig. 4.—Relationship of flow rate to hopper orifice diameter cubed. Key: A, calcium sulfate dihydrate granulation; B, 10% starch granulated aspirin; C, 18–25 mesh glass beads.

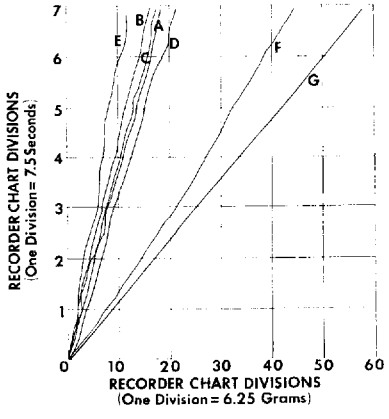

Fig. 5.—Flowometer recording of chewable hexavitamin tablet formulations. Key: A, original formula; the following ingredients were omitted from the original formula: B, magnesium stearate; C, thiamine; D, riboflavin; E, sodium ascorbate; F, niacinamide. Formula G was the reformulated product.

Glass beads, 18–25 mesh, a calcium sulfate granulation, aspirin crystals, and anhydrous dicalcium phosphate powder were used in order to ascertain precision of the instrument over a broad range of flow rates. Flow rates and corresponding standard deviations are shown in Table I.

The results indicate that the instrument is capable of monitoring flow of materials flowing at widely divergent flow rates and of giving good reproducibility. The recorder tracing provides continuous data on such flow with time, a major advantage of this instrument. Fluctuating or irregularly flowing materials can be readily identified. The pulsating character shown by the dicalcium phosphate powder represents alternating periods of flooding and starving. From Fig. 3, it can also be noted that the relative magnitude of the flooding may be estimated from the amplitude of the pulse pattern. This could prove valuable in solving problems relative to tablet weight variation.

The instrument appears to be applicable to basic studies in powder flow. The relationship originally established by Ketchum (5) indicated that the flow rate is directly proportional to the product of a constant, the density of the material, and the cube of the diameter of the orifice. This proved to be an oversimplification, primarily because other factors which affect the rate of discharge were not included (6). However, Fig. 4, although presented only to indicate a potential use for the instrument, does seem to show this relationship between the cube of the orifice diameter and flow rate.

Application of the instrument as an aid in solving flow problems either during the developmental stage of a new product or during production of an existing product appears promising also. To illustrate this point, chewable hexavitamin tablet N.F. formulations containing coated niacinamide, riboflavin, and thiamine, together with fine granular sodium ascorbate, magnesium stearate powder, granules of vitamins A and D, and mannitol were studied. The powder formulation has poor flow properties as illustrated by tracing A in Fig. 5. To analyze the powder mixture for the effect of individual ingredients on flow, five formulations in which a different ingredient was excluded from each were prepared. There appeared to be no significant differences in the flow patterns of the complete formula and of those formulations without either magnesium stearate, thiamine, or riboflavin. The formulation in which sodium ascorbate was omitted, tracing E, had a slower flow rate than the complete formula, indicating that this ingredient had a beneficial effect on the flow rate. When niacinamide was omitted from the formula, recording F, the material had a significantly faster flow rate, thus identifying niacinamide as that ingredient primarily responsible for the poor flow of the original formula. The product was then reformulated with a granulated niacinamide and the resulting formulation, tracing G, showed a marked improvement in flow rate. This example indicates the relative ease by which a poorly flowing formulation may be analyzed and improved through the use of this instrumented approach to the problem. This would be a formidable problem if approached by existing methods of flow evaluation.

The relationship of static charge to flow rate was studied by means of the flowometer modified with an ionostat. Flow rates and static charges of selected organic acids are listed in Table II. The results obtained with two different hopper orifices are the averages of nine determinations. The static charge, read directly from the ionostat, represents the maximum charge resulting from the flow of 500 Gm. of material. Although presented only to illustrate another potential application of the apparatus, it is interesting to note that the increase in flow rate from the larger orifice (15.0 mm.) effected an increase in static charge.

TABLE II.—FLOW RATES AND STATIC CHARGES OF SELECTED ORGANIC ACIDS AS OBTAINED WITH DIFFERENT HOPPER ORIFICES

	Orifice Diam., 8.0 mm.		Orifice Diam., 15.0 mm.	
	Flow Rate, Gm./sec.	Static Charge, −v./cm.	Flow Rate, Gm./sec.	Static Charge, −v./cm.
Aspirin	4.77	239	37.77	600
Ascorbic acid	4.41	472	36.11	744
Citric acid, anhyd.	3.77	533	44.22	1039
Tartaric acid	5.99	1306	49.37	2289

SUMMARY

A new instrumented approach applicable to the analysis of flow properties of powdered or granular materials has been described. The instrument, a recording powder flowometer, consists basically of various hoppers with or without vibration, a strain gauge balance, and a recorder. A major advantage of the apparatus is the recorder tracing from which the flow rate can be calculated and from which fluctuating or inconsistently flowing materials can be readily detected. In addition, incorporation of an ionostat into the flowometer permits simultaneous measurement of the static charge with the flow rate.

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Quantitative Recovery of Radioactivity from ^{14}C-Pentaerythritol Tetranitrate Administered to Rats

By MALCOLM C. CREW, CLAUDE B. COUTINHO, and FREDERICK J. DICARLO

A single dose of ^{14}C-labeled pentaerythritol tetranitrate (PETN) was administered orally to fasted rats, and the level of radioactivity was determined 24 hr. later in the blood, liver, gastrointestinal tract (GIT), urine, and carcass. One group of treated rats was housed to permit collection of expired CO_2 which was also assayed for ^{14}C. It was possible to account for all of radioactivity administered. More than 40 per cent of the drug was absorbed, and about 25 per cent of the ^{14}C was excreted into the urine. The blood, GIT, and urine, were also assayed quantitatively for PETN and its metabolites. The blood contained pentaerythritol and its mono- and dinitrates. Urine contained these metabolites and also a trace of pentaerythritol trinitrate. The GIT contained PETN and pentaerythritol.

A PREVIOUS STUDY (1) examined the absorption and subsequent metabolism of ^{14}C-pentaerythritol tetranitrate (PETN) administered in ligated sections of the rat gastrointestinal tract. The present experiment was undertaken to evaluate the necessity of monitoring the respiratory carbon dioxide in a more general metabolic study of normal (*i.e.*, nonligated) rats. This experiment provided, furthermore, the opportunity to evaluate the efficacy of the extraction methods used in the estimation of PETN and its metabolites.

In the metabolism of PETN the nitrate groups are removed to form PE-trinitrate, PE-dinitrate, PE-mononitrate, and finally pentaerythritol (PE); no breakdown products from the PE have been found (2–5). Although no evidence of expired $^{14}CO_2$ was found in a comparable study using mice (3), this determination was necessary in rats to plan the procedures for animal management in a more complete metabolic study.

For the present experiment, ^{14}C-PETN was administered to six rats housed in glass metabolic cages in such a manner as to provide for the collection of the expired carbon dioxide. After 24 hr. the rats were sacrificed and the ^{14}C content of the carbon dioxide assayed. The blood and some of the tissues were extracted, and the radioactivity of both the extract and the residue was measured to evaluate the efficacy of the extraction. The distribution of the metabolites of PETN in the extracts and the urine was also determined.

METHODS

Radioactive PETN.—^{14}C-PE labeled at C-1 and C-2 was synthesized from acetaldehyde with specific activity of 1.6 mc./mmole and was employed to prepare ^{14}C-PETN, m.p. 140–141.5°. To minimize the danger of working with explosive material, the labeled PETN was mixed with 7 parts by weight of chemically pure lactose. The activity of the lactose–PETN mixture was 0.59 mc./Gm.

CO_2 Collection.—Six white female Wistar rats weighing approximately 180 Gm. each were fasted for a period of 24 hr. prior to the experiment. The rats were dosed orally with 1.8 ml. of a 8.0 mg./ml. suspension of PETN–lactose in propylene glycol (10 mg. ^{14}C-PETN/Kg. body weight) and housed in glass metabolic cages connected to a gas absorption train.

The gas absorption train consisted of a drying tower containing Drierite and Ascarite connected to a source of low-pressure air, followed by a gas-scrubber containing water for rehumidifying the air. The air from the scrubber was passed to a manifold to distribute the air to the six metabolic cages. The effluent air from the cages was recombined in a second manifold and bubbled successively through three containers of 10% sodium hydroxide to trap the expired CO_2 before exhaustion to the atmosphere. A total of 4 L. of 10% NaOH was used in the three collection flasks.

After 24 hr. of CO_2 collection, the combined NaOH solutions were treated with a saturated solution of barium chloride until the precipitation of barium carbonate was complete and crystallization of barium hydroxide was evident. The precipitate was filtered, washed with 4 L. of water, and dried.

The animals were removed from the cages and sacrificed immediately. The blood, liver, gastrointestinal tract, and carcass were extracted with dioxane, and the residues from the extractions assayed for ^{14}C by combustion analysis. The barium carbonate was assayed by gas evolution analysis.

Radioactivity Counting.—Quantitative assays of the urine and the dioxane extracts for ^{14}C were conducted by scintillation spectrometry in a Packard Tricarb using a dioxane solvent for the scintillation solution. The combustion analyses of the residues and the gas evolution analysis of the barium carbonate were conducted by the New England Nuclear Corp.

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Thin-Layer Chromatography.—The assay of the urine and the dioxane extracts for metabolites of PETN was carried out by means of thin-layer chromatography and radioscanning as previously described (1).

RESULTS AND DISCUSSION

Several hours prior to the expiration of the experiment one of the six rats died. The urine and tissue used for this study were, therefore, taken only from the five survivors. The results of the assay for total ^{14}C are given in Table I. The distribution of PETN metabolites in the blood, urine, and gastrointestinal tract is given in Table II. The carcass extract was not assayed qualitatively. The liver extract proved too intractable to produce a satisfactory chromatogram.

TABLE I.—RECOVERY OF ^{14}C 24 hr. AFTER ORAL ADMINISTRATION OF ^{14}C-PETN

Sample	Dose, % Extract	Residue	Total
Blood	0.61	0.02	0.63
Liver	0.24	0.08	0.32
GIT	54.73	3.92	58.65
Urine	25.21	—	25.21
Carcass	15.73	1.20	16.93
CO_2[a]	0.28	—	0.28
	96.8	5.2	102.0

[a] Value from "5.5" rats.

TABLE II.—DISTRIBUTION OF PETN METABOLITES AT 24 hr. POSTADMINISTRATION

Sample	Compn., %[a] PE	PE-Mono-nitrate	PE-Di-nitrate	PE-Tri-nitrate	PETN
Blood	45	27	28	—	—
GIT	58	—	—	—	42
Urine	51	30	17	2	—

[a] Calculated on a molar basis.

The small amounts of radioactivity found in the residues from the tissue extraction confirm the efficacy of the extraction procedure. The one notable exception is the liver extraction which removed only 75% of the radioactivity from the tissue.

The barium carbonate collected weighed 108.6 Gm. A blank with the apparatus using no rats produced 2.9 Gm. of barium carbonate. The difference of 105.7 Gm. of barium carbonate, corresponding to 23.6 Gm. of carbon dioxide, represents the air expired by "5.5" rats in the 24 hr. On this basis it is estimated that each young rat (fasted for 48 hr. as described) exhaled about 24 Gm. of carbon dioxide per kilogram body weight. This quantity is in agreement with data reported by Benedict and MacLeod (6).

From the finding (Table I) that the rats exhaled $^{14}CO_2$ to the extent of only 0.28% of the ^{14}C-PETN administered, it is evident that there was no significant conversion of PETN or its metabolites to carbon dioxide. This observation confirms the finding in mice (3). One possible source of the $^{14}CO_2$ might have been some trace quantity of radioactive impurity in the PETN. Another possibility is that the bacterial flora of the intestine degraded a small quantity of pentaerythritol. There seems to be no information on this point. In their review on the metabolism of tetritols, Carr and Krantz (7) indicated that some microorganisms degrade erythritol. Erythritol, of course, contains two secondary alcohol groups which may render it more vulnerable to enzymatic attack than is the completely symmetrical, primary alcohol-containing pentaerythritol.

Kutscher (8) studied the chronic feeding of pentaerythritol to rats and reported almost 90% of the compound to have been excreted without structural alteration. Considering that Kutscher did not employ radioactive material, his recovery was very high, and inclines one to accept the generalization (9) that compounds containing four alcohol groups are not metabolized by mammals.

More than one-half of the radioactivity of the initial dose was found in the gastrointestinal tract. Of this activity one-third was PETN and two-thirds pentaerythritol; none of the intermediate organic nitrates was detected. One might speculate that the PETN was located in the upper intestinal tract since it was shown previously (1) that the large intestine or its flora is capable of a measurable degradation of the PETN which would result in the detection of the intermediates. The pentaerythritol in the gastrointestinal tract may be the result of this action in the large intestine, although it could be found in the small intestine as a result of bile recirculation or in the stomach as a result of coprophagy. Since the design of the metabolic cages permitted coprophagy, it is not surprising that after 48 hr. of starvation, there were no feces available for collection.

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Effects of Some Psychotropic Agents on Peripheral Nerve Conduction Rate

By JACK K. PRUETT* and BYRON B. WILLIAMS

Impulse conduction rates in isolated frog sciatic nerves were reduced significantly by immersion of the nerves in solutions of chlordiazepoxide, 3.4×10^{-3} μm./ml. and prochlorperazine, 5.5×10^{-3} μm./ml. Promazine at a concentration of 3.5×10^{-3} μm./ml. did not significantly alter conduction rates. For chlordiazepoxide the conduction rate reduction increased with increase in drug concentration.

Most of the studies of psychotropic agents center around their effects on the central nervous system. While it has been established that the major component of psychotropic effect involves one or more central sites, there are side effects produced by drugs in this group which are suggestive of peripheral action, *e.g.*, tingling of extremities and impaired association movements (1–4). Plexus anesthesia in frogs induced by chlorpromazine (5) is indicative of the capacity of such a drug to affect peripheral nerve function.

Krivoy (6) reported peripheral nerve effects of several analgetic agents and speculated concerning the relationship of such effects to drug action mechanism. Tasaki (7) has shown that a 1.2% urethan–ringer solution reduced the conduction rate of a large motor fiber approximately 30% after exposure of less than 5 min. Although it may be argued that transmission reduction of such a magnitude by a drug solution at such a concentration would hardly explain the usual pharmacological effects of urethan, it does seem likely that the effects of urethan in large doses might reflect to some extent this peripheral action. It seems reasonable that psychotropic drugs also in high doses might have some of their nervous system activity attributed in part to an effect on peripheral nerve function.

This project was designed to provide preliminary information on the possible effect of some commonly used psychotropic drugs on impulse conduction rate in peripheral nerves. The drugs chosen, chlordiazepoxide,[1] prochlorperazine,[2] and promazine[3] represent two different structural categories.

EXPERIMENTAL

The sciatic nerve of the green frog, *Rana pipiens*, was used in this investigation. The nerve was dissected from the spinal cord to a point near the termination of the peroneal nerve. Care was taken to retain the eighth and ninth roots of the sciatic nerve, and the tibial nerve was trimmed away at the bifurcation of peroneal and tibial nerves. The dissected nerve was maintained in a beaker of frog ringer solution at pH 7.2 for 1 hr. after which time a general state of stability was attained (7). The nerve was then suspended from silver electrodes mounted in the nerve chamber. The proximal end of the nerve trunk was placed on a pair of stimulating electrodes, and the distal end on a pair of recording electrodes. The stimulating electrodes were 53 mm. from the recording electrodes.

Biphasic stimuli were employed to initiate impulses. Stimuli were provided by a Harvard electronic stimulator, model 340, isolated from the nerve chamber by an isolation transformer. The stimulus intensity was 0.5 v. and of 800 μsec. duration. The voltage constituted a submaximal stimulus for A fibers. The recording and measurement of impulse conduction rates were accomplished by standard electrophysiological techniques essentially like those of Krivoy (6) and Stacy (9) with instrumentation differences as described below. An all-purpose conductor-coupled Mark III preamplifier and a d.c. amplifier, both manufactured by E and M Instrument Co., were used in conjunction with a Lavoie Laboratories TS-239 A/UP oscilloscope. A model 800 Polaroid camera with close-up lens was mounted on the oscilloscope to allow photographic recording of the traces. Type 47, 3000 speed Polaroid film was used to insure high-contrast prints. The sweep generator of the oscilloscope was synchronized with the stimulator. An impulse from the stimulator served to start the sweep at the same time the nerve received a stimulus. The nerve, suspended on the electrodes as described above, was stimulated, and the trace with the resulting action potential was recorded photographically by opening the shutter for the duration of the trace. Conduction time was measured from the end of the stimulus artifact to the apex of the action potential spike. Time markers superimposed on the trace eliminated need for separate time signals. Time signals appeared on all traces and were imposed every 100 μsec. along the trace as shown in Fig. 1.

Nerve conduction rates were determined for the treatment groups and one control group. The treatment groups included chlordiazepoxide, 3.4×10^{-3} μm./ml.; prochlorperazine, 5.5×10^{-3} μm./ml.; and promazine, 3.5×10^{-3} μm./ml. Nerves from treatment groups were checked for normal conduction rate then immersed in drug-ringer solution for a period of 60 min., after which they were

Fig. 1.—Oscilloscope trace of action potential. Arrow indicates stimulus artifact.

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[1] Marketed as Librium Hydrochloride by Roche Laboratories, Nutley, N. J.

[2] Marketed as Compazine by Smith Kline & French Laboratories, Philadelphia, Pa.

[3] Marketed as Sparine by Wyeth Laboratories, Philadelphia, Pa.

TABLE I.—REDUCTION IN IMPULSE CONDUCTION RATE IN ISOLATED FROG SCIATIC NERVE

Treatment	Concn., μm./ml.	Variates, No.	Mean % Reduction	S.E.	*t* Test Probability
Control		18	1.04	1.25	
Chlordiazepoxide	3.4×10^{-3}	25	20.7	3.2	0.01
Prochlorperazine	5.5×10^{-3}	12	16.17	5.1	0.01
Promazine	3.5×10^{-3}	11	6.1	4	0.2

TABLE II.—VARIANCE ANALYSIS OF CHLORDIAZEPOXIDE CONDUCTION RATE EFFECT AT DIFFERENT CONCENTRATIONS

Component	Sum of Sq.	Degrees of Freedom	Variance	*F*	*F* at *P* = 0.01
Among groups	5158.91	3	1719.63	31.29	4.64
Within groups	1429.87	26	54.95		
Total	6588.78	29			

removed from the drug solution and a second rate recording made. Nerves in control groups were subjected to the same procedure except that no drug was added to the frog ringer solution. The chlordiazepoxide group comprised 25 nerves, the prochlorperazine group 12, the promazine group 11, and the control group 18.

To establish dose-effect relationship, four higher concentrations of chlordiazepoxide were used. Since some of these concentrations lowered the pH of the drug-ringer solutions, control nerves for these concentrations were treated with frog ringer solutions adjusted to the altered pH values. Drug treatment groups included from six to 12 nerves and all control groups included six nerves. The same general procedure was used as described above except that the period of immersion was 30 sec.

RESULTS

Single Concentrations.—Per cent change from normal conduction rate was determined for each treated nerve, and these data were subjected to statistical analysis. Table I presents data from tests in which single concentrations of each drug were used. Probability values indicate that the conduction rates were significantly lowered in comparison with controls by direct exposure of nerves to chlordiazepoxide and to prochlorperazine. Promazine treated nerves did not differ significantly from control nerves in conduction rate change at the drug concentration used.

Concentration-Effect Relationship.—Table II presents variance analysis data from tests in which four higher concentrations of chlordiazepoxide were used in an attempt to evaluate the concentration-effect pattern. Experimental *F* values were found to exceed table *F* values at 1% probability and, thus, indicated statistically significant difference between effects of the several drug concentrations. Comparison of control data at pH values corresponding to those of drug-ringer solutions revealed that the hydrogen-ion concentration of the solution used did not appreciably affect the conduction rate. Statistical analysis also indicated a significant difference between drug treated and control nerve conduction rates. Figure 2 graphically presents the relationship between concentration and per cent reduction in conduction rate. Data points as well as calculated regression points are shown. Regression analysis indicated no significant deviation from linearity at the 5% probability level.

DISCUSSION

Preliminary determinations of effects of three psychotropic agents on peripheral nerve impulse conduction rates revealed a significant rate reduction by chlordiazepoxide and prochlorperazine. Promazine at the concentration used failed to significantly alter the conduction rate. An investigation of the concentration-effect pattern for chlordiazepoxide indicated that within the limits of concentration used there was evidence of increase in rate reducing effect with increase in concentration of drug.

Although change in conduction rate of the magnitude revealed at these concentrations (10 to 30% reduction) would hardly be expected to account for the major pharmacological effects of these drugs, it would not be unreasonable to assume that there might be some contribution by such an axon effect to the total activity of these drugs at high dose levels. Evidence of this type may be considered of importance also as an indication of a capacity of some psychotropic drugs to exert general effects on nervous tissue in addition to their rather specific central nervous system effects. Such evidence may point also to a need for the consideration,

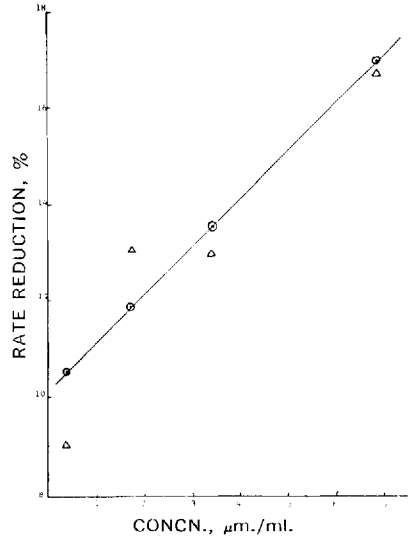

Fig. 2.—Relationship between conduction rate and concentration of chlordiazepoxide. Key: ⊙, calculated point; △, data point.

in mechanism studies, of the possibilities of conductional as well as junctional sites of action for psychotropic agents.

The need to determine site and mechanism of the conduction rate alteration is apparent. The drug concentrations used in this study were near the concentration of chlorpromazine found by Nathan and Friedman (8) to alter the permeability of resting cells of *Tetrahymena pyriformis*. They concluded that this alteration had a lipid site of action. Tasaki (7), in his experiments with saponin, has demonstrated that pharmacological alteration of lipid can change impulse conduction rate. He reported that this agent, by an effect on lipid of the myelin sheath, increased the time required for impulse transmission across the internodal segment. He found, however, that urethan altered conduction rate by a nodal rather than an internodal effect. A study of conduction parameters to provide information as to the site of the rate alteration by these psychotropic agents is planned.

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Ion-Exchange Separation and Ultraviolet Spectrophotometric Determination of Dextromethorphan in Pharmaceutical Products

By K. O. MONTGOMERY* and M. H. WEINSWIG

A method for the isolation and determination of dextromethorphan is presented. The dextromethorphan is extracted with strong cation exchange resin, AG 50W-X4, and is subsequently eluted with 1 *N* hydrochloric acid in 60 per cent methanol in water. The dextromethorphan is determined in the eluate by ultraviolet spectrophotometry. The assay is used successfully on several commercial products.

THE POPULAR acceptance of dextromethorphan as an antitussive agent has brought numerous pharmaceutical products to the consumer with this as the main ingredient. There has been very little information reported on the analytical procedures for the determination of dextromethorphan concentrations in liquid dosage forms.

Saques (1) was able to obtain satisfactory results using ultraviolet spectrophotometry and nonaqueous titration on the pure compound but control blanks had to be utilized to overcome interferences from excipients. Lee (2) demonstrated the use of the classical reineckate precipitation and colorimetric determination on the pure compound. Horioka (3) tested a dye complexation and extraction. The official method (4) for the assay of the pure compound and for the official tablets utilizes nonaqueous titration. Ultraviolet spectrophotometry and paper chromatography (5, 6) have also been studied.

The official assay procedure for the syrup requires an involved immiscible solvent extraction and reextraction followed by the determination using ultraviolet spectrophotometry.

The present paper utilizes the ability of strongly acidic cation exchange resins to separate an amine such as dextromethorphan from common dosage form ingredients prior to determination on a spectrophotometer. A weakly acidic solution is used prior to the use of the strongly acidic solution to remove traces of aromatic amines from flavors or coloring agents that may be present in pharmaceutical products. This type of separation using ion-exchange resins has been employed in the determination for phenylephrine by Kelly and Auerbach (7) and by Blake and Nona (8) in the determination for ephedrine salts.

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Abstracted from a thesis submitted by K. O. Montgomery to the Graduate Division, Butler University, Indianapolis, Ind., in partial fulfillment of Master of Science degree requirements.
* Present address: Pitman-Moore Division, The Dow Chemical Co., Indianapolis, Ind.

EXPERIMENTAL

Apparatus.—Glass column 20 cm. × 1 cm. with stopcock made of Teflon and containing built in needle valve for control of flow rate. The column is also fitted with a reservoir with a capacity of 250 ml.

A suitable recording ultraviolet spectrophotometer such as the Beckman DK-2A or Spectronic 505 which records in absorbance units.

Reagents.—Cationic exchange resin AG 50W-X4 100–200 mesh in hydrogen form available from Bio-Rad Laboratories, Richmond, Calif. Enough resin, about 3 Gm., is added in the form of a slurry to the glass column and rinsed with water. Hydrochloric acid, 0.05 *N* in 60% methanol in water. Hydrochloric acid, 1.0 *N* in 60% methanol in water.

Standard Solution.—Weigh exactly 150 mg. of dextromethorphan hydrobromide N.F. reference standard and transfer to a 100-ml. volumetric flask. Dissolve and adjust the volume with distilled water to prepare the desired stock standard.

Pipet exactly 10 ml. of the stock standard into a 200-ml. volumetric flask and adjust the volume with 1.0 *N* hydrochloric acid in 60% methanol in water.

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Pipet exactly 10 ml. of the stock standard into a 200-ml. volumetric flask and adjust the volume with 1.0 *N* hydrochloric acid in 60% methanol in water.

TABLE I.—ANALYSIS OF PRODUCTS CONTAINING DEXTROMETHORPHAN HYDROBROMIDE

Dextromethorphan Hydrobromide Product No.	Amt. Claimed	Amt. Found	% Label Claim
1[a]	0.9 Gr./fl. oz.	0.906 Gr./fl. oz.	100.7
2[b]	15 mg./5 ml.	14.93 mg./5 ml.	99.5
3[c]	5 mg./5 ml.	5.18 mg./5 ml.	103.6
4[d]	7.5 mg./5 ml.	7.76 mg./5 ml.	103.5

[a] Marketed as Cheracol D by The Upjohn Co., Kalamazoo Mich. [b] Marketed as Robitussin D M by A. H. Robins, Richmond, Va. [c] Marketed as Thorexin by Isodine Pharmacal Corp., New York, N. Y. [d] Marketed as Actin by Chesebrough-Pond's, Inc., New York, N. Y.

Sample Treatment.—Pipet a sample equivalent to 15 mg. of dextromethorphan hydrobromide into the reservoir, rinse the pipet with distilled water, and add to the reservoir.

Add distilled water to the sample to make the volume approximately 100 ml. and mix well.

Allow the sample solution to flow through the resin bed at the rate of 2–3 ml./min. Wash the column by adding 100 ml. of distilled water and allow it to flow through the resin at the rate of 5 ml./min.

Traces of aromatic amines from flavors or coloring agents are removed by allowing 50 ml. of 0.05 *N* hydrochloric acid in 60% methanol in water to flow through the column at 5 ml./min.

Position a 200-ml. volumetric flask under the column and add 190 ml. of 1.0 *N* hydrochloric acid in 60% methanol in water. Allow this to flow through the column at the rate 3 ml./min. The volume is adjusted with 1.0 *N* hydrochloric acid in 60% methanol in water.

Determination.—The ultraviolet spectrum of the sample effluent and of the working standard is recorded with a suitable spectrophotometer. Absorbance units should be used.

Using the baseline technique determine the absorbance at the maximum at about 278 mμ. Calculate the amount of dextromethorphan hydrobromide present from the standard values obtained at the same time the sample is analyzed

DISCUSSION AND RESULTS

Standard solutions, when subjected to the above procedure, yield an average recovery or accuracy of 99.04% with a standard deviation of ±0.52% based on ten determinations. The data for the analysis of marketed products are shown in Table I.

The analytical grade resin used was found to be satisfactory without pretreatment except for washing with a simple water rinse.

A slightly raised baseline was noted with some products where a small portion of the coloring agent was held and eluted with the sample. This did not cause any problem since the baseline technique was used in the calculations.

Other ingredients present in the various formulations included potassium guaiacolsulfonate, ammonium chloride, tartar emetic, sodium citrate, glyceryl guaiacolate, white pine and wild cherry bark extractives, and various common ingredients in syrups and elixirs.

SUMMARY

A method for the isolation and determination of dextromethorphan has been presented. The procedure has been used successfully on commonly available liquid dosage forms and the results are accurate and reproducible.

REFERENCES

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Effect of Red Cedar Chip Bedding on Hexobarbital and Pentobarbital Sleep Time

By HUGH C. FERGUSON*

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In the course of a screening program, control results changed abruptly in one of the author's standard tests. Investigation of all possible varia-

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TABLE I.—EFFECT OF BEDDING ON SODIUM HEXOBARBITAL AND SODIUM PENTOBARBITAL SLEEP TIME

Time, hr.	Sodium Hexobarbital Sleep Time ± S.E., min.		Sodium Pentobarbital Sleep Time ± S.E., min.	
	Corncob	Red Cedar Chips	Corncob	Red Cedar Chips
24	32.4 ± 2.7	31.2 ± 2.1	107 ± 8.1	110 ± 9.2
48	34.6 ± 3.1	16.8 ± 1.5	117 ± 9.8	58.3 ± 2.8
72	31.7 ± 2.1	15.9 ± 1.3	105 ± 10.2	62.2 ± 5.8
96	31.1 ± 2.4	14.1 ± 0.9	121 ± 8.7	55.0 ± 2.4
120	34.5 ± 2.3	15.3 ± 1.1	111 ± 11.0	60.0 ± 3.8
144	34.3 ± 1.9	15.0 ± 1.4	109 ± 9.2	57.1 ± 2.8
168	32.8 ± 2.2	16.1 ± 1.5	125 ± 8.7	61.4 ± 4.5

bles showed that the only alteration was in the bedding material employed. Prior to the erratic results, mice were housed in plastic cages with a ground corncob bedding, and this had been changed to red cedar chips. Since further investigation verified the fact that changing the bedding material altered the pharmacologic response, the following study was undertaken.

Similar results were obtained in two different laboratories: Distillation Product Industries, a Division of Eastman Kodak, and the School of Pharmacy, University of Georgia. Two different sources of male albino mice and red cedar chips were employed in these experiments.

MATERIALS AND METHODS

Male albino mice, obtained from Blue Spruce Farms and the National Laboratory Animal Co., weighing 20–25 Gm. were used in all tests. Each animal was used only once and then sacrificed. Upon receipt of a shipment of animals, half were housed in ground corncob bedding and half housed in red cedar chip bedding in a separate room. Animals were allowed a 24-hr. adjustment period before tests were started. The red cedar chips were obtained from a local pet supply store.

Sleep time, for a group of 10 animals, was measured from the time of administration of sodium hexobarbital (100 mg./Kg. i.p.) or sodium pentobarbital (80 mg./Kg. i.p.) until the return of the righting reflex, indicated by two spontaneous righting responses within a 30-sec. period. The average sleep time and the standard error were determined daily for each group of mice, after the 24-hr. adjustment period.

To determine if the effects were reversible, animals that had been housed in red cedar chips for 120 hr. were then changed to the corncob bedding and sleep time again determined daily employing both sodium hexobarbital and sodium pentobarbital.

RESULTS

Table I shows typical results obtained with sodium hexobarbital and sodium pentobarbital in both control group and those housed in red cedar chips. In both cases there is a significant difference between animals housed in the two bedding materials. The sleep time for mice housed in red cedar chips is approximately 50% less than control groups. This difference is evident at 48 hr. and shows no significant change up to 168 hr. Tests run at 24 hr. were not significantly different from control results.

The results in Table II indicate that the effect produced by red cedar chips on sleep time is reversible. An approach to normal is observed sometime between the 24th and 48th hr. after the mice are removed from the red cedar chip environment; thus the change develops with about the same speed as it is lost.

TABLE II.—EFFECT OF SODIUM HEXOBARBITAL AND SODIUM PENTOBARBITAL ON SLEEP TIME AFTER REMOVAL FROM RED CEDAR CHIPS

Time After Removal, hr.	Sleep Time ± S.E., min.	
	Sodium Hexobarbital	Sodium Pentobarbital
0	16.3 ± 1.3	58.8 ± 2.9
24	21.6 ± 1.9	60.8 ± 4.1
48	32.6 ± 3.1	102.6 ± 7.6
72	33.8 ± 3.2	119.0 ± 6.9
96	33.6 ± 3.2	121.0 ± 7.1

DISCUSSION

These results point to another controllable variable that might possibly exist in pharmacologic evaluations. It is conceivable that one might extrapolate to all phases of pharmacologic screening and wonder whether the manner in which these test animals were housed alters other responses and determinations. Further investigation along these lines is in progress, *e.g.*, effect on other classes of drugs and alterations in metabolism and detoxification. Additional experiments are also being carried out to determine if the volatile material in red cedar chips might be responsible for the change reported.

Thus, investigators should be cautious about the use of red cedar chips in animal rooms for the sole purpose of minimizing odors.

SUMMARY

Male albino mice, in a red cedar chip environment, show a decreased sleep time to sodium hexobarbital and sodium pentobarbital. The effect appears to be reversible.

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Solubility of Acetanilide and Several Derivatives in Sucrose Solutions

By BHOGILAL B. SHETH, ANTHONY N. PARUTA, and FRED C. NINGER

The solubilities of acetanilide, acetoacetanilide, and the *p*-methyl, *p*-ethoxy, *p*-hydroxy, and *p*-amino derivatives of acetanilide were determined in aqueous sucrose solutions. The solubilities of all solutes were found to change significantly with changes in the concentration of sucrose solutions. The solubility curves indicate the changes probably involve solvent polarity, as indicated by dielectric constant of the solvent, and decrease in the activity of water. The solubility changes obtained for acetanilide and the several *para* derivatives were found to show a fair correlation with respect to the magnitude of solubility in water. This indicates a possibility of predicting solubility changes for the remainder of the compounds if the solubility change for one of the compounds is known.

SEVERAL STUDIES have been carried out in these laboratories to investigate solubility phenomena and their implications in the formulation of pharmaceutical dosage forms. Previous studies indicated the solubility of a given solute in syrup could be significantly different from that in water. This was demonstrated for sulfanilamide, quinine, phenobarbital, and *p*-aminobenzoic acid (1), for xanthines, antipyrine and derivatives (2), and for the *p*-hydroxybenzoic acid esters (3). The fact that increased as well as decreased solubilities were noted and the nature of the solubility curves obtained indicated these changes were probably of complex mechanism. It was thought that the mechanism involved changes in solvent polarity, as indicated by the dielectric constant, and decreased activity of water due to an additive, sucrose, with strong dependence also on the nature of the solute and solvent.

This is the third in a series of investigations of sucrose solutions as solvents of considerable pharmaceutical interest. Solutes were selected for this study to yield information for predicting solubility changes in addition to the solubility curves obtained in the conventional manner. Solubilities of antipyrine and its 4-amino and 4-dimethylamino derivatives (2) in water and in various sucrose solutions indicated a possibility that solubility changes might be predictable.

EXPERIMENTAL

Materials.—The compounds used in this study were as follows: acetanilide N.F. (Mallinckrodt); acetoacetanilide (Eastman No. 1239); *p*-acetotoluidide (Eastman No. 425); phenacetin U.S.P. (Nepera Chemical Co.); acetaminophen N.F. (Nepera Chemical Co.); 4-aminoacetanilide (Eastman No. 13); sugar, granulated U.S.P. These materials were used without further purification.

Solubility Determinations.—Solubility determinations of each material were made in water and in the following sucrose solutions: 18.6, 31.6, 46.0, and 63.4% w/w sucrose. The 63.4% sucrose solution corresponds to syrup U.S.P. Deionized water was used throughout this study. The dielectric constants of these solutions ranged from 78.5 for water to 58.5 for syrup U.S.P. The solubility determinations were made as described previously (4–6). Equilibration time was 72 hr. All determinations were made at 25°. Sample volumes diluted for analysis were 1 ml. for 63.4% sucrose solutions and 5 ml. for all other samples. After appropriate dilutions, all samples were analyzed spectrophotometrically. The final dilutions and absorbance maxima for each compound were as follows: 1:1000 dilution at 237 mμ for acetanilide, 1:100 dilution at 241 mμ for *p*-acetotoluidide, 1:100 dilution at 245 mμ for phenacetin, 1:1000 dilution at 241 mμ for acetaminophen, 1:2000 dilution at 250 mμ for 4-aminoacetanilide, 1:1000 dilution at 240 mμ for acetoacetanilide.

Dielectric Constant.—The dielectric constants of saturated solutions of the compounds investigated in this study were measured on a WTW Multidekameter, model DK-06 (Kahl Scientific Instrument Corp.), as previously described (2). Previously determined values of the dielectric constants of the sucrose solutions were used, as these were in agreement with the values reported by other workers (7, 8).

RESULTS AND DISCUSSION

The solubility data of the solutes investigated in this study are shown in Table I. The values represent an average of three determinations in all cases, except phenacetin, where two values were determined.

The solubility ratios, *i.e.*, solubility in 63.4% sucrose solution relative to that in water, are shown in Table II. It is noted that significant solubility changes were obtained in going from water to 63.4% sucrose solution. For drawing solubility curves, the solubility is plotted against both sucrose concentration and dielectric constant of the corresponding sucrose solution used as solvent.

In general, the dielectric constant scale squeezes in the *x*-axis. The solubility curves are shown in Figs. 1–6.

Acetoacetanilide shows the largest relative change in solubility, with a solubility ratio of 0.5. Its solubility curve is linear in relation to both sucrose concentration and dielectric constant (DEC) of the solvent. The decrease in solubility is 0.25 mg./ml./DEC unit. The solubility curves of acetanilide and the *p*-methyl derivative (*p*-acetotoluidide) are smooth, nonlinear functions. However, it is interesting to note that for the *p*-ethoxy derivative (phenacetin) and the *p*-hydroxy derivative (acetaminophen), there is a considerable change in solubility going from 46.0 to 63.4% sucrose. The solubility ratio of the *p*-aminoacetanilide is 0.88, showing the least change in this series of solutes.

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TABLE I.—SOLUBILITY OF ACETANILIDE AND SEVERAL DERIVATIVES IN SUCROSE SOLUTIONS

Solvent	Solubility, mg./ml. Acetanilide	*p*-Methyl-acetanilide	Phenacetin	Acet-aminophen	*p*-Amino-acetanilide	Aceto-acetanilide
Water	6.38	1.05	0.93	13.85	15.98	9.87
18.6% Sucrose	5.74	0.95	0.87	13.08	16.04	8.34
31.6% Sucrose	5.41	0.90	0.82	12.62	15.68	7.59
46% Sucrose	4.63	0.81	0.76	11.63	14.74	6.17
63.4% Sucrose	4.25	0.77	0.59	9.80	14.02	4.96

TABLE II.—SOLUBILITY RATIOS FOR ACETANILIDE AND SEVERAL DERIVATIVES

Solute	Solubility Ratio: (mg./ml. in 63.4% w/w Sucrose Soln.)/(mg./ml. in Water)
Acetanilide	0.66
p-Methylacetanilide	0.73
p-Ethoxyacetanilide	0.63
p-Hydroxyacetanilide	0.71
p-Aminoacetanilide	0.88
Acetoacetanilide	0.50

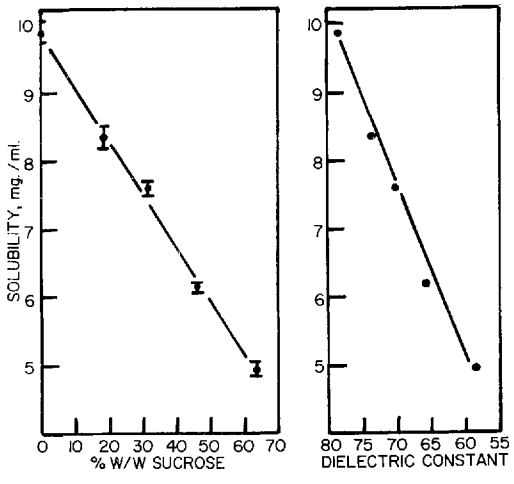

Fig. 1.—Plot of solubility of acetoacetanilide, mg./ml. at 25°, as a function of sucrose concentration and dielectric constant of solvent.

This series of solubility curves is generally in agreement with previous findings (2, 3). The curves indicate the observed changes in solubility probably involve complex mechanisms.

The decrease in solubility, going from water to 63.4% sucrose solution, is shown in Table III, for acetanilide and the *p*-methyl, *p*-ethoxy, *p*-hydroxy, *p*-amino, and the aceto derivatives. It is seen that the solubility change is fairly constant relative to the solubility in water for the first four compounds. These results indicate the possibility of predicting solubility and are in agreement with similar observations made for antipyrine and two derivatives (2). Thus, knowing the solubility change that occurs for acetanilide, the expected change for the derivatives could be approximately calculated.

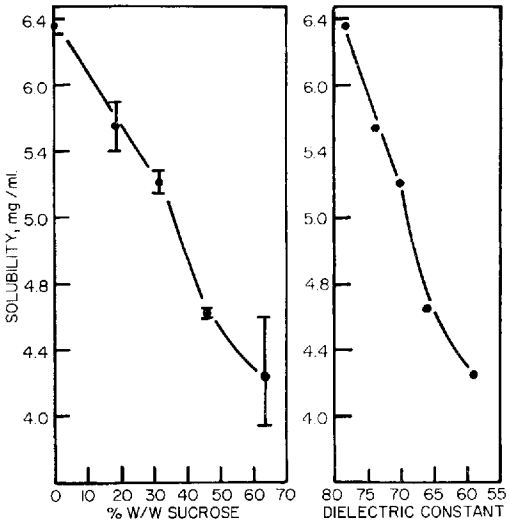

Fig. 2.—Plot of solubility of acetanilide, mg./ml. at 25°, as a function of sucrose concentration and dielectric constant of solvent.

The *p*-amino derivative differs from the rest of the solutes in this regard. This may be due to the fact that the amine substitution may cause a much stronger change in the polarity of the molecule.

It should also be noted that the *p*-amino derivative has the highest magnitude of solubility in this series of solutes. Acetoacetanilide, showing about a 50% decrease in solubility, was not expected to correlate since the substitution was directly on the functional group of the parent compound.

It again indicates the dependence on the nature of the solute and solvent. However, the apparent correlation noted in the change in solubility with two sets of derivatives may be of value if it is further substantiated.

The dielectric constants of the saturated solutions followed, in general, the shape of the dielectric constant curves of the solvents. There was no indication of any specific correlation. This may partly be due to the low solubilities of the solutes.

SUMMARY AND CONCLUSIONS

The solubilities of acetanilide and five related compounds were determined in water and in sucrose solutions containing from 18.6 to 63.4% sucrose. The solubility of all solutes changed significantly in going from water to syrup. The solubility curves obtained in this study again indicate that observed changes are of complex mechanism probably in-

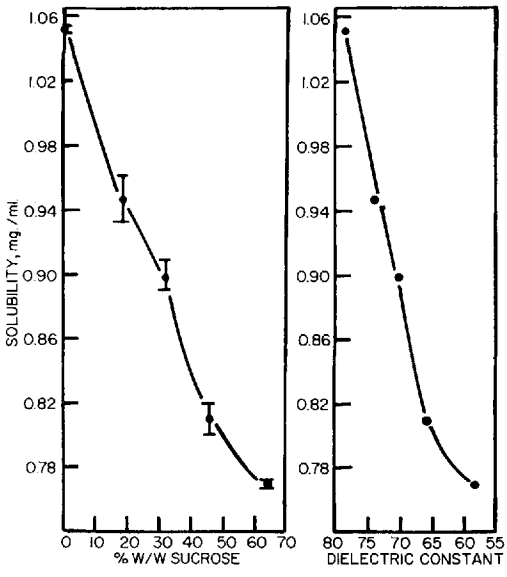

Fig. 3.—Plot of solubility of *p*-methylacetanilide, mg./ml. at 25°, as a function of sucrose concentration and dielectric constant of solvent.

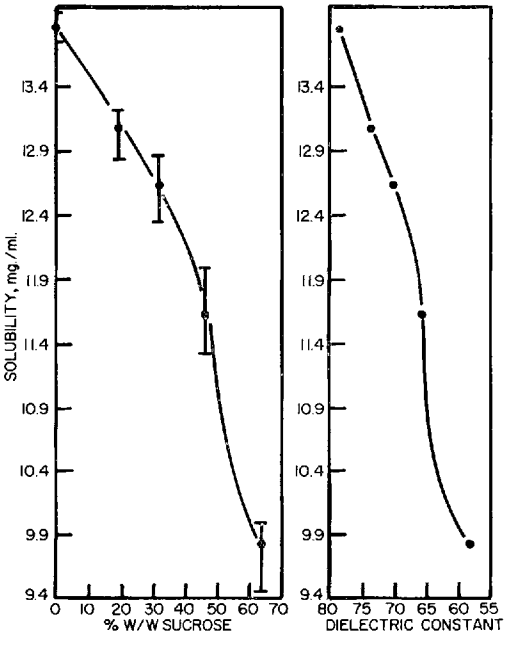

Fig. 4.—Plot of solubility of *p*-hydroxyacetanilide, mg./ml. at 25°, as a function of sucrose concentration and dielectric constant of solvent.

volving solvent polarity, as indicated by the dielectric constant of the solvent, and decrease in the activity of water due to the additive sucrose. A strong dependency on the nature of the solute and the solvent is also indicated.

The decrease in solubility relative to the solubility in water, in going from water to syrup, was found to be fairly consistent for several of the derivatives studied. This further indicated the possibility of predicting solubility changes. These conclusions should be considered within the type and range of systems investigated.

This and previous studies show that the formulator, in considering solubility or applying solubility data, needs to take into account the solvent as represented by the total system of interest. It is the authors' view that an appropriate recognition of solubility phenomena is necessary if the formulator is to use solubility differences of the type reported in this and other studies either to his ad-

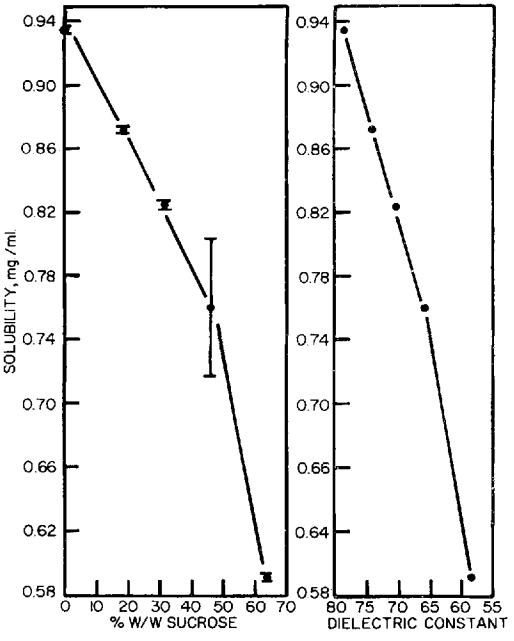

Fig. 5.—Plot of solubility of *p*-ethoxyacetanilide, mg./ml. at 25°, as a function of sucrose concentration and dielectric constant of solvent.

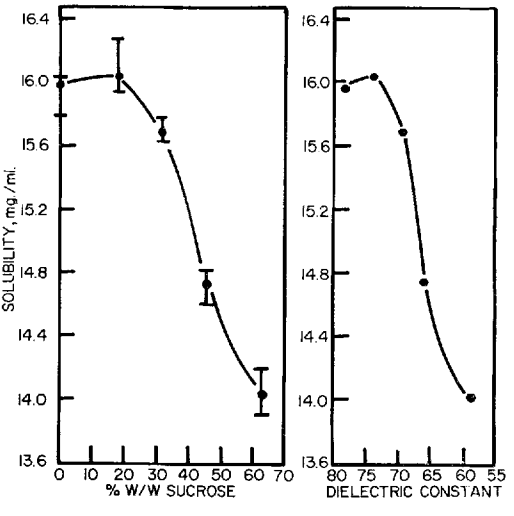

Fig. 6.—Plot of solubility of *p*-aminoacetanilide, mg./ml. at 25°, as a function of sucrose concentration and dielectric constant of solvent.

TABLE III.—SOLUBILITY CHANGE, PER CENT DECREASE RELATIVE TO SOLUBILITY IN WATER, FOR ACETANILIDE AND SEVERAL DERIVATIVES, IN GOING FROM WATER TO 63.4% SUCROSE SOLUTION

Solute	Solubility in Water, mg./ml.	Solubility in 63.4% Sucrose Soln., mg./ml.	Decrease, %
Acetanilide	6.38	4.25	33
p-Methylacetanilide	1.05	0.77	27
p-Ethoxyacetanilide	0.93	0.59	36
p-Hydroxyacetanilide	13.85	9.80	29
p-Aminoacetanilide	15.98	14.02	12
Acetoacetanilide	9.87	4.96	50

vantage in achieving solubility or in avoiding problems due to increased or decreased solubility.

Although there is no sweeping involvement of the dielectric constant as either the parameter of choice or the mechanism involved, it may be a useful tool to the formulator in considering solubility problems.

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Metabolism of ^{14}C-Labeled Glutamic Acid and Pyroglutamic Acid in Animals

By WINTHROP E. LANGE and EDWARD F. CAREY*

A useful method has been developed for the paper chromatographic separation and identification of certain amino acids found in biological fluids. A synthetic route to ^{14}C-labeled pyroglutamic acid was developed. The ^{14}C-labeled glutamic acid and pyroglutamic acid were given orally to mice and rabbits. The drug concentration in various tissues was determined utilizing a chromatogram scanner or a liquid scintillation counter. Experimental data indicated that the metabolic products formed following glutamic acid therapy were pyroglutamic acid, γ amino butyric acid, and glutamine. Animals to which the labeled pyroglutamic acid had been administered showed radioactivity present as γ amino butyric acid and glutamic acid.

GLUTAMIC ACID is an important amino acid. Although it is not essential for growth, it has been known for many years to be a major constituent of body protein and to take part in many metabolic processes. In clinical therapy the monosodium salt has been used in place of the free acid because L-glutamic acid is only slightly soluble in water and is absorbed slowly by ingestion. On the other hand the monosodium salt, which is soluble to the extent of over 70% at room temperature is readily absorbed (1, 2). Monosodium glutamate has been tested in many types of neurological and psychiatric cases with positive results (3, 4) and with negative results (5). However, glutamic acid therapy is a problem from two points of view. First, large doses are required; and second, its taste is difficult to mask.

In 1944, Ratner demonstrated the formation of D-pyroglutamic acid in rats fed DL-glutamic acid (6). Wilson and Koeppe determined labeled carbon dioxide excretion, pyroglutamic acid formation, and tissue glutamic acid concentrations after the administration of D- and L-glutamic acid-2-^{14}C and DL- or D-glutamic acid-5-^{14}C (7). They found that when labeled D-glutamic acid was administered in small doses, intraperitoneally or by stomach tube, more than 50% of the radioactivity was excreted in the urine in 24 hr., most of it as D-pyroglutamic acid. There has been a great deal of investigation of the metabolism of pyroglutamic acid in animals and man. Bethke and Steenboek found that it was converted to glutamic acid and postulated that it was an enzymatic transformation (8). However, no reports of the presence of pyroglutamic acid in brain tissue were found. Thus, it is proposed to develop a micro-method which could accurately determine the distribution of glutamic acid or a metabolic product in various tissues following oral administration of glutamic and pyroglutamic acid. If the oral administration of pyroglutamic acid shows the same compound or compounds are crossing the blood–brain barrier then possibly it can be used in place of glutamic acid to give a similar therapeutic response at a lower dosage.

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Abstracted from a thesis submitted by Edward F. Carey to the Massachusetts College of Pharmacy in partial fulfillment of Master of Science degree requirements.
This investigation was supported in part by a research grant from The Purdue Frederick Co.
* Present address: Atlantic Gelatin, Division of General Foods Corp., Woburn, Mass.

METHODS AND PROCEDURES

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dried and developed by the descending techniques in chromatographic tanks containing a solution consisting of methyl ethyl ketone, propionic acid, and water (75:25:20). The strips were developed at 25° for 8 hr., removed from the tanks, and air dried. The strips were then sprayed with a 0.2% solution of ninhydrin in alcohol and activated in an oven at 80°. All of the amino acids appeared as characteristic pink to purple colored spots on the paper except for pyroglutamic acid. The strips were placed in an atmosphere of chlorine vapors for 5 min., allowed to hang in a hood overnight, and then sprayed with a starch–potassium iodide solution to show the pyroglutamic acid as a blue-black spot on a light blue background (10). The R_f values calculated for the various amino acids of interest were:

DL-Glutamine	0.22
DL-, L-, and L(+) Glutamic acid	0.29
γ Amino butyric acid	0.52
Glutathione	0.55
Pyroglutamic acid	0.68

Synthesis of Labeled Pyroglutamic Acid.—A measured solution of glutamic acid-1-^{14}C, buffered to a pH of 2.0 with hydrochloric acid, was sealed in a glass ampul, and placed in an oven at 130° for 72 hr. After the ampul was cooled, it was opened and a sample subjected to paper chromatographic examination. The paper strips, after chromatography, were run through a chromatogram scanner, model RSC-160 by Atomic Accessories, Inc., at a rate of 1.5 in./min. with a constant flow of Geiger gas. The rate meter attachment was a Tracermatic by Tracer Labs. Standard curves of glutamic and pyroglutamic acid were obtained by plotting for a scan of a given number of counts per minute the area under the curve in centimeters against the number of gamma's of isotope spotted. The conversion of glutamic acid to pyroglutamic acid was nearly quantitative.

Liquid Scintillation Detection.—To increase the sensitivity for the detection of micro quantities of the amino acids in the tissue samples, the paper strips were cut into small portions and placed in scintillation fluid. The fluid used consisted of 0.1 Gm. of 1,4-di-2-(5-phenyloxazolyl)-benzene, 14 Gm. of 2,5-diphenyloxazole, 100 Gm. of naphthalene (C.P.), and sufficient dioxane to make 2 L. The liquid scintillation counter was from Baird Atomic, Inc. From the R_f values for the various amino acids, the activity found in various portions of the paper strips could be identified.

Comments on the Analytical Procedures.—It was found that the paper strip could not be chlorinated before spraying with ninhydrin if the ninhydrin-positive amino acids were to be detected. It was also noted that the chlorination step could not be speeded up without a loss of sensitivity. Thin-layer chromatography was tried. Satisfactory separation of the amino acids was not obtained.

BIOLOGICAL TESTING

Investigation with Rabbits.—A fasted rabbit was fed 200 μl. of glutamic acid-1-^{14}C, which contained 20 μc. of activity in 20 Gm. of glutamic acid carrier. After administration of the drug, 2-ml. samples of blood were obtained from the rabbit by cardiac punctures at 0.5, 1, 2, 3, 4, 6, 18, and 24-hr. intervals. One milliliter of a 10% solution of trichloroacetic acid was added to the blood sample, the suspension mixed, centrifuged, and the clear supernatant fluid separated. A 100-μl. sample of the supernatant was spotted on a strip of Whatman No. 1 chromatography paper and the strips chromatographed in the previously described developing system. The strips were allowed to develop for 6 hr., dried, and run through the chromatogram scanner with a rate meter attached. A 250-μl. sample of the supernatant fluid was added to 15 ml. of scintillation fluid in low ^{40}K vials, the mixture shaken, and counted in a liquid scintillation counter to give the total activity for the sample. Portions of the chromatographed strips were treated with scintillation fluid and the resulting solutions counted as before. From the R_f values found for the various amino acids, the source of radioactivity found in the samples could be identified.

The pyroglutamic acid-1-^{14}C, which had been prepared from the labeled glutamic acid, was fed to a 2.5-Kg. rabbit. The procedure followed was the same as for the labeled glutamic acid study except that 300 μl. of pyroglutamic acid-1-^{14}C in a 3-Gm. carrier solution of pyroglutamic acid was used as the dose.

Investigation with Mice.—Three white mice, each weighing approximately 20 Gm., were fed 1 ml. of a glutamic acid carrier solution which contained 1 μc. of glutamic acid-1-^{14}C. After 3 hr., the mice were heparinized and sacrificed. The blood from the three mice was pooled, the protein precipitated with trichloroacetic acid, the mixture centrifuged, and the supernatant fluid separated. The brain was enucleated from each mouse and washed with three portions of sterile saline solution at 5°. The brains from the three mice were pooled, homogenized with 2 ml. of cold saline solution, the mixture centrifuged, and the clear liquid collected. The kidneys of each mouse were treated in a similar manner. Control tissues were similarly prepared from untreated mice. Fifty microliters of the three different tissue samples were spotted on strips of Whatman No. 1 chromatography paper. The strips were developed in the previously described manner. The chromatographed strips after drying were run through the chromatogram scanner. No clearly defined areas of radioactivity could be detected. The strips were then cut into sections and placed in ^{40}K free glass vials, each containing 15 ml. of scintillation fluid. Radioactivity was detected in some of the vials. Knowing the area (R_f) of the

TABLE I.—SERUM LEVELS OF LABELED AMINO ACIDS FOLLOWING ADMINISTRATION OF LABELED GLUTAMIC AND PYROGLUTAMIC ACIDS TO RABBITS[a]

	Glutamic Acid[b]	Pyroglutamic Acid[c]
Glutamic acid	36[d]	60
Glutamine	28	..
γ Aminobutyric acid	..	60
Pyroglutamic acid	32	20

[a] Radioactivity obtained from a liquid scintillation counter of 100 μl. of a 3-hr. serum sample chromatographed on paper and extracted with scintillation fluid. [b] Dose of 250 μl. in 20 Gm. of carrier solution (20 μc. of activity). [c] Dose of 300 μl. in 2 Gm. of carrier solution (20 μc. of activity). [d] Average number of counts/min. for six animals.

TABLE II.—TISSUE LEVELS OF LABELED AMINO ACIDS FOLLOWING ADMINISTRATION OF LABELED GLUTAMIC AND PYROGLUTAMIC ACID TO MICE[a]

	Glutamic Acid[b]			Pyroglutamic Acid[b]		
	Serum	Brain	Kidney	Serum	Brain	Kidney
Glutamic acid	42[c]	40	46	. . .	29	. .
Glutamine	. .	28	36	. . .	. .	. .
γ Aminobutyric acid	. .	. .	. .	. . .	. .	82
Pyroglutamic acid	30	. .	. .	310	. .	. .

[a] Radioactivity obtained from a liquid scintillation counter of 100 μl. of a 3-hr. tissue sample chromatographed on paper and extracted with scintillation fluid. [b] Dose of 1 ml. of glutamic or pyroglutamic acid carrier with 1 μc. of activity. [c] Average number of counts/min. for three groups of three animals/group.

strip associated with the vials having radioactivity, it was possible to predict the amino acids involved.

The entire procedure was repeated with groups of three mice being fed pyroglutamic acid-1-^{14}C. The dosage was the same as for the glutamic acid study.

RESULTS AND DISCUSSION

In the synthesis of the labeled pyroglutamic acid from the labeled glutamic acid, it was found that the 72-hr. heating at 130° gave better than a 98% conversion of glutamic acid to pyroglutamic acid. Heating for 24 hr. at the same temperature gave about a 95% conversion, while heating at 100° for 24 hr. gave only about an 80% conversion.

Tables I and II summarize the distribution of the different labeled amino acids found in the various tissues after the oral administration of labeled glutamic and pyroglutamic acid to rabbits and mice. No attempt was made to determine absorption rates. However, when the total concentration of labeled pyroglutamic acid and labeled glutamic acid in the tissues studied are compared it can be seen that at the 3 hr. sampling time, despite a lower dose being given, the pyroglutamic acid is more completely absorbed.

The paper strips which contained the chromatographed samples from the blood of the rabbits fed labeled glutamic acid showed only the presence of glutamic acid and pyroglutamic acid when they were run through the chromatogram scanner. The acids were identified by their R_f values on the paper, as well as their color following spraying of the strips with the described indicator solutions. The presence of labeled glutamine in the blood samples was not detected until the strips were cut into sections and examined with the liquid scintillation counter. The highest radioactivity levels in the blood were found between 2 and 4 hr. after oral administration of the labeled glutamic acid. These activity levels were found to be equivalent to approximately 4% of the administered dose of labeled drug. The chromatogram scanner did not differentiate the radioactivity source on paper strips prepared from the blood of rabbits fed the labeled pyroglutamic acid. It was possible, however, to differentiate the amino acids containing the radioactivity using the liquid scintillation counter.

In the mice, the highest tissue concentration of radioactivity was found to be between 2 and 4 hr. after administration of the labeled acids. In all of the studies with mice, the chromatogram scanner was not able to differentiate the sources of radioactivity in the tissues examined. After 3 hr., the mice which had been fed the labeled glutamic acid were found to have the greatest concentration of radioactivity in the kidney, while after pyroglutamic acid adminstration the highest activity was found in the blood.

Attempts to obtain autoradiograms from contact of the chromatographed strips with X-ray film were not successful. Various concentrations of the tissue fluids were chromatographed as well as various development times were tried for the X-ray film. It was thought that the autoradiograms might give a second method for checking R_f values for the labeled amino acids.

SUMMARY

It has been found that the metabolism of labeled glutamic acid and pyroglutamic acid in mice can be followed using a combination of paper chromatography and liquid scintillation counting. Labeled pyroglutamic acid was found to be absorbed after oral administration and converted to glutamic acid. The labeled glutamic acid was further found to be present in brain tissue while the pyroglutamic acid was not found in the brain tissue at any time. Thus, it might be possible to obtain a therapeutic response similar to that of glutamic acid after oral administration of pyroglutamic acid.

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Alkaloids of *Scopolia carniolica*

By SANTO W. ZITO and JOHN D. LEARY

The alkaloid composition of the rhizomes of *Scopolia carniolica* Jacq. has been investigated and the identification of six alkaloids is reported. Three of these alkaloids have been reported previously and are confirmed in this report. Moreover, the authors report here for the first time the presence of 3-α-tigloyloxytropane in the genus *Scopolia;* in addition, pseudotropine and cuscohygrine are reported in this plant for the first time. One other alkaloid could not be identified at this time.

SCOPOLIA CARNIOLICA Jacq., a member of the family *Solanaceae*, is well known for its content of the tropane ester alkaloids hyoscyamine and scopolamine, and for the hydramine, tropine (1). Evidence for the existence of alkaloids other than those mentioned is without firm foundation. Bendik *et al.* (2) detected the presence of scopine in addition to the previously mentioned bases, and Schreiber (3) reported on the presence of solanidine in this plant as well as in many other members of the nightshade family; neither of these bases could be confirmed in this work. Another report suggests the presence of alkaloids which were not identified (4).

The dried rhizomes were extracted by two different methods, subjected to preliminary purification, and were analyzed for the presence of alkaloids by paper partition and thin-layer chromatographic procedures. Examination of the chromatograms revealed the presence of seven alkaloids, three of which were previously identified.

EXPERIMENTAL

Extraction.—The dried rhizomes[1] were extracted by two different procedures. The first method involved continuous extraction with methanol in a Soxhlet apparatus (5) until devoid of alkaloids when tested with Mayer's reagent. The methanol extract was concentrated to a viscous solution at 40° *in vacuo*, diluted with water, and acidified to pH 2 with hydrochloric acid. The aqueous solution was exhaustively extracted with chloroform (fraction A). The aqueous phase was then adjusted to pH 10 with 20% sodium hydroxide solution and extracted anew with chloroform (fraction B). Each of these fractions was submitted to analysis by paper and thin-layer chromatography.

In the second method the drug was moistened overnight with water and was then intimately mixed with calcium hydroxide according to the method of Evans and Stevenson (6). The basified drug was stirred with solvent ether during the next 2 hr.; the supernatant liquid was decanted, and the marc was further percolated with solvent ether until the percolate gave a negative test for the presence of alkaloids. The ether extracts were combined and concentrated at 40° *in vacuo;* the viscous extract was fractionated on a buffered kieselguhr column.

Column Partition Chromatography.—The concentrated extracts from Evans' method were placed on a column of kieselguhr (30 Gm.) loaded with phosphate buffer (15 ml.; pH 6.6). Elution was carried out with successive portions of petroleum ether, solvent ether, chloroform, and chloroform saturated with concentrated ammonium hydroxide solution. Petroleum ether gave an oily material which gave a questionable Dragendorff-positive spot of high R_f value on paper and thin-layer chromatograms (see below). Ether eluted hyoscine followed by 3-α-tigloyloxytropane; chloroform removed hyoscyamine and an unidentified material. The ammoniacal chloroform afforded tropine, pseudotropine, and cuscohygrine. Identifications were based on elution patterns on the buffered column which coincided with previously reported results (6), as well as by comparison of the extracts with known compounds in the chromatographic systems mentioned later.

Paper Partition Chromatography.—Whatman No. 1 paper was impregnated with 0.5 *M* KCl solution, air dried, and used for the separation of the various extracts and reference compounds,[2] used singly and in mixtures. Development of the chromatograms was achieved with the solvent system of Rother *et al.* (7) which consisted of *n*-butanol–concentrated hydrochloric acid (98:2) saturated with water. Revelation of the alkaloids was effected with modified Dragendorff's reagent (8). Two other partition systems were used but were not as successful in complete resolution of the mixtures.

Thin-Layer Chromatography.—A matrix of aluminum oxide G (Brinkmann) was prepared by the standard procedure (0.250 mm.), and the plates were activated at 110° for 30 min. Extracts of the plant in addition to reference compounds, singly and admixtures, were developed with a solvent system of benzene–methanol (9:1); extracts suspected of containing cuscohygrine were developed with a mixture of benzene–methanol–diethylamine (99:1:5). Revelation of the alkaloids was achieved with modified Dragendorff's reagent.

The data for the chromatographic studies are summarized in Table I.

RESULTS

Extracts of the rhizomes of *S. carniolica* obtained by two different methods have yielded fractions which were analyzed by several paper and thin-layer chromatographic procedures. Maximum resolution was achieved in the two systems which are reported. The development of purple spots in the case of tropine and pseudotropine-containing extracts aided materially in their identification. Identification was effected by means of elution patterns on a kieselguhr-phosphate buffer column,

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[1] The *S. carniolica* Jacq. rhizomes used in this study were generously supplied by S. B. Penick Co., New York, N. Y.

[2] Hyoscyamine and scopolamine (Merck) were obtained commercially; cuscohygrine, tropine, pseudotropine, and 3-α-tigloyloxytropane were supplied by Dr. A. E. Schwarting University of Connecticut, Storrs; a second sample of the latter was supplied by Dr. W. Evans, University of Nottingham, Nottingham, England.

TABLE I.—CHROMATOGRAPHIC DATA FOR THE ALKALOIDS OF *S. carniolica*

Alkaloid	Paper[a]	Thin-Layer[b]
Cuscohygrine	0.08	0.00; 0.63[c]
Pseudotropine	0.15	0.13
Scopolamine	0.26	0.52
Unidentified alkaloid	0.31	0.63
Tropine	0.34	0.22
Hyoscyamine	0.48	0.43
3-α-Tigloyloxytropane	0.84	0.63

[a] Whatman No. 1 paper (0.5 *M* KCl); *n*-butanol–HCl (98:2) water-saturated. [b] Aluminum oxide G; benzene–methanol (9:1). [c] Aluminum oxide G; benzene–methanol–diethylamine (99:1:5).

comparison of R_f values of compounds in extracts when analyzed by paper and thin-layer chromatographic procedures with authentic compounds, and color reactions.

The data indicate the presence of at least seven alkaloids. Identification of hyoscyamine, scopolamine, and tropine corroborates the studies of previous workers. This work has succeeded in the tentative identification of 3-α-tigloyloxytropane, the first reported occurrence of this compound in the genus *Scopolia;* it extends the distribution to still another genus of the family *Solanaceae* along with *Datura* (6), *Withania* (5), and *Physalis* (9). It also broadens the knowledge of the existence of cuscohygrine in plants containing tropane alkaloids. Pseudotropine was the sixth alkaloid to be identified. One other alkaloid remains to be identified. Failure to confirm the presence of scopine and solanidine in this sample was due to a lack of authentic reference compounds.

Work is continuing and complete details on the extraction, isolation, and characterization will be published at a later date.

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Synthesis of *N,N'*-Haloacyl Analogs of *p,p'*-Oxydianiline as Potential Antineoplastic Agents

By WILLIAM D. ROLL

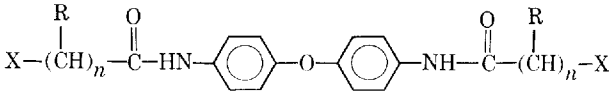
A series of eight new haloacetyl and halopropionyl derivatives of *p,p'*-oxydianiline have been synthesized for evaluation of anticarcinogenic activity.

AS AN INTEGRAL part of this continuing cancer chemotherapy research project, another series of bis-haloamide analogs of a parent diamine molecule have been prepared. Based upon the screening data obtained in this laboratory (1–3) and that from others in the field (4–7), the chemotherapeutic activity of such compounds is deserving of further study. This report concerns itself with the synthesis of a series of bis-haloacetyl and bis-halopropionyl derivatives of *p,p'*-oxydianiline (I).

This type of alkylating agent may inhibit the growth of cancer cells through selective inhibition of vital metabolic activities within tumor cells (8–15). By varying the carrier moieties of these active, relatively nontoxic compounds, it is hoped that some insight will be gained as to structure–activity relationships as regards their alkylating abilities.

DISCUSSION

An anhydrous chloroform solution of the diamine, *p,p'*-oxydianiline[1] (I), was treated with a chloroform solution of chloroacetyl chloride, 2-chloropropionyl chloride, and 3-chloropropionyl chloride to form,



X = Br, Cl, and I
R = H, n = 1,2
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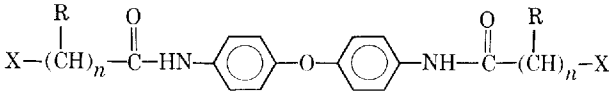

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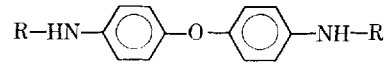
TABLE I.—*N,N'*-HALOACYL ANALOGS OF *p,p'*-OXYDIANILINE



Compd.	R	M.p., °C.	Yield, %	Anal. Calcd.	Anal. Found	Infrared ν cm.$^{-1}$ (KBr) (C=O amide)
II	$-COCH_2Cl$	231–232	80	C, 54.40 H, 4.00 Cl, 20.08 N, 7.93	C, 54.45 H, 3.96 Cl, 19.98 N, 7.99	1670
III	$-COCH(Cl)CH_3$	235–236	71	C, 56.70 H, 4.76 Cl, 18.60 N, 7.36	C, 56.68 H, 4.79 Cl, 18.52 N, 7.45	1670
IV	$-CO(CH_2)_2Cl$	227–228	75	C, 56.70 H, 4.76 Cl, 18.60 N, 7.36	C, 56.73 H, 4.80 Cl, 18.51 N, 7.30	1660
V	$-COCH_2Br$	216–217	75	C, 43.46 H, 3.19 Br, 36.14 N, 6.34	C, 43.39 H, 3.25 Br, 36.20 N, 6.29	1650
VI	$-COCH(Br)CH_3$	247–248	69	C, 46.98 H, 3.94 Br, 34.73 N, 6.08	C, 46.95 H, 3.93 Br, 34.80 N, 6.13	1660
VII	$-CO(CH_2)_2Br$	225–226	74	C, 46.98 H, 3.94 Br, 34.73 N, 6.08	C, 46.94 H, 3.92 Br, 34.70 N, 5.99	1650
VIII	$-COCH_2I$	239–240	70	C, 35.78 H, 2.62 I, 47.26 N, 5.22	C, 35.79 H, 2.61 I, 47.20 N, 5.18	1650
IX	$-CO(CH_2)_2I$	226–227	78	C, 38.32 H, 3.22 I, 44.98 N, 4.96	C, 38.40 H, 3.24 I, 45.03 N, 5.00	1660

respectively, *N,N'*-bis(chloroacetyl)-*p,p'*-oxydianiline (II), *N,N'*-bis(2-chloropropionyl)-*p,p'*-oxydianiline (III), and *N,N'*-bis(3-chloropropionyl)-*p,p'*-oxydianiline (IV). Similarly, the reaction of I with bromoacetyl bromide, 2-bromopropionyl bromide, and 3-bromopropionyl chloride gave the corresponding diamides, compounds V, VI, and VII, respectively. Compounds V and VII were converted into the iodoamides, compounds VIII and IX, respectively, by treating the former with sodium iodide in acetone.

EXPERIMENTAL

The procedure used for the synthesis of these compounds has been previously related (2). Reference may be made to Table I for the results of this synthetic work and properties of the compounds prepared. The melting points were determined with a Fisher-Johns melting point apparatus and are corrected. The infrared spectra were obtained with a Perkin Elmer Infracord.

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Growth of Callus Tissue of *Catharanthus roseus* in Suspension Culture

By DAVID P. CAREW

The successful suspension culture of *Catharanthus roseus* (L.) G. Don callus tissue for an indefinite period of time has been accomplished. The growth rates of the suspension cultures, which were grown in media with and without growth substances, are reported. Alkaloids were detected in both the tissue and the media.

THE IMPORTANCE of *Catharanthus roseus* (L.) G. Don (*Vinca rosea* L.) as a source of anticancer alkaloids is well established (1). The report of the 1964 Symposium on the Chemistry and Biological Activity of Catharanthus, Vinca, and Related Indole Alkaloids (2) provides considerable information on this subject. The literature also contains reports on the growth of *C. roseus* as a tissue culture (3–7) and of the presence of alkaloids in *Catharanthus* callus tissue. Previous tissue culture studies of *C. roseus* have been concerned with growth on solid media. This paper reports a study of *C. roseus* tissues grown in suspension culture over a period of several months.

EXPERIMENTAL

Plant Tissues and Media.—Callus cultures of *C. roseus* which had been growing on solid media for more than 3 years provided the source of tissue for initiation of suspension cultures. The solid nutrient medium was a modified White's formula containing 15% coconut water and 3 mg./L. of 2,4-dichlorophenoxyacetic acid (2,4-D) (3). A preliminary study revealed that while a modified White's medium provided excellent nutrition for the growth of *C. roseus* tissues cultured on solid media, a modified Wood and Braun medium (5) was preferable for suspension culture. The modified Wood and Braun medium as reported previously (5) was used as a basal medium throughout this study.

Inoculation, Growth, and Transfer Procedures.—Suspension cultures of *C. roseus* were initiated by aseptically inoculating 100 ml. of Wood and Braun's modified medium contained in 500-ml. conical flasks, with approximately 3 Gm. of callus tissue which had been growing on solid media. The inoculated flasks were placed on an Eberbach rotating shaker which operated at 160 r.p.m. on a 1.5 in. circle. The shaker was located in a dark room maintained at a temperature of 26°. Once suspension cultures were successfully established these tissues were used as inocula for initiating continuing suspension culture studies. A circular metal scoop with a perforated base, 1 cm. in diameter and 0.7 cm. in depth, was constructed and used to transfer suspension cells to fresh liquid media. The scoop was attached to the handle of a conventional inoculating needle and the unit was easily sterilized by flaming. Numerous transfers of tissue were made and their weights determined in order to find the approximate average weight of tissue contained in one scoop. The average figure was found to be 255 mg.

In an attempt to determine the effect of naphthaleneacetic acid (NAA) and kinetin on the growth of continuing suspension cultures, two media formulations were studied. One medium consisted of Wood and Braun's basal medium without the presence of either kinetin or NAA. A second formula contained the basal medium plus NAA, 1 mg./L., and kinetin, 0.5 mg./L.

The procedure for inoculating and subsequently continuing growth of the suspension cultures was the following. The appropriate medium was prepared and adjusted to pH 5.8 and placed in conical flasks (100-ml./500-ml. flask). The flasks were plugged with cotton and sterilized by autoclaving for 15 min. at 15 p.s.i. One hundred milliliters of media was inoculated by the aseptic addition of five scoops of suspended cells (approximately 1.275 Gm.). The inoculated flasks were placed on a rotating shaker. The suspension cultures were observed daily and at the end of the growth period the flasks were removed from the shaker. At that time some of the suspended cells were aseptically removed for the purpose of inoculating new media. The remainder of the suspended cells were collected on filter paper as the cell-media suspension was passed through a Büchner funnel. The fresh weight of the tissue from each flask was determined and recorded. In each case the tissue which had been removed for inoculation of new media was included in the total fresh weight figure. The tissue which was collected on the filter paper was wrapped in aluminum foil and frozen for later chemical investigation. The results of the growth rate study are recorded in Tables I and II. As noted in these tables, five series of suspension cultures, each series consisting of from 10 to 20 cultures, were grown on media with NAA and kinetin, while 10 series of cultures were grown on the basal medium without the addition of any growth substance.

In a separate experiment suspension cultures of *C. roseus* were grown in 250-ml. conical flasks, some of conventional design and others containing three baffles in the base of each flask. The baffled flasks are a product of Bellco Glass Co. The purpose of this experiment was to determine if increased agitation and aeration of cells would have any effect on the growth rate. The 250-ml. conical flasks each contained 50 ml. of sterile Wood and Braun medium with kinetin, 0.5 mg./L., and NAA, 1 mg./L. A suspension culture inoculum of approximately 765 mg. was aseptically placed in each flask of sterile medium. Both the conventional and the triple-baffled flasks were placed on an Eberbach rotating shaker which operated at 160 r.p.m. Two growth cycles were evaluated and the growth rates of tissues

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TABLE I.—MODIFIED WOOD AND BRAUN MEDIUM WITHOUT GROWTH SUBSTANCES

Series	Flasks Inoculated	Flasks Contaminated	Days Cultured, No.	Av. Fresh Wt./Flask, Gm.[a]
1	20	3	47	19.361
2	10	5	53	15.324
3	20	8	48	17.913
4	10	3	41	18.534
5	20	7	41	24.936
6	20	6	53	19.899
7	20	0	41	12.920
8	10	2	41	21.578
9	20	0	53	11.415
10	20	4	54	14.581

[a] Growth rates were not determined if flasks were contaminated.

TABLE II.—MODIFIED WOOD AND BRAUN MEDIUM CONTAINING KINETIN AND NAA[a]

Series	Flasks Inoculated	Flasks Contaminated	Days Cultured, No.	Av. Fresh Wt./Flask, Gm.[b]
1	10	5	53	19.834
2	10	5	47	24.249
3	20	7	47	15.935
4	10	1	53	15.631
5	20	8	53	10.044

[a] Kinetin in concentration of 0.5 mg./L. and NAA in concentration of 1 mg./L. [b] Growth rates were not determined if flasks were contaminated.

cultured in the two types of flasks were compared. In each case the tissue was harvested after 54 days.

RESULTS AND DISCUSSION

It has been demonstrated that *C. roseus* tissue cultures can be grown under submerged conditions and it appears, that providing proper subculturing procedures are performed, growth can be carried on indefinitely. In the experiment employing growth substances, the concentration used in liquid media was considerably less than that employed previously in solid media. In fact, it was found in one experiment with suspension cultures that the tissue required no added growth substance in the medium. The mean fresh weight, per flask, of tissue grown in a medium without NAA and kinetin was 17.646 Gm. while the average fresh weight of tissue grown on a medium with these growth factors was 17.938 Gm. In each instance the weight of inoculum was about 1.3 Gm. Several previous attempts to maintain *C. roseus* tissue cultures on solid media without growth substances met with only limited success. The tissue grew very slowly, especially in comparison to tissue grown on media with growth substances.

In the flasks of media without growth substances there was never evidence of tissue differentiation. Furthermore, on several occasions suspension cells were transferred to solid nutrient media without growth substances and in no instance was there evidence of differentiation which has been reported under similar circumstances with other tissues.

Despite the fact that the suspension cultures were conditioned to the media over a considerable period of time, very little growth appeared in the first 3 weeks after cells were transferred to new media. After this 3-week "lag" period growth occurred at a rapid rate for approximately 3 weeks. In flasks in which the cells remained as clumps or clusters, as opposed to those flasks in which the cells were finely dispersed, there was much slower growth. In a few flasks very little growth occurred and the clumps of cells at the end of the growth period appeared about the same as at the beginning. Problems of microorganism contamination were encountered as are indicated in Tables I and II. No growth rates were determined for flasks of tissue which became contaminated.

In the experiment concerned with cultures in conventional and baffled flasks it was found that suspension cultures in conventional conical flasks grew at a significantly faster rate than did cultures in the triple-baffled flasks. Apparently the increased agitation and aeration does not improve the growth rate but in fact may be detrimental to growth.

A qualitative examination of the tissues and the spent media from this study revealed the presence of several alkaloids in both the tissue and the media. The suspension culture of *C. roseus* is presently being scaled up from 500-ml. flasks to multiliter fermentors.

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Molecular Interaction of Stereoisomers When in Solid State

By A. H. BECKETT and N. H. CHOULIS

Studies of acid-base association of asymmetric molecules in the solid state, involving the determination of melting points of the formed diastereoisomeric salts, their infrared spectra, and for a number of examples, X-ray diffraction powder photography, have been carried out. In these examples, in which crystalline salts were obtained using optically active acids and bases, the diastereoisomeric salts prepared from components of the same configuration had higher melting points than those of different configuration. Although infrared measurements could not be used to indicate configuration of the components, definite differences in the spectrum of salts formed from isomers of like configuration from those of unlike configuration have been observed; likewise, the X-ray diffraction pattern was different.

JAROWSKI AND Hartung (2), working with the mandelates of ephedrine-type molecules, showed some correlation between chemical structure and physiological properties; they also reported the melting points of the salts formed. However, no attempt was made to interpret the differences in melting points in terms of differences in the geometry of the components of these diastereoisomeric salts.

Ricci (3) discussed the principles of the quasi-racemic method of configurational assignment and concluded that molecular interactions in a system of two substances of opposite configuration cause negative deviation from ideality as compared to the phase equilibrium in the corresponding system between related configurations.

Differences observed in infrared spectra of diastereoisomeric salts have already been the subject of another paper (1).

The X-ray method has been used by Bijvoet *et al.* (4) to deduce configurations and also by examining X-ray photographs (5–7), the degree of association of the molecules within the diastereoisomeric salts.

In the present investigation, melting point measurements, infrared spectra evaluations, and X-ray diffraction powder photographs of a number of different diastereoisomeric compounds have been examined.

EXPERIMENTAL

Materials.—The optically active acids and bases used are these recorded in Table I.

The signs D- and L- refer to the absolute configuration of the compounds used.

Preparation of the Salts.—Equimolar quantities (about 0.5 Gm.) of the appropriate acid and base were dissolved in an appropriate solvent (*e.g.*, methyl or ethyl alcohol, ether, etc.) under reflux. The solution was cooled and placed in the refrigerator until crystals separated. These crystals were filtered off, dried under vacuum, and melting points were taken; these salts were recrystallized to constant melting points.

The substance (small quantity in a melting point tube) was placed in the melting point apparatus when the temperature was about 10° below the melting point. Heating at a rate of about 2°/min. was then carried out.

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This work was carried out at Chelsea School of Pharmacy, London, England, as partial requirement for a Ph.D. thesis under a grant from the Greek Foundation Scholarships.

For the infrared spectra Nujol mulls of the salts were prepared (1). The same salts were sent for X-ray diffraction powder photographs.[1]

Instruments.—The following instruments were used: (*a*) the Townson and Mercer melting point apparatus and (*b*) the Unicam S.P. 200 spectrophotometer.

RESULTS

Melting Point Measurements.—The results (Table I) indicate that salts formed from optically active acids and optically active bases, and possessing the same configuration, had a higher melting point than those formed from the corresponding acids and bases of opposite configuration. The latter salts in some cases could only be obtained in the form of oils.

As expected, the melting points of D-acid/D-base salts were similar to those of the corresponding L-acid/L-base salts; L-acid/D-base salts also had the same melting points as D-acid/L-base salts.

X-Ray Diffraction Powder Photography.—The results (Table II) indicate that *d*-values, calculated from the application of Bragg's law (Eq. 1), were not the same for both diastereoisomers (*i.e.*, between D/D and D/L or between L/L and L/D).

$$n\lambda = 2\,d \sin \vartheta^\circ \qquad \text{(Eq. 1)}$$

where

n = order of reflection
λ = wavelength
d = interplanar spacing
ϑ° = angle of deviation from the direct X-ray beam.

DISCUSSION

The results indicated that in the series studied, diastereoisomeric salts, formed from compounds of the same configuration (*e.g.*, D-amphetamine–D-mandelate or L-amphetamine–L-mandelate) have a different arrangement of the molecules, and consequently a difference in association between the isomers, from that of diastereoisomeric salts from compounds of unlike configuration (*e.g.*, D-amphetamine–L-mandelate or L-amphetamine–D-mandelate).

This dissimilarity between the D/D (or L/L) and D/L (or L/D) diastereoisomeric salts was observed in differences in melting points (*e.g.*, D-amphetamine–D-mandelate, m.p. 166°, D-amphetamine–L-mandelate, m.p. 162°), differences in their spectra, and also

[1] The X-ray diffraction powder photographs were carried out by Mr. G. H. Hunt, Overseas Geological Surveys, London, England.

TABLE I.—MELTING POINTS OF VARIOUS SALTS FORMED FROM OPTICALLY ACTIVE ACIDS AND OPTICALLY ACTIVE BASES OF KNOWN CONFIGURATION

Acids	Bases			
	D-Isomers	Salts, M.p.	L-Isomers	Salts, M.p.
D-Mandelic	Amphetamine	166.2	Amphetamine	162.0
L-Mandelic	Amphetamine	162.0	Amphetamine	166.2
D-Mandelic	Adrenaline	139.0	Adrenaline	oil
L-Mandelic	Adrenaline	oil	Adrenaline	139.0
D-Mandelic	α-Phenethylamine	177.4	α-Phenethylamine	104.7
L-Mandelic	α-Phenethylamine	104.7	α-Phenethylamine	177.4
D-Tartaric	Amphetamine	169.7	Amphetamine	145.2
L-Tartaric	Amphetamine	145.2	Amphetamine	169.7
m-Tartaric	Amphetamine	199.1	Amphetamine	199.1
L-Tartaric	Adrenaline	144.0	Adrenaline	150.5
D-4-CH_3-POP[a]	Amphetamine	187.2	Amphetamine	156.0
L-4-CH_3-POP[a]	Amphetamine	156.0	Amphetamine	187.2
D-NOP[b]	Amphetamine	104.5	Amphetamine	oil
L-NOP[b]	Amphetamine	oil	Amphetamine	104.5
D-4-Cl-POP[a]	Amphetamine	178.5	Amphetamine	147.5
L-4-Cl-POP[a]	Amphetamine	147.5	Amphetamine	178.5
D-POB[c]	Amphetamine	153.0	Amphetamine	148.1
L-POB[c]	Amphetamine	148.1	Amphetamine	153.0
D-POP[a]	Amphetamine	150.5	Amphetamine	146.7
L-POP[a]	Amphetamine	146.7	Amphetamine	150.5
D-2-OCH_3-POP[a]	α-Phenethylamine	120.5	α-Phenethylamine	oil
L-2-OCH_3-POP[a]	α-Phenethylamine	oil	α-Phenethylamine	120.5
D-4-Br-POP[a]	α-Phenethylamine	155.2	α-Phenethylamine	124.7
L-4-Br-POP[a]	α-Phenethylamine	124.7	α-Phenethylamine	155.2

[a] Phenoxy-α-propionic. [b] Naphthoxy-α-propionic. [c] α-Phenoxybutyric.

TABLE II.—*d*-VALUES OF SOME DIASTEREOISOMERS AS DETERMINED BY USING THE X-RAY DIFFRACTION POWDER PHOTOGRAPHIC TECHNIQUE

D-Amphetamine–D-Mandelate	D-Amphetamine–L-Mandelate	D-α-Phenethylamine–D-Mandelate	D-α-Phenethylamine–L-Mandelate
14.5 Å.	14.5 Å.	12.8 Å.	12.8 Å.
5.1	5.15	6.35	6.35
4.65	4.9	5.2	5.2
4.35	4.57	4.51	4.60
3.86	4.2	4.09	4.4
3.59	3.70	3.54	3.60
2.75	2.93	3.31	...

differences in *d*-values in X-ray diffraction powder photographs.

Because of the observed influence of the geometry of the components of diastereoisomeric salts on the melting points of the latter, the use of melting point differences of salts to assign configuration to acids and bases may become possible. For example, the configuration of a base isomer may be determined if an isomer pair of acids of known configuration are available and crystalline salts can be prepared from the base and these two isomeric acids; the higher melting point diastereoisomeric salt will have components of the same configuration (*i.e.*, if a mandelic acid isomer forms a salt, m.p. 166°, with D-amphetamine and one, m.p. 162°, with L-amphetamine, the mandelic acid isomer used would have the D-configuration).

The infrared spectra also showed significant differences between the diastereoisomeric salts formed from stereoisomers of unlike and of like configuration.

However, infrared spectra cannot be used to allocate the configuration of the components (1).

X-Ray diffraction powder photography also showed differences between diastereoisomeric salts, formed from stereoisomers of unlike and of like configuration (*e.g.*, D-amphetamine–D-mandelate and D-amphetamine–L-mandelate) (Table II).

Since these differences were observed between the interplanar spacing (*d*) of the diastereoisomeric salts, it is suggested that there is a difference of packing between the stereoisomers in the crystals.

Smaller *d*-values for the diastereoisomeric salt formed from stereoisomers of like configuration as compared with the diastereoisomeric salt with stereoisomers of unlike configuration, suggested closer packing of the crystals and, therefore, better association. However, the number of examples studied for the X-ray method was not enough to show that this method could be used for configurational assignment and further work is needed.

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REVIEWS

Methods in Drug Evaluation. Proceedings of the International Symposium on Methods in Drug Evaluation, Milan, Italy, September, 1965. Edited by P. MANTEGAZZA and F. PICCININI. North-Holland Publishing Co., Amsterdam, The Netherlands, 1966. xii + 580 pp. 16 × 23 cm. Price $16.80.

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Reviewed by W. J. Kinnard, Jr.
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Textbook of Biochemistry. 9th ed. By BENJAMIN HARROW and ABRAHAM MAZUR. W. B. Saunders Co., West Washington Square, Philadelphia, Pa., 19105, 1966. ix + 648 pp. 17 × 25.5 cm. Price $9.

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Husa's Pharmaceutical Dispensing. 6th ed. Edited by ERIC W. MARTIN. Mack Publishing Co., Easton, Pa., 1966. vii + 903 pp. 18 × 26 cm. Price $12.00.

More than 30 pharmaceutical authorities have participated in the revision adding new material to make this sixth edition nearly 200 pages longer than the fifth. The format of the book and the topics included are similar to those of the previous edition. However, new information has been incorporated in those areas where it has become available. A section on emulsion stability has been added, several new suspension stabilizers are evaluated, the information on flavors has been expanded, an entire new chapter covers aerosols, and the chapter on drug stability has been updated. Adverse drug reactions are recognized and discussed from the viewpoint that they are a therapeutic incompatibility. Specific organic incompatibilities are included for many recently marketed drugs. Like its predecessor, Husa's sixth edition is a useful reference for both student and practicing pharmacist.

Reaction Mechanisms in Organic Chemistry. By BIANCO TCHOUBAR. American Elsevier Publishing Co., Inc., 52 Vanderbilt Ave., New York, N. Y. 10017, 1966. 197 pp. 14 × 22 cm. Price $8.75.

This work is intended as an introduction to this special field of organic chemistry. It was published originally in France by Dunod and titled "Les Mecanismes Reactionnels el Chimie Organique," and was modified slightly; certain areas have been amplified. The first section of the book covers fundamentals of electronic theory of chemical structure. This includes modern theory of molecular structure, covalency and electron density, polarity and polarizability of covalent bonds, conjugation or mesomerism, and acids and bases. The second part of the book covers reaction mechanisms and rates and deals with the application of electronic theory to the study of reaction mechanisms.

Experimental Pharmaceutical Technology. 2nd ed. By E. L. PARROTT and WITOLD SASKI. Burgess Publishing Co., 426 S. Sixth St., Minneapolis, Minn. 55415, 1965. iv + 276 pp. 21 × 28 cm. Price $5.75. Paperbound.

To bridge the gap between a pharmaceutical preparations course and physical chemistry, the authors have designed a manual which correlates the two. It is designed to acquaint the student with the theoretical, mathematical, and physical relationships, and the practical skills and techniques which characterize various pharmaceutical systems. Solids, solutions, polyphasic systems, and plastic systems are presented. Within each physical state classification, the traditional pharmaceutical classes are considered. As an example—in preparing a suspension, particle size, interfacial tensions, adsorption, stability, viscosity, Stoke's law, and drug solubility must all be considered. The authors use this manual for a three-semester course in pharmaceutical technology, preceded by an orientation course and followed by dispensing pharmacy.

NOTICES

Dictionary of Organic Compounds. Second Supplement. 4th ed. Edited by R. STEVENS. Oxford University Press, 417 Fifth Ave., New York, N. Y. 10016, 1966. 221 pp. 20 × 26.5 cm. Price $28. For a review of the 5-volume *Dictionary* see *J. Pharm. Sci.*, **55**, 357 (1966).

Current Drug Handbook 1966–68. By M. W. FALCONER, H. R. PATTERSON, and E. A. GUSTAFSON. W. B. Saunders Co., W. Washington Square, Philadelphia, Pa. 19105, 1966. ix + 207 pp. 18 × 26 cm. Price $3.50. Paperbound.

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Review Article

Drug Effects on Animal Performance and the Stress Syndrome

By HERBERT BARRY, III, and JOSEPH P. BUCKLEY

CONTENTS

INTRODUCTION

STRESS is the response of an organism to a variety of challenging and threatening events which inevitably occur and recur throughout life. Each individual's health and survival depend on effective physiological and behavioral responses to stressors on repeated occasions. Many compounds, including those classified as tranquilizers, sedatives, and antidepressants, are used for the purpose of aiding an adaptive response to stressors. The actions of these drugs, administered under stress, may depend partly on their interactions with endocrine and other systems which are stimulated by stressors.

Stressors and Stress Responses.—The environmental stimulus which constitutes the stressor is generally distinguished from the physiological reactions which have been described as the stress syndrome or general adaptation syndrome (1–3). The existence of a stressor is generally inferred from the strength of the noxious stimulus and confirmed by observation of the stress reaction. All environmental events and changes are stimuli which threaten the organism's state of biological equilibrium or homeostasis (4). Those stimuli which merely require slight and well-established adjustments are not considered to be stressors, but any stimulus, if sufficiently intense, may evoke the stress syndrome. Many types of stressors have been classified as biological drives, including hunger, thirst, pain, and excessive heat or cold. The physiological reaction of fatigue, during or after exertion, and the mental state of fear or anxiety, aroused by

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realistic or unrealistic anticipation of a threatened stressor, likewise are stressors. Deprivation of environmental stimulation for several hours may also be stressful (5). Different types of stressors require different behavioral responses and physiological changes to maintain or restore homeostasis. However, all stressors, if sufficiently intense and prolonged, evoke the general adaptation syndrome, with the successive stages of alarm reaction, adaptation, and exhaustion.

The various physiological and endocrinological changes in different stages of the general adaptation syndrome are accompanied by behavioral responses which the stressed animal learns and performs. The alarm reaction generally includes vigorous muscular movements of attack or flight to destroy or escape the stressor. If the stress situation cannot be terminated by the initial violent reaction, the animal eventually resumes a more normal response, thus conserving energy and prolonging resistance to exhaustion by means of behavioral as well as physiological adaptation. The initial agitated behavior tends to improve the animal's ability to destroy or escape the stressor but curtails its survival in a situation of severe, inescapable stress. The choice of a therapeutic compound may depend on whether it is needed to enhance the initial alarm reaction or to prolong resistance to exhaustion.

Purposes of This Review.—The present paper attempts to review and evaluate the experimental techniques which have been used to test effects of compounds on behavioral responses to pain, threat of pain, and other stressors. The most frequently used test situations may be classified as measures of avoidance, escape, or approach-avoidance conflict. The physiological and endocrinological reactions involved in the stress syndrome are also summarized and related to the effects of drugs on behavioral performance during stress. The principal purpose of this review is to identify the features of the test situations which influence drug effects on behavioral performance. In order to enable such comparisons, emphasis is placed on techniques which have been used most frequently, such as the conditioned avoidance response, and on those compounds, notably chlorpromazine, whose effects have most commonly been tested with these techniques.

A series of excellent reviews of behavioral effects of drugs (6–10) have included various stressful test situations. Their coverage of the literature was necessarily selective and abbreviated. A comprehensive review of drug effects on the conditioned avoidance response (11) was limited to this one major technique and was primarily concerned with identifying the effects of a wide variety of drugs rather than comparing the various techniques with respect to their measurement of drug effects. Other articles (12–14) have described and evaluated certain selected techniques for testing drug effects on behavioral performance but without any attempt at a comprehensive coverage. Many of the studies described or cited in these prior articles are included in the present review, which aims to provide a new summary and interpretation of accumulated findings rather than merely bringing the literature up to date. Two collections of abstracts, available from the U. S. Public Health Service, have provided a number of useful references: "Psychopharmacology Handbook," vols. 1–3, for publications in 1954–1961, and "Psychopharmacology Abstracts," vols. 1–4, for publications in 1961–1964.

In spite of the fact that the preponderance of studies on behavioral drug effects have been published since 1953, the high and rapidly accelerating rate of output since that time has accumulated several thousand articles, only a small proportion of which can be cited in the present paper. The main criterion for including an article in this review was the description and use of an important behavioral technique for measuring drug effects in a stressful situation. Preference is given to large-scale studies, testing a number of doses of several compounds on a sizable number of animals. Most of the studies have tested drug effects on performance of a previously established avoidance, escape, or conflict response, but some have tested drug effects on acquisition of the response or on persistence of the avoidance during extinction, when removal of the threatened stressor has made it unnecessary for the animal to continue responding to the warning signal. Painful electric shock has been used as the stressor in most studies; objective records of performance have been ensured by automatic recording in almost all of the studies cited, and automatic programming of the experimental events was also used in many of them. The majority of the experiments were performed on rats, but many other species have been used, including mice, dogs, cats, and monkeys in a substantial number of the studies. This review excludes the few pertinent studies on humans. The use of infrahuman animals permits much greater control of the stress conditions and also has the advantage that the data are free from variations due to verbal learning and cultural expectations of the

subjects. The behavioral tests on animals were usually designed to measure general features of motivation and performance which are common to all species, including humans. Certain types of stressful situations have been discussed in the Review Article in the October 1966 issue of *J. Pharm. Sci.* (15) and are not included here. These include the relatively mild stress of exposure to a novel environment, measured by defecation and locomotor activity in the open field test, and test methods which involve manually pinching or otherwise stimulating the animal to elicit a reaction which is observed and rated rather than being automatically recorded. Measurements of forced locomotor activity, also included in this prior review (15), are stressful situations but have generally been used as tests of muscular coordination.

The present review is expected to be particularly useful for those who conduct, direct, or evaluate experiments on animal behavior. The comparisons among commonly used techniques are intended to help in the choice of experimental methods and in the interpretations of the findings, whether the purpose is basic scientific knowledge or screening for clinically useful compounds. However, the authors hope that this review will also be read with appreciation by those with a purely clinical interest in the drugs and by those with a scientific desire for further understanding of the interrelations between drugs and behavior in stressful situations.

MANIPULATIVE RESPONSE

A frequently used test situation is a chamber (Skinner box) equipped to deliver painful electric shocks to the grid floor; the animal is trained to escape or avoid the stressful shocks by means of an "operant" manipulative response, usually pressing a lever or rotating a wheel attached to one of the walls. This situation is generally used for testing drug effects on performance in test situations which last several hours, after the avoidance or escape response has been thoroughly established in a number of prior sessions. Most animals readily learn to press a lever to escape the shock, but many fail to perform the same response consistently in order to avoid the shock. The manipulative avoidance response must compete with an immobile, crouching response to the threat of shock. The test session comprises an inescapable, chronic stress situation in which crouching is a strong behavior tendency; thus, the animal's normal performance and the drug effects show the outcome of a conflict between opposing response tendencies rather than measuring the strength of a simple avoidance response.

Continuous Avoidance.—The procedure of continuous avoidance without a warning signal is one of the most recent of the commonly used behavioral tests. It was devised by Sidman (16) in 1953, at approximately the same time as the beginning of the recent upsurge in rate of publications on behavioral drug effects. The animal receives a brief electric shock at a fixed interval of once every few seconds. Each lever press postpones the next shock by a fixed interval, so that the animal can avoid the shock indefinitely by pressing this lever before the expiration of the fixed interval between shocks. Several different studies on rats (17–20) showed that a low dose of chlorpromazine (CPZ) substantially decreased the rate of avoidance lever presses, thus increasing the number of shocks received. The number of shocks is a more valid measure of the drug effect than the avoidance rate; one of these studies (18) showed an increase in shocks and an increase in the rate of lever presses with a low CPZ dose, because of a tendency for the animal to make a rapid burst of lever presses after each shock. Low doses of reserpine or tetrabenazine likewise markedly impair avoidance, thus increasing the number of shocks received by rats (21, 22) and by monkeys (23). The doses at which these compounds prevent avoidance are too small to cause any marked analgesia or ataxia; however, it is possible that the drugs intensify the immobile, crouching response which competes with the lever-pressing avoidance. Relatively high doses of chlordiazepoxide (17) and alcohol (24) caused only a moderate decrement in lever pressing by rats, and half the anesthetic dose of pentobarbital was required to impair avoidance in monkeys (23).

The avoidance performance of rats improved after injection of adrenocorticotropic hormone (ACTH) or dexamethasone (25). Lysergic acid diethylamide (LSD) improved performance at a low dose and impaired it at higher doses (26). Administration of amphetamine or one of its isomers, at low or moderate doses, increases the rate of lever pressing under a variety of continuous avoidance conditions (19–21, 27–30). A toxic depression of responding is produced by doses only slightly above those inducing maximal stimulation of lever pressing. Even at low doses, there is generally little or no decrease in the number of shocks received; an analysis of lever-pressing inter-response times (21) showed that amphetamine increased the incidence of responses in rapid succession while decreasing the incidence

of the more effective avoidance responses at times shortly before the next shock was scheduled. Two alternative schedules, requiring rats to lick a water tube at a high or low rate for shock avoidance, were used to demonstrate that low doses of amphetamine improved avoidance performance on either schedule, whereas higher doses caused the animals to lick at a faster rate on either schedule (31). Amphetamine greatly increased the rate of lever pressing during nonshock time-out periods which were designated by a visual signal (29). In a procedure with omission of shocks for 0.5-hr. periods without any signal (19), response rates decreased during the nonshock period in nondrug tests but not under dextroamphetamine. Three anticholinergic compounds (scopolamine, atropine, and benactyzine) generally produced an elevation in lever-pressing rate with no consistent effect on the number of shocks received (19, 20); thus, as in the case of amphetamine, efficiency of performance was impaired. Likewise, monkeys responded to scopolamine and atropine with a marked increase in avoidance lever presses and in unnecessary responses during a signaled nonshock period. Much larger doses of methyl scopolamine and methyl atropine were required for equivalent activity, suggesting that the behavioral effects were mediated by central rather than peripheral anticholinergic action (32).

The fact that the continuous avoidance schedule does not require any signals for experimental events facilitates the use of this procedure as one component of a multiple schedule, in which different experimental events are associated with different signals, at different times during the same session. This has the advantage of permitting a comparison of shock avoidance with food-rewarded or other types of performance, in the same animal and session. Drug effects on a continuous avoidance component of a multiple schedule appear to agree well with drug effects reported in other studies on a simple avoidance schedule. CPZ impaired avoidance in rats (33) and in dogs (34) at doses which produced no decrement in other components of the multiple schedule. Doses of amphetamine which greatly increased lever presses during the food-reinforced and time-out stages of the multiple schedule had no consistent effect on the number of shocks received during continuous avoidance (33). A high dose of pentobarbital was required to increase the number of shocks received by rats (33) and by rhesus monkeys (35). Low doses of scopolamine increased the rate of avoidance lever presses, and high doses depressed food-reinforced much more than shock-avoidance lever pressing, in rats (36) and in monkeys (35).

A method for differentiating avoidance from escape performance is through the use of a two-lever system whereby depression of the escape lever terminates the shock, while the separate avoidance lever is ineffective during this shock period. In tests with a variety of compounds (37), low doses of CPZ consistently increased the number of shocks received, indicating impairment of avoidance. Other compounds reliably increasing the occurrence of shocks at low doses include morphine and chlordiazepoxide, whereas high doses were required for detrimental effects of barbiturates, alcohol, and meprobamate. The animals always escaped the shock in nondrug sessions, and much higher doses of CPZ, morphine, and chlordiazepoxide were required to cause escape failures than to impair avoidance, whereas the compounds which required a high dose to impair avoidance were found to cause escape failures at slightly higher doses. An increase in rate of avoidance responses was produced by low doses of dextroamphetamine, cocaine, and anticholinergic compounds. In general, the drug effects in this study (37) agree well with the findings obtained with other continuous avoidance procedures. Detailed analysis of inter-response times, shock escape latencies, and other measures of performance on this two-lever avoidance schedule is feasible with a recently reported system for punched paper tape records and computer analysis (38). With the use of this system, the probability of avoidance responses shortly before shock is scheduled was greatly decreased by CPZ at doses which had little effect on the bursts of response in rapid succession (39).

Warning Signal for Avoidance.—If the continuous avoidance schedule is modified by presentation of a signal several seconds before the shock, rats (40, 41) and monkeys (42) generally do not perform the response until the signal appears. In most studies, the warning signal is presented at fixed or varied intervals, according to a schedule determined by the experimenter rather than by the animal, and lever presses during the intertrial interval have no effect. Generally, the same response which terminates the signal and avoids the shock also terminates the shock if the animal fails to avoid, so that it is possible to compare the drug dosage which impairs avoidance with the higher dose which impairs escape. A crouching tendency interferes with the lever-pressing avoidance response, so that typically only a minority of animals acquire consistent

avoidance performance (43), but the warning signal apparently has a stimulating effect which results in greater resistance to disruption by drugs than is found in continuous avoidance. Six depressant compounds (tetrabenazine, CPZ, chlordiazepoxide, diazepam, meprobamate, and pentobarbital) all impaired continuous avoidance at a lower dose than that which impaired the avoidance response to a warning signal (44, 45). The differential sensitivity of these testing methods was apparently greatest for chlordiazepoxide and smallest for meprobamate and pentobarbital (45). The fact that CPZ and thiopropazate, another phenothiazine, had similar magnitudes of effect on continuous avoidance and on avoidance with a warning signal (46) might be explained by the fact that the continuous avoidance was measured throughout 90 min. after drug administration, whereas avoidance with a warning signal was measured only for the 30 min. of maximum drug effect. Doses of scopolamine and atropine causing a large increase in shocks received by monkeys in a continuous avoidance schedule had less effect on avoidance by monkeys which were required to perform or inhibit an avoidance response by discriminating between two warning signals (32). The drugs produced a much greater detrimental effect with warning signals which were more difficult to discriminate.

Differential drug effects on avoidance with a warning signal in general appear to agree well with findings in continuous avoidance situations. A wheel-turning avoidance response was impaired at much lower doses of CPZ than secobarbital (47) or pentobarbital (48), and the dose required to prevent shock-escape was much greater than the avoidance-blocking dose for CPZ but not for the barbiturates. Morphine appeared to be intermediate in these respects (49). The ratio between escape-blocking and avoidance-blocking doses was reported to be highest for CPZ, intermediate for chlordiazepoxide, diazepam, and meprobamate, and lowest for pentobarbital (45). Avoidance in response to a warning signal was decreased by low doses of anticholinesterase drugs (50, 43, 51) and was increased by dextroamphetamine (52) at a dose which generally caused a toxic decrement of responding in the continuous-avoidance situation. A "trace" avoidance procedure consists of following the 5-sec. warning noise by 5 sec. of silence before the shock is delivered. A dose of chlordiazepoxide, diazepam, and meprobamate, which prevents avoidance during the signal, permits avoidance in the postsignal period, indicating that these drugs tend to delay rather than block the response to the warning signal, whereas in CPZ, pentobarbital, and nondrug tests, the animal generally responds either during the warning signal or not at all (45).

A lever-pressing avoidance response to a warning signal has been used as one component of a multiple schedule, compared with a milk-reinforced approach response in the same sessions. Low doses of CPZ, which greatly decreased avoidance in rats, had little effect on the approach response. Similar differential effects, requiring rather high doses, were induced by meprobamate and reserpine, whereas a high dose of pentobarbital had almost equal effects on avoidance and approach (53). In contrast, LSD, mescaline, serotonin, dextroamphetamine, and iproniazid impaired approach with a much smaller detrimental effect on avoidance (54). In a similar schedule, reserpine had a much greater inhibitory effect on lever pressing by cats for shock avoidance than for milk reward (55). These findings with a multiple schedule agree well with each other and with drug effects on avoidance in other situations.

LOCOMOTOR RESPONSE

Animals may avoid or escape shocks by the more naturally occurring response of running or jumping. Such a procedure has the advantage of enabling quicker and easier training of the avoidance response, with few animals being discarded due to insufficient performance. Drug effects on various locomotor responses have been investigated in many studies.

Avoidance by Running.—A test apparatus which has been widely used, for many years prior to its recent extensive application to drug research, is the two-compartment shuttle box. The animal avoids or escapes the shock by running into the other compartment, usually through a door or across a hurdle; successive trials can be programmed automatically, shifting the shock from one compartment to the other. In a shuttle box for continuous avoidance by mice, low doses of CPZ were found to decrease performance, with one of three strains tested being much more resistant to the drug effect than were the other two (56). A number of investigators have tested drug effects on shuttle-box avoidance by rats or other species in response to a warning signal. Low or moderate doses of CPZ decreased avoidance in rats (57–59), mice (60), and monkeys (61), with much higher doses being required to affect escape. The same doses of CPZ had a greater inhibitory effect on the escape response when shocks were delivered on the same schedule

without the warning signal (indicating a function of the signal in arousing the animal and thus facilitating the escape response) at doses which prevented the avoidance response (57). Reserpine likewise produced a great decrement in shuttle-box avoidance of mice (62, 63), rats (62, 64), cats (65–68), and monkeys (69). A very high, ataxia-inducing dose is required for ethyl alcohol (70) or pentobarbital (61) to impair avoidance. Amphetamine or its congeners effectively improved shuttle-box avoidance of rats (71–73) and cats (68), apparently by decreasing the crouching tendency (72). Performance was also improved by a low dose of benactyzine or LSD (71) and by a high dose of benactyzine, which reduced various rated measures of tension (74), but not by scopolamine, which likewise reduced tension (75). A general excitatory or disinhibitory effect of some compounds which improve avoidance is indicated by the finding that amphetamine and several anticholinergic compounds increased the frequency of incorrect, shuttling responses to a second warning signal which indicated punishment if the animal crossed to the other compartment, whereas it would not be shocked if it remained in the same one (76). Another procedure required animals to remain motionless on the grid floor in order to avoid shock; this response was readily learned in the nondrug condition, and activity was increased by CPZ, imipramine, and methylphenidate in test sessions when shocks were omitted (77). Nearly all other experiments have used some form of active behavior as the avoidance response, so that it is difficult to distinguish the specific drug effect on avoidance performance from a general stimulant or depressant effect.

In general, the shuttle-box avoidance seems to be more readily improved by stimulants and less easily impaired by depressants than is a lever-pressing avoidance response. A higher dose of CPZ was required to block a shuttle-box than lever-press avoidance response in rats (78). Contrary to this finding, the same doses of CPZ, secobarbital, and morphine appeared to produce a greater decrement in avoidance and escape for a shuttle-box than for a wheel-turning response, perhaps because the wheel-turning response was extensively trained to a high level of performance (79). Avoidance performance in the shuttle-box may be impaired by the fact that the animals on each trial are required to return to the compartment in which they previously received shock (13). A four-compartment box (80), permitting the animals to progress in a clockwise or counterclockwise direction, has been shown to improve performance. A still more effective method for increasing the attractiveness of the escape or avoidance response might be a safety compartment where the animals are never shocked, from which they are manually removed before being placed into the starting compartment at the start of each trial.

Rats (81) which were manually placed in the same compartment of a two-compartment shuttle-box, thus never receiving shock in the other compartment, required rather high doses of CPZ and reserpine for inhibition of avoidance. The same conclusion appears to be valid for other studies on effects of CPZ and reserpine in rats (82) and mice (83). Barbiturates, anticholinergics, and meprobamate were even less active in this situation. Drug-induced inhibition of avoidance may have been enhanced in one of these studies (82) by the use of a long, 60-sec. interval before shock, without any warning signal other than placement in the starting compartment of the test box. Rats which have learned to avoid shock by running to a safe compartment at the end of an alley are highly resistant to inhibiting effects of CPZ (84, 85) and other compounds (85). Different groups were trained to run down an alley for food reward, shock escape, or shock avoidance, using a higher shock intensity for the avoidance than escape group in order to equalize nondrug running speed. Most of the compounds had similar effects on the three groups, but amobarbital caused the greatest decrease in avoidance and the smallest decrease in approach speed (85). CPZ does appear to inhibit avoidance performance at relatively low doses in locomotor response situations involving a multiple schedule or a discriminative choice. CPZ greatly impaired avoidance with very little effect on approach, whereas reserpine impaired approach with very little effect on avoidance, in rats trained to avoid shock or approach food during different trials in the same runway (86). Effects of compounds have been studied in a situation with a visual stimulus identifying the correct exit for avoidance or escape from shock (87–91). CPZ decreased avoidance at a low dose, with a higher dose being required to decrease the percentage of correct choices during escape. Benzquinamide, chlordiazepoxide, meprobamate, and hydroxyzine likewise had greater effects on avoidance than on discrimination. In contrast, reserpine and pentobarbital affected both measures of performance almost equally, and alcohol had a greater detrimental effect on discrimination than on avoidance. All of the

compounds required higher doses to suppress escape from the shock than to inhibit avoidance.

Avoidance by Jumping.—When shock is delivered to a grid floor, a response of jumping up to a safe area may provide quicker escape than running across the electrified grid. A rather high dose of CPZ but a fairly low dose of meprobamate was required to inhibit an avoidance response of rats trained to jump onto a stand (92). A relatively high dose of CPZ was required to inhibit a similar avoidance response in rats (93). In mice, an avoidance response of jumping onto a net was not affected by amphetamine and required high doses of CPZ to impair this response (94). Other studies on mice indicated that a jumping avoidance response was more resistant to inhibition by meprobamate and barbiturates than by CPZ, reserpine, and chlordiazepoxide (95–97).

Many investigators have tested drug effects on an avoidance response of jumping onto a vertical pole, which is usually constructed of wood with a rough surface, so that the animal can cling to it and must be removed manually. A number of investigators have shown that rather high doses of CPZ were required to inhibit the pole-jumping avoidance response of rats (98–103). The dose which prevented escape from shock was generally much higher than the avoidance-inhibiting dose. A comparison of pole-jumping with shuttle-box avoidance has been reported in *Peromyscus maniculatus gracilis*, an arboreal species of mice (104). Animals trained in the pole-jumping apparatus acquired a higher percentage of avoidances and were more resistant to suppression of avoidance by CPZ and pentobarbital than animals trained in the shuttle-box. A similar conclusion may be drawn from the report (105) that a much higher dose of a cholinesterase inhibitor was required to suppress avoidance by rats in a pole-jumping than lever-pressing situation.

Pole-jumping avoidance was inhibited in rats by cholinergic compounds (106) and by benzoquinolizine derivatives, monoamine oxidase inhibitors, and catecholamines (107), generally at much lower doses than those required to prevent the escape response. Morphine has also been reported to inhibit the pole-jumping avoidance at a much lower dose than escape (98, 99, 103), whereas pentobarbital and meprobamate impair both avoidance and escape at very high doses, which usually also induce ataxia (98, 99). In general, pole-jumping avoidance was highly resistant to the effects of the above compounds, reserpine (99), and other drugs (103). However, the "secondary conditioned response" of jumping onto the pole as soon as the animal was placed into the chamber, prior to the warning signal, was inhibited by much lower doses, especially by CPZ, meprobamate, and morphine (99). The drug effect on this unnecessarily early avoidance response may be primarily an index of general central nervous system depression.

Avoidance trials have been programmed automatically at regular intervals, using a metal (108) or plastic (109) pole which causes the animals to slide down to the grid floor. With this procedure, avoidance is inhibited by CPZ at lower doses, reserpine at approximately the same doses, and pentobarbital only at higher doses in comparison with brief pole-jumping sessions (108, 109). A finding that CPZ at low doses inhibited pole-jumping avoidance, with very brief intertrial intervals (110), suggests that rapidly repeated trials, in addition to a continuous session, may enhance the inhibitory effect of CPZ. However, even under these conditions a jumping avoidance response seems to be more resistant to inhibition by CPZ than is a lever-pressing avoidance response.

Flinching and Fighting.—A leg-flexion avoidance response by dogs requires only a slight movement, is performed very reliably, and is highly resistant to inhibition by CPZ, meprobamate, phenobarbital, and morphine (111). The dose necessary to prevent shock-escape is much higher than the avoidance-inhibiting dose for CPZ but not for meprobamate and phenobarbital, indicating differential effectiveness of these drugs on performance despite the high dose of each compound necessary to inhibit avoidance. An increase in heart rate during the warning signal (found in nondrug tests) was blocked by the doses of chlorpromazine, phenobarbital, and meprobamate which suppressed avoidance. Morphine, which failed to suppress avoidance, also failed to alter the heart rate response to the warning signal (112). A similar leg-flexion avoidance response in cats (113) was inhibited by cholinergic drugs, and this technique was described as showing an all-or-none effect in comparison with a shuttle-box avoidance response in a different group of cats. When an i.v. injection of *l*-epinephrine was used as the warning signal 30 sec. before shock (114), the leg-flexion avoidance response in dogs was inhibited by a low dose of chlorpromazine which did not block the usual physiological effects of the epinephrine. The conditioned avoidance response may have been weakly established, with the use of a drug as warning signal and the unusually long interval until shock.

Pain threshold is generally measured by gradually increasing the intensity of a painful stimulus until the animal performs an escape or other response. It is difficult to specify whether a drug alters the motivation, the intensity of the stimulus, or the capability for performance, in this as in other behavioral tests. A number of compounds require higher doses to inhibit the initial flinch response to electric shock on a grid floor (115, 116) or foot-licking response on a hot plate (117) than to inhibit the subsequent response of jumping. The effects of several doses of the same compounds have been compared in a test of pain threshold (response to electric stimulation of the tail root) and pole-jumping avoidance in rats (118, 119). Much higher doses of CPZ were required to increase the pain threshold than to decrease the probability of avoiding. A similar differential effect was found with pentobarbital, bulbocapnine, and reserpine. Morphine, and to some degree dextroamphetamine, showed opposite differential effects, with higher doses being required to decrease the probability of avoiding than to increase the pain threshold. Several cholinergic compounds had a similar magnitude of effect on both measures. In mice, a much higher dose of CPZ than morphine was required to inhibit the response of squeaking when electric shock was applied to the tail (120) and to inhibit the reaction when heat was applied on a hot plate (100) or to the tail (121). Contrary to these findings of analgesic effects of morphine, higher doses of morphine were required to inhibit the reaction to these two types of heat stimulation in rats than to inhibit the pole-jump avoidance response (122). Rats which had been trained to terminate a progressively increasing tail shock by turning their head in one direction required a higher dose of morphine than CPZ to cause an elevated shock intensity threshold at which this response occurred (123). In general, tests of pain threshold appear to be rather insensitive to drug effects, requiring high doses of morphine and even higher doses of most other compounds to produce reliable changes. However, analgesic effects with low doses of morphine have been reported using grid shock (124) and ultrasonic pain stimulation (125) in rats and with the jaw-jerk response to electrical stimulation of the tooth pulp in dogs and cats (126). Sympathomimetic compounds have shown analgesic effects, with ACTH and cortisone causing an elevated pain threshold, measured by thermal stimulation of mice on a hot plate (127), and with amphetamine and norepinephrine (NE) likewise causing an elevated pain threshold, measured by the inflamed-foot method in rats (128) and by electric shock to the tooth pulp in guinea pigs (129). Most tests of analgesia have been based on a motor response of flinching, withdrawal, or vocalization. However, a well-trained lever-pressing response has been used successfully in several experiments. With progressively increasing shock on a grid floor, the threshold for a lever-pressing escape response by rats (130) or for an active motor reaction by mice (124) was increased by moderate doses of morphine, sodium salicylate, and acetylsalicylic acid, whereas a sizable dose of pentobarbital had no effect (130). A similar procedure likewise showed analgesic effects with low doses of morphine in monkeys (131).

The startle response to a loud noise associated with painful shocks may be a sensitive measure of anxiety or fear, but there have been few tests of drug effects on this response because of the technical difficulties in constructing and using an appropriate measuring device. Alcohol (13) and amobarbital (132) reduced the motor response to a loud sound when it occurred during a visual warning signal for shock, at doses which had little effect on the startle response to the sound alone. Amobarbital was even more effective in reducing the startle response to shock, without loud noise or warning signal; however, fear rather than pain may have been the principal reaction to the mild shock used (132). A variety of severe stressors have been used for the measurement of drug effects on escape. The speed with which rats escaped from electric shock in a runway was slightly increased by dextroamphetamine and slightly decreased by pentobarbital and chlordiazepoxide (133, 134). Swimming has been used as an escape response, and drug effects may be influenced greatly by variations in the procedures. Barbital (135) and amphetamine (136) greatly slowed swimming of rats to an escape ramp when they were required to pull a weight, at doses with little effect on swimming time under normal conditions. CPZ and meprobamate also decreased swimming speed at fairly low doses, especially when the animals were required to pull a weight, with generally smaller effects on a shuttle-box avoidance response tested in the same rats during the same session (137). Escape of rats from audiogenic stimulation was inhibited by fairly low doses of CPZ but not by high doses of phenobarbital and meprobamate (138, 139). Rats housed and tested in isolation generally fail to escape audiogenic stressors, and amphetamine or other stimulant drugs enabled a substantial proportion of them to escape (140). A lever-pressing escape response has also been used in tests of drug

effects. Amphetamine increased the rate of lever pressing by rats to terminate loud noise (141) and moderate doses of amphetamine increased, CPZ decreased, and pentobarbital briefly decreased rate of lever pressing by rats to turn on a heat lamp in a cold environment (142).

Painful electric shocks on the grid floor may induce pairs of animals to attack each other, indicating that a stressor is likely to elicit aggression if the test situation permits this response. Fighting in rats was inhibited by high doses of CPZ and benactyzine; however, high doses of meprobamate and reserpine had no effect (143, 144). Fighting of mice in response to grid-floor shocks is suppressed by meprobamate and to a lesser degree by CPZ, barbiturates, and chlordiazepoxide (145–147). Another method for inducing aggressive behavior in mice is to house the animal in isolation for several weeks prior to testing with another mouse. Suppression of attack behavior was found at approximately the same dose of CPZ for both techniques, but much higher doses of phenobarbital and meprobamate were necessary to inhibit isolation-induced than shock-induced fighting (148). Isolation-induced aggression of mice was also suppressed by a moderate dose of benactyzine and a very high dose of reserpine (149). A comparison of aggression with analgesia and other behavioral measures showed that isolation-induced fighting was suppressed at a lower dose by CPZ, other phenothiazines, and morphine, but not by barbiturates and meprobamate (150). In a comparison of aggression with shuttle-box avoidance, isolation-induced attack was suppressed at a lower dose by chlordiazepoxide, at the same dose by CPZ, and at a higher dose by pentobarbital and meprobamate (97). The doses of pentobarbital and meprobamate required to suppress aggression also prevented escape from shock in the shuttle-box. Isolation-induced attack has been reported to be inhibited by high doses of LSD, psilocybin, and mescaline (151, 152). The aggressive response to stressors generally appears to be highly resistant to effects of most compounds, even in one study (97) in which it was characterized as being weak and unstable in nondrug tests. A different type of attack behavior, found in a minority of rats, is to kill a mouse placed into the rat's cage. This has been described as aggression but may be related to predatory or feeding behavior, and is highly resistant to drugs. A variety of depressant compounds inhibited this behavior only at severely ataxic doses; hydroxyzine was the only drug tested which abolished the mouse-killing response at a moderate dose (153, 154). However, low doses of several antidepressant compounds effectively inhibit the mouse-killing response (155).

APPROACH–AVOIDANCE CONFLICT

A conflict may readily be established by punishing the animal for responses which procure a desired goal. The stress of the punishment is augmented by the thwarted need for food or other reward formerly obtained, and the conflict itself may be an additional stressor (13). The strength of the opposing approach and avoidance tendencies can be measured only in relation to each other, but this disadvantage is offset by the advantage that the opposing tendencies are likely to be affected equivalently by any drug-produced changes in activity or freezing. This equivalence is not complete, because the approach response is generally acquired first, is more strongly established, and requires more active behavior. Whereas the active lever-pressing or locomotor response is motivated by fear of shock in the usual avoidance test situation, in the conflict test it is the suppression of an active response which is motivated by fear. Therefore, the conflict situation may indicate whether CPZ and other compounds suppress performance of an avoidance response during a warning signal because they intensify an incompatible freezing tendency or because they decrease the fear-producing effect of the signal.

Manipulative Response.—One technique for measuring conflict is to present a signal, terminated by inescapable electric shock, while an animal is pressing a lever for food reward. The "conditioned emotional response" to this stimulus includes "conditioned suppression" of the lever-pressing response. Reserpine, in doses which decreased normal lever-pressing rate, increased the number of responses during the aversive stimulus in rats and monkeys (156, 157) but not in guinea pigs (158). Procedures which caused almost complete suppression of responding by rats during the signal (159–161) prevented any substantial recovery under reserpine. On the other hand, when rats were trained to press a lever in response to a signal that food was available, instead of on the usual free-operant schedule, reserpine greatly increased the rate of suppressed lever pressing during a concurrent signal for inescapable shock (162). Lauener (163) trained rats on a fixed-interval schedule, with water reward obtained by the first lever press 5 sec. or more after the last reward, instead of using the more customary variable-interval schedule. The high, stable performance rate generated is very resistant to disruption by drugs and thus is advantageous for

testing drug effects on conditioned suppression. The responding during the signal was greatly increased by chlordiazepoxide, several barbiturates, and to a lesser degree by meprobamate, but not by CPZ, morphine, ethanol, and amphetamine. Morphine has been reported to increase suppressed responding (164), but most of Lauener's findings are supported by other studies, which show that suppressed responding was greatly increased by amobarbital (165, 166), increased by meprobamate in one study (167), but not in another (162), not increased by CPZ (160, 162), and decreased by amphetamine (156, 157).

A more direct conflict procedure is to punish the animal only when it presses the lever, so that the aversive shock is associated specifically with the food-rewarded lever-pressing response. Comparisons between these procedures have given evidence that reserpine increases responding during a signal for inescapable shock but not when the animal is punished for pressing the lever (156, 157); whereas, meprobamate increases responding during a signal for punishment but not inescapable shock (168). Effects of several compounds have been tested in a conflict situation where lever presses by rats are rewarded on the average of once every 2 min. on an unpredictable, variable-interval schedule, and at periodic intervals a tone is presented for 3 min., during which every lever press is punished by shock and rewarded by food (169–172). The number of lever presses during the conflict signal was increased greatly by meprobamate, substantially by barbiturates and chlordiazepoxide, slightly by reserpine, and decreased by CPZ and morphine, at doses which had little effect on the lever-pressing rate during the unpunished portion of the schedule. Another conflict procedure (173), for rats trained to press a lever for milk in response to a signal, is to accompany this reward signal with an additional stimulus indicating that each of the next four lever presses will be rewarded and also punished. The number of lever presses during the conflict signal was increased by meprobamate and pentobarbital and not by CPZ and reserpine. Punished responding was likewise increased by a barbiturate (amobarbital) but not by CPZ in pigeons pecking a key for food reward on a variable-interval schedule and punished by shock for every response during the conflict signal (174).

When every response is punished during the conflict period, as in the above studies, drug effects might be due to changes in the aversiveness of the shocks, based on the immediately preceding experience with shocks in the same session, rather than being due to changes in fear or avoidance of the threatened shocks. A procedure for measuring fear rather than pain, by omitting shocks during the conflict signal in some test sessions, showed a large increase in lever pressing during the conflict signal under amobarbital but not under the other compounds tested (175). Contrary to the failure of CPZ to increase suppressed responding, in several of the above studies, CPZ produced a slight but reliable increase in lever pressing during the conflict signal in this situation with shocks omitted (175). CPZ produced a large increase in lever pressing during the conflict signal in a study using similar procedures (176) but with a more prolonged duration of the conflict signal.

Drug effects on conflict have been tested in situations without a signal for punishment. Alcohol and amphetamine decreased rate of rewarded and punished lever pressing by rats (177), in agreement with the effects of these drugs during the conflict signal in another study (175). Morphine greatly increased the number of punished water-drinking responses by rats during a prolonged conflict session (178); a similar effect of CPZ during a prolonged conflict signal (176) suggests that the duration of the conflict period may be a factor in the drug effect. In two other studies on rats (179, 180), the frequency of punished drinking responses was increased by meprobamate, amobarbital, and methylpentynol, decreased by CPZ, and not significantly changed by benactyzine. A measure of agitated approach-withdrawal responses showed a decrease under benactyzine (179).

Locomotor Response.—Conditioned suppression of a running response was tested in rats previously trained to obtain water reward by shuttling back and forth in a two-compartment box (181). In this situation, the signal for an inescapable shock caused slightly less suppression of the running response in animals injected with CPZ than in a control group. However, conflict has generally been induced by direct punishment of the locomotor approach response. The use of a long runway permits measures of speed and distance of approach, and provides a test of drug effects on fear of punishment as well as on the immediate effects of punishment. Conger (182) showed that alcohol restored the approach response in rats which had been shocked at the food cup. Measures of strength of pull in the same apparatus showed that alcohol greatly reduced the vigor of running in a shock-avoidance group but not a food-approach group. A further demonstration of the avoidance-reducing effect of alcohol was with a method of omitting shocks after alcohol injection for one group and after

placebo for another group; approach in the nonshock condition was more rapidly learned by the group for which shocks were omitted under alcohol rather than placebo. Barry and Miller (183) devised a "telescope alley" in which progressive changes in runway length signaled increases in shock intensity delivered at the food or water cup, during several trials of the same day. This technique measured drug effects on normal approach speed and on the intensity of punishment or fear of punishment required to prevent the approach response, with repeated tests of the same animals under different drugs on successive test days. Amobarbital and alcohol generally decreased approach speed in the initial safe trial of the day but consistently increased speed and probability of approach in the series of trials with increasing shocks, whether the shocks were delivered or omitted (183–185). Other compounds tested (CPZ, morphine, cocaine, methamphetamine) tended to decrease approach speed, generally with a greater effect in the safe trial than in the conflict test (183). One study gave evidence that CPZ increased approach during trials with shock but decreased approach during trials when shocks were omitted (184).

Drug effects on conflict in a runway or other locomotor situation have also been studied in other species. Amobarbital effectively restored the approach response of cats in a runway (186). In a more complex situation, designed to induce neurotic behavior (187), alcohol restored food-approach responses of cats and had other beneficial effects in the conflict test. A series of studies on cats and monkeys in the same situation (188) showed even greater beneficial effects of barbiturates but little or no effect of reserpine, CPZ, and mephenesin. A similar procedure (189) was used to test effects of several compounds (190) on conflict behavior of cats after food deprivation of only a few hours instead of 24 hr. Approach responses were increased by benactyzine, by related anticholinergic agents, and by alcohol, with no beneficial effect of CPZ and scopolamine. Cats which were punished by electric shock when they seized a mouse resumed the punished response under the influence of a low dose of meprobamate, but high doses of benactyzine and CPZ were required to elicit the response (191). Dogs resumed a punished food-approach response under the influence of barbiturates, alcohol, and meprobamate but not benactyzine (192).

The most consistent finding in the locomotor conflict studies is that barbiturates and alcohol increased approach performance of all species tested, in all of the situations which included tests of these compounds. The failure of alcohol to increase approach in a lever-pressing conflict (175, 177) may be due to the greater detrimental effect of alcohol-induced ataxia on this type of response. CPZ fails to increase approach in both lever-pressing and locomotor conflicts, with a few exceptions (175, 176, 184, 191). Other tranquilizing agents (meprobamate, benactyzine) apparently increase approach responding in some situations but not in others.

Other Conflict Tests.—An approach-avoidance conflict occurs during "experimental extinction," when the cessation of rewards for a learned approach response results in a conflicting avoidance response motivated by the aversive experience of frustration (193, 194). In this situation, the inhibited approach response is increased by amobarbital (193, 195) and by alcohol (193); this drug effect has been attributed to a reduction in frustration-motivated avoidance (193, 194). Likewise, amobarbital gives evidence of counteracting inhibition due to a frustrating schedule in which many of the responses are not rewarded (194, 196, 197) or due to a portion of a schedule associated with nonreward (198). These findings have been reported for locomotor responses of rats (193–197) and for lever pressing by pigeons (196, 198). Responding of rats inhibited by a signal for nonreward was increased by amobarbital in a runway but not in a lever-pressing situation (166), indicating a stronger drug effect for the locomotor response. A temporary increase in lever pressing by rats during the first few minutes of nonreward, attributed to frustration-produced emotionality, was enhanced by CPZ (199). A comparison of this compound with phenobarbital (200) showed that the barbiturate elicited a larger number of unrewarded responses, following a smaller initial increase in lever pressing at the beginning of extinction. Scopolamine and other anticholinergic compounds have been shown to increase preservation and to retard the inhibition caused by nonreward under a wide variety of conditions (201–203). However, scopolamine gave no evidence of diminishing the aversive effects of punishment (201), and the effects of anticholinergic drugs were attributed to a specific antagonism of the inhibitory effects of nonreward (203).

Drugs may help or hinder performance in a conflict situation by affecting the specific motor actions which are required. An example is found in the requirement that a pigeon hold its head for a specified duration in a restricted spot, intersecting two photocell beams, in order to receive food reinforcement (204). The birds were observed to be very excited and agitated in this situation, and the time they were able to remain sufficiently immobile was increased by CPZ and decreased by

pentobarbital. This conflict situation, in which the obstacle to the necessary response is the animal's own motor activity, is one of the few instances in which CPZ has been found to improve the performance of animals.

Drug effects on performance of rats in a complex conflict situation have been reported in a series of studies (205–209). The Lashley jumping apparatus is used to induce a maladaptive, perseverative-choice response during a long series of test sessions. The hungry animal jumps from a platform to one of two windows in a situation where the chosen window has an equal probability of opening to give access to food reward or of punishing the choice by failing to open so that the animal falls into a net below. An electric shock, delivered to the platform after 30 sec., forces the animal to make a choice and adds a further stressor to the situation. These procedures are highly stressful as shown by frequent urination and defecation on the platform and by the fact that the hungry animal usually does not eat on the trials when the door opens to make the food available (205, 206). The maladaptive, perseverative-choice response developed in this situation is highly resistant to therapeutic modification by drugs, but chlordiazepoxide (205, 206) and diazepam (206) gave evidence of reducing emotionality and improved the performance of some animals after a number of days of drug treatment. Under certain conditions, however, the reduced motivation under the influence of chlordiazepoxide prevented animals from acquiring an adaptive choice response (207). The other compounds tested did not have any therapeutic effect in this situation; high doses of CPZ, reserpine, and meprobamate but not phenobarbital gave evidence of reducing avoidance of the shock on the platform by causing the animals to delay jumping to one of the windows under some conditions until the shock was administered (205, 208). The therapeutic effectiveness of a guidance method for breaking a maladaptive, perseverative choice was apparently enhanced by amphetamine and retarded by CPZ and meprobamate (209). The maladaptive, perseverative-choice behavior seems to resemble certain types of neurotic behavior in humans, but this test situation has the disadvantage of being extremely complex, and the behavior was apparently resistant to the rather high drug doses used in these studies.

PERSISTENT BEHAVIOR ALTERATION

Most of the studies reviewed thus far tested the acute effect of a single drug administration on performance which had previously been well established and stabilized. Drug effects on acquisition, extinction, and relearning of responses are also of interest, especially for potential applications to therapy in humans.

Drug Effects on Acquisition.—CPZ (82, 210–214) and reserpine (82, 212) impair performance during acquisition of various types of avoidance responses in rats, but the magnitude of these drug effects does not appear to exceed their inhibitory effects on a well-established avoidance response. The magnitude of the drug effects may depend partly on the test situation; for example, CPZ caused slight decrement in acquisition of runway avoidance, but when the test was made more difficult by requiring the animal to select the lighted one of two adjacent compartments, this compound produced a much greater decrement (213). Likewise, CPZ but not pentobarbital reduced the percentage of rats learning to make the correct choice in a swimming escape situation which was made more stressful by forced immersion for 30 sec. prior to the start of each trial (215).

A variety of depressant drugs have been found to facilitate acquisition of an avoidance response in rats. These include reserpine at low doses (216), amobarbital (212, 217), meprobamate (218), alcohol (219, 220), chlordiazepoxide (221), and benactyzine (222). Some of the drugs which improved shuttle-box avoidance during the warning signal also were shown to increase the frequency of intertrial crossings from one compartment to the other (217–219) indicating that these ordinarily depressant compounds apparently decreased the tendency for immobile, freezing behavior in this stressful situation. These drug effects are influenced by certain characteristics of the test situation. Acquisition of a pole-jumping avoidance response was impaired by amobarbital (223); another pole-jumping situation where amobarbital facilitated acquisition (212) differed in several procedural conditions, including a longer interval between onset of the warning signal and the shock, and a longer intertrial interval. The same dose of benactyzine which improved acquisition of a shuttle-box avoidance response (222) impaired acquisition of a lever-pressing continuous avoidance response (224). On the other hand, a dose of scopolamine which improved acquisition of a lever-pressing continuous avoidance response (225) impaired acquisition of a pole-jump avoidance response (226). Various stimulant compounds have been shown to facilitate acquisition of avoidance, including amphetamine (212

223), pipradol (224), epinephrine (227), and ACTH (228–230).

Acquisition of an approach-avoidance conflict response was studied with the use of a signal that a lever press would deliver water reward to the thirsty rat, with a painful shock being delivered 15 sec. after onset of the signal (231). Inability to control the shock duration apparently enhanced its stressful effect, as indicated in a comparison of rats which escaped the shock by pressing a lever with matched animals which received the shock for the same length of time; after several days of training the latency of drinking was much shorter for the escapable-shock animals than for their paired inescapable-shock controls. A phenothiazine (thioridazine), administered chronically throughout training, substantially decreased the latency of responding, with a greater effect on the inescapable-shock than on the escapable-shock animals. This technique thus gives evidence for a tranquilizing effect of a phenothiazine not usually found with the more commonly used tests of conditioned suppression.

Drug Effects on Extinction.—An animal which always makes the avoidance response when the warning signal is presented will continue to respond unnecessarily even if failure to avoid is no longer punished by shock. Therefore, occasional failures to avoid serve an adaptive purpose, and when the warning signal is repeatedly presented without shocks, in a test of extinction of the avoidance response, excessive persistence of the learned response is maladaptive. However, if animals acquire the avoidance response in a nondrug condition followed by extinction trials under a drug, their performance may be influenced not only by the drug itself but also by the novelty of their drugged condition. It is necessary to have separate drugged and nondrugged groups in acquisition, so that the effects of the drug and of a change in condition can be equalized by changing half the animals of each group to the other condition at the start of extinction (13). The change in condition may have an important effect, as indicated by an experiment in which rats, following punishment for a lever-pressing food-rewarded response, resumed pressing and obtaining food without punishments under the influence of amobarbital but failed to continue pressing the lever in a subsequent placebo test (232). A decrease in avoidance response, due to a change from drugged to placebo or from placebo to drugged condition, has been shown in rats with amobarbital (185), phenobarbital (233), chlordiazepoxide (221), and CPZ (214).

A dose of CPZ which produced a moderate decrement in acquisition of avoidance response also moderately decreased the number of extinction trials before the animals stopped responding to the warning signal (210). CPZ decreased the probability of an avoidance response to the warning signal during extinction (234); all of the animals were in the nondrug condition throughout acquisition, so that the drug effect was associated with a change in condition, but phenobarbital had no effect on extinction performance after nondrug acquisition. CPZ had little effect on extinction of a runway avoidance response (213), but the requirement of a choice response almost completely eliminated avoidance responses during extinction under CPZ. A dose of phenobarbital which had no effect on speed of shock escape in a runway decreased persistence of the response during extinction, when shock was omitted (233). Other compounds have the opposite effect of increasing persistence of avoiding after shocks are omitted. Extinction of shuttle-box avoidance was greatly retarded by a dose of ACTH which had very little effect on acquisition (228). A dose of demeton,[1] which greatly reduced brain cholinesterase also retarded extinction of a platform-jumping avoidance response, with little effect on acquisition (235). Anticholinergic drugs have likewise been shown to increase persistence of avoidance responses in a variety of situations (202, 203).

A passive instead of active avoidance response may be tested by placing a rat in a box which it has previously explored without shock and measuring the amount of time spent in an adjoining, smaller compartment where it previously received painful shock (236–238). Drug effects have been reported with a similar procedure adapted for mice (239). This technique has generally been used as a measure of impairment in the passive avoidance response, presumably due to loss of memory, after administration of anticholinergic drugs. However, prolonged or repeated test sessions would provide a measure of extinction of avoidance.

There have been some studies of drug effects on extinction of avoidance in conflict situations. Rats trained to press a lever for water and punished for this response by shocks normally resumed pressing the lever in subsequent nonshock tests but not if ACTH was administered during punishment and subsequent test sessions (240). A higher level of performance found in animals punished under ACTH and tested without drug than in the placebo group might be due to the

[1] Marketed as Systox by the Chemagro Corp., New York, N. Y.

change in condition (13). Jumping or running was measured as the response to a signal for inescapable shock in mice (241), shocked under CPZ or placebo and all given nonshock extinction trials under placebo. Fewer extinction trials were required to abolish this active response to the signal in the animals which had been given acquisition under CPZ. This might indicate a tendency for CPZ to cause the acquisition of a freezing rather than active response to the signal for inescapable shock. Drug effects on learning to reverse a choice response were tested in rats which were trained to turn their head in one direction to turn off a gradually increasing shock, followed by trials in which only the opposite direction of head-turn escaped the shock (123). The reversal learning was greatly impaired by phenobarbital (123) and by meprobamate (242).

Prolonged Drug Effects.—Most of the studies have investigated the acute effects of a single dose of a compound. The chronic effects of repeated administrations may result in decreased drug effect on behavior, indicating tolerance, or else increased effect, indicating sensitization. Behavioral tolerance to the effect of a high dose of CPZ is shown in the finding (243) that there was progressively less suppression of a locomotor avoidance response on successive test days under the drug. A progressive development of tolerance is indicated by the finding that CPZ produced less decrement in a lever-pressing continuous avoidance response (244) if progressively higher doses were given, starting with a very low dose, than if the doses were given in a descending sequence. A more acute instance of behavioral tolerance is shown by the finding (245) that CPZ caused less decrement in a shuttle-box avoidance response if the test session began immediately after injection, providing a gradual onset of drug effect, rather than at the time of peak drug action. These and other factors influencing behavioral tolerance or sensitization to drug effects may alter the results of experiments, especially those which use repeated administrations of a drug. Furthermore, test compounds themselves are stressors if given in high doses or if they impair the performance of avoidance or escape in a stressful situation. Thus, the development of behavioral tolerance to the drugs may represent the stage of resistance to a chronic stressor.

A different type of response, suitable for measurement of the chronic effects of a prolonged stressor, is an increase or decrease in voluntary consumption of certain drugs. A number of the behavioral tests are designed to assess drug effects in alleviating or intensifying anxiety in a stressful situation. If animals could be trained to consume a drug in order to relieve their anxiety, this might confirm the tranquilizing drug effect and also provide a method for measuring the stress response to various experimental situations. A tendency for an increase in choice of an alcohol solution, during or after stress, has been found in cats (187), rats (246, 247), and monkeys (248). This behavioral response generally seems to be slight, with no resemblance to the human alcoholic's craving for liquor, but it apparently does occur in several species of animals in spite of the obstacles of the delayed pharmacological effect after drinking, the unpleasant effects of excessive quantities, the unpalatability of alcohol solutions except at low concentrations, and the difficulty of inducing consummatory behavior in stressful situations. A technique for self-injection, which may overcome most or all of these obstacles, was used in a study with rats, showing that inescapable electric shocks caused an increase in rate of lever presses which injected amobarbital into their jugular vein (249). Diminution of this response after several 1-hr. sessions indicates the possibility of habituation to the drug or aversive physiological effects of the injected substance. Tests in rats showed that stress failed to increase the choice of a solution containing reserpine (246) and actually decreased the choice of a solution containing chlordiazepoxide (250).

THE STRESS SYNDROME

Selye (1–3) has postulated that organisms subjected to alarming stimuli will respond in a given manner, which he termed "the general adaptation syndrome" or "stress syndrome." Briefly, the general adaptation syndrome (GAS) can be divided into three distinct stages. The first is the alarm reaction, associated with the discharge of adrenocorticotropic hormone (ACTH), cortical steroids, and catecholamines, plus various other physiological changes. The second is the stage of resistance, in which adaptation to the stressor results in a diminished reaction and thus increased resistance. The third is the stage of exhaustion, during which adaptation can no longer be maintained because of prolonged overexposure to the stressor. Different homeostatic adjustments may be aroused by various types of stressors, such as the contrasting stimuli of excessive heat or excessive cold. However, in the case of severe stressors the universal, nonspecific stress syndrome is generally the most prominent response.

Acute Exposure to Stress.—The following physiological responses to stress have been suggested by Selye (3). The stressor (stimulus) acts on the target (the body or some part of it) directly and by way of the pituitary and adrenals. An immediate discharge of ACTH stimulates the release of corticoids from the adrenal cortex. If the stress is extremely severe, the adrenal cortex shows morphologic changes characteristic of hyperactivity. Simultaneously, the animal's corticoid requirement markedly increases and there is an increase in the blood concentration and urinary excretion of corticoids and their metabolites. There is a general stimulation of the sympathetic division of the autonomic nervous system and the splanchnics induce the adrenal medulla to discharge epinephrine (E) and norepinephrine (NE), thus increasing the discharge of NE at various peripheral receptor sites and causing the cardiovascular responses of vasoconstriction and hypertension. Other marked physiological changes include alterations in water and electrolyte metabolism, gluconeogenesis and increased blood sugar levels, alteration in both red and white blood cell counts, and increased renin production by the kidney.

Some of these components of the stress syndrome have been measured in animals which were subjected to painful electric shocks in behavioral test situations. Elevated plasma 17-hydroxycorticosteroid (251) and NE levels (252) have been found in monkeys after sessions of pressing a lever on a continuous shock-avoidance schedule. There was also an increase in plasma steroid and NE levels after sessions of pressing a lever for food rewards in which no shocks were delivered but a conditioned emotional response was aroused by presentation of a clicking noise previously associated with shocks. Aceto *et al.* (109) reported that rats subjected to the pole-climbing avoidance test developed hypertension within 4 weeks. A recent study (253) showed that corticosterone concentration is elevated in rats at the end of a session of inescapable shocks, and an adaptive behavioral function of this physiological response is suggested by the further finding that the animals successfully acquired a shuttle-box avoidance response if they were trained immediately following their exposure to the warning signal paired with inescapable shocks; the animals did not acquire this response if the avoidance training began 1 to 4 hr. afterward, at which time the corticosterone had dropped to a normal level. Inescapable shocks delivered to the grid floor at regular intervals, for 1.5 hr., caused a 38% reduction in brain NE of guinea pigs (254). Another measure of sympathetic activation is the skin resistance of the paws, which was lowered in rats by a severe, single 5-sec. shock to the grid floor (255).

Compounds Altering Acute Stress Response.—There is potential clinical value as well as basic scientific information to be gained by identifying compounds which alleviate or aggravate the components of the stress syndrome. Several compounds have shown evidence of protective effects, with somewhat conflicting findings for CPZ. The large decline in brain-stem NE in rats, resulting from the stressor of inescapable electric shocks on the grid floor, was partly reversed by large doses of CPZ and pentobarbital but not by morphine (256). In another study, the decrease in adrenal ascorbic acid in rats due to the stressor of excessive heat or cold was partly counteracted by a moderate dose of CPZ and by a low dose of reserpine (257). A large CPZ dose had a similar effect on rats subjected to restraint at room temperature (258). The increase in blood glucose after rotation stress was partly counteracted by methylpentynol but not by CPZ (259). Sedative doses of CPZ and other phenothiazines have been reported to stimulate secretion of ACTH, mimicking the effect of exposure to cold temperature (260).

Stress increases the urinary excretion of catecholamines, especially E (261–263). Although E is the main catecholamine excreted in the urine during stress, a more important component of the stress response might be the general stimulation of the sympathetic nervous system and the increased discharge of NE at the peripheral receptor sites. Since the uptake of NE at the peripheral receptor site is very rapid, a more prolonged physiological change may be preferable as a measure of the stress response. Maickel *et al.* (264) reported that adipose tissue lipase was stimulated and plasma free fatty acid (FFA) elevated in rats by catecholamines, ganglionic stimulants, and exposure to cold, but plasma FFA was unaffected by cold exposure in the absence of a functional sympathetic nervous system. Gilgen *et al.* (265) found that an intact sympathetic nervous system was essential for increasing the output of FFA and glucose on exposure to cold and concluded that NE at peripheral nerve endings was essential for this reaction. Plasma FFA levels in rats are significantly increased by inescapable electric shocks delivered to the grid floor, and the degree of increase in plasma FFA is proportional to the duration of the stress. The effect of the stressor (electric shock) on plasma FFA was either blocked

or markedly attenuated by CPZ and meprobamate (266). The elevation of plasma FFA in rats by a similar schedule of inescapable electric shocks was effectively blocked by several tranquilizers (reserpine, benzquinamide, CPZ, meprobamate, hydroxyzine, and chlordiazepoxide). Two sedative compounds (pentobarbital and ethanol) were only partially effective; however, rather small doses were given. Two stimulants (dextroamphetamine and caffeine) elevated plasma FFA, in both shocked and nonshocked rats (267).

Since the stress syndrome may have an adaptive function in preparing the animal to resist a stressor, compounds which diminish the physiological reactions do not necessarily have beneficial effects. A more valid criterion for a protective effect of a compound might be a prolonged survival time during exposure to an acute stressor which is severe enough to cause rapid death. CPZ prolonged survival of rats subjected to combined heat and vibration stress (268) and of pigs subjected to combined heat and restraint stress (269). Survival of mice subjected to rapid mechanical vibrations was prolonged after pretreatment with large doses of chlordiazepoxide, reserpine, pentobarbital, and phenobarbital, and curtailed after large doses of iproniazid, dextroamphetamine, and morphine (270). Swimming time of mice in agitated cold water was prolonged by meprobamate (271) and by morphine (272), with no beneficial effect of hexobarbital or of several stimulants (271).

The generalized increase in sympathetic outflow, occurring in the alarm reaction to acute stressors, rapidly elicits a reciprocal stimulation of the parasympathetic nervous system. This reciprocal activation is not usually included in descriptions of the stress syndrome, but it adds a high level of internal stimulation to the effects of the original stressor and in some situations may be the immediate cause of the sudden lethal effect sometimes observed in cases of intense, acute stress. Richter (273) described this type of reaction, which is almost invariably lethal when wild rats are forced to swim in a vertical position with their whiskers clipped. In this situation, there is a marked slowing of heart rate, accompanied by decreased respiration and hypothermia, and at the time of death the heart is stopped in diastole, indicating a massive overstimulation of the parasympathetic nervous system. The lethal effect is aggravated by cholinergic drugs and retarded by atropine. This stress reaction may be influenced by the animal's experience with the situation, producing variations in the arousal of hopelessness or helplessness as a component of the perceived situation. Animals are much more resistant to the lethal effects if they are allowed to escape from the situation a few times instead of being maintained in the situation continuously.

Another consequence of excessive parasympathetic stimulation during stress may be the development of gastric ulcers. Reserpine has been shown to increase the incidence of ulcers in rats subjected to physical restraint for a number of hours (274–277). In one study (277), pretreatment with reserpine for several days prior to the restraint lowered the incidence of ulcers to the level of the nondrugged controls; this effect of more extensive premedication was attributed to the tranquilizing action of the drug. The incidence of ulcers after restraint stress has been found to be decreased by imipramine (275, 276, 278), thalidomide (279), cortisone (280), and a variety of other compounds, including anticholinergics, barbiturates, and CPZ (276, 281). A method for inducing ulcers without physical restraint is to immobilize rats for 24 hr. by punishing every motion with electric shock; ulcers were prevented by an extremely low dose (5 mg./Kg.) of meprobamate, but higher doses (10 and 20 mg./Kg.) were less effective, perhaps because they reduced the animal's ability to remain sufficiently immobile to avoid the shocks (282).

Chronic Exposure to Stress.—The alarm reaction cannot be maintained for long, and the process of adaptation or habituation enables most of the physiological reactions to return to their normal homeostatic level of functioning, even if the stressor continues unabated. This is identified as the stage of resistance, which continues until the stressor ceases or until exhaustion overcomes the adaptation. Mice forced to swim in cold, agitated water (272) were described as showing within the first 6 min. the agitated reactions of alarm reaction followed by the slower, energy-conserving behavior of the resistance stage and finally exhaustion when they sank beneath the surface. However, a much longer time span is generally required as a criterion for the stage of resistance.

Not all of the components of the stress response appear to return to normal levels of functioning during this stage. Aldosterone production increases, whereas corticosterone production is normal (283, 284), giving evidence that the renin–angiotensin II–aldosterone complex is involved in the response of the organism to chronic stress. Investigators (285–287) have

shown that the kidney is the source of an aldosterone-stimulating hormone and that the renin–angiotensin II system is involved in the stimulation of aldosterone production by the zona glomerulosa of the adrenal cortex. Miller (288) has shown that the glomerular zone increases in weight and hypertrophies by stress in hypophysectomized rats. Feldberg and Lewis (289) have reported that angiotensin is one of the most potent compounds inducing a release of catecholamines from the adrenal medulla, and other investigators (290–294) have provided evidence that there is an interrelationship between the activity of angiotensin II and the sympathetic division of the autonomic nervous system.

In a chronic stress situation, gastric ulcers have developed in monkeys performing a continuous shock-avoidance lever-pressing response for 6-hr. sessions, alternating with 6-hr. rest periods (295). The ulcerogenic effect was apparently a consequence of the chronic rather than the acute stress situation, because the gastric acid secretion was suppressed during the test sessions and greatly elevated in the rest periods. In rats, a chronic approach-avoidance conflict situation lasting 18 days gave rise to gastric ulcers which were greatly increased in animals given reserpine (296).

Gastric ulcers are not the only consequences of chronic stress. Friedman and Ader (297, 298) delivered inescapable electric shocks to the grid floors of the home cages of mice for 15 hr. per day, during a span of 7 days. The most stressful experimental condition, as indicated by the greatest loss in body weight (297) and the greatest susceptibility to the effects of injected coxsackie virus (298), was the presentation of the 2-sec. shock once every 15 min., at regular instead of irregular intervals, and with a stimulus light being presented for 15 sec. immediately before each shock instead of at different times. These conditions are similar to the typical schedule for a conditioned avoidance response. In another study (299), reduced weight gain and enhanced susceptibility to a toxic virus (herpes simplex) were found in mice after 28 days of 6-hr. sessions in a shuttle-box conditioned avoidance response. Animals tested for 1 or 14 days did not differ significantly from nonstressed controls. Measurements of blood pressure in rats tested for 42 weeks in a pole-climbing conditioned avoidance response (109) showed a rapid hypertensive response within the first few weeks on this schedule, persisting at approximately the same elevated level thereafter.

Rats subjected to a chronic variable stress program consisting of visual, auditory, and mechanical stimulation (flashing bright light, noxious intermittent sounds, and 120 oscillations per minute) for 4 hr. per day developed hypertension and had a high incidence of mortality within 20 weeks (300). Rosecrans *et al.* (301), using a similar stress protocol, also induced experimental hypertension in rats and found significant increases in both urinary NE and E following a single stress exposure, with a return to normal range by the eighth week of chronic stress. After the initial increase in secretion of adrenal E, adaptation occurred within 8 weeks. In contrast to acclimation of the sympathetic nervous system, the pituitary-adrenal axis appeared to continue to function maximally throughout the study, as indicated by high plasma steroid levels. The authors suggested that adrenal medullary activity appeared to be more important in acute stress situations, whereas the pituitary-adrenal axis appeared to play a more important role in adaptation during the long-sustained phase of chronic stress.

In spite of the great clinical importance of identifying protective or harmful drug effects in chronic stress, little research has been reported on drug effects in prolonged stress situations. Moderate doses of reserpine and CPZ failed to counteract the hypertension induced by chronic stressors but, on the contrary, potentiated the lethal effects of the stressors, apparently by the action of these compounds on the anterior pituitary-adrenocortical system (302). When reserpine treatment was begun after the seventh week of stress, blood pressures dropped to the control level but there was some indication of a higher mortality rate among the reserpinized than nondrugged animals (303). Acetylsalicylic acid failed to reduce blood pressures and greatly increased mortality of rats subjected to this chronic stress program; the deaths were apparently due to perforated gastric ulcers (303). The stress of physical restraint for 3 hr. per day caused 50% mortality within 32 days in rats pretreated with a large dosage of reserpine compared to successful adaptation and no mortality in nondrugged animals (304).

Drug Effects Modified by Stress.—In view of the physiological alterations involved in the stress response, the effects of some exogenous compounds may be expected to differ, depending on whether they are administered to a stressed or tranquil animal. Such differential effects have been found in a behavioral test situation in which rats turn a wheel to terminate a progressively increasing electric shock delivered to the grid

floor. There was an exaggerated stimulant effect of methamphetamine and caffeine and an exaggerated depressant effect of CPZ on escape performance of a group of animals previously given severe, inescapable shocks in the same apparatus. In contrast, alcohol had less depressant effect on the stressed animals than on the control group (305, 306). Another study has also provided evidence that the stress reaction potentiates the effects of stimulant drugs and of CPZ. With the use of a method of rating various measures of fright in rats introduced to a novel situation, animals whose fear was aroused by loud noises and by a bright, flashing light showed a greater increase in fright under the influence of E and a greater decrease under the influence of CPZ than did the low-fear controls (307). The finding that stress counteracted the depressant effect of alcohol (306) is convincingly supported by a report that rats under the influence of alcohol were better able to cling to a tilted plane after the stress of forced swimming or, to a lesser degree, after exposure to inescapable electric shocks or loud noise compared to nonstressed control animals. Amphetamine and E also improved performance under the influence of alcohol. Forced swimming likewise improved the performance of hypophysectomized rats, indicating that the depressant effect of alcohol was counteracted by a general arousing mechanism rather than by adrenocortical secretion activated in the stress reaction (308).

Reports have shown that stress may markedly alter the activity and toxicity of compounds. The survival time of guinea pigs administered emetine hydrochloride (a cardiotoxic agent) was reduced in animals which had been trained in a shuttle-box conditioned avoidance response and was further reduced in animals which, after training, had been subjected to conflict by being punished with shock when they made the conditioned avoidance response (309). The LD_{50} for amphetamine is less than one-tenth the dosage for mice or rats after receiving a brief severe inescapable shock every 8 or 10 sec. for 3 hr. than for nonshocked control animals (310). Rats being trained in a lever-pressing avoidance may be killed by normally sublethal doses of dextroamphetamine (19). Aggregation in a confined space may be a stressor, and there have been many replications of the original report (311) that aggregation greatly increases toxicity of amphetamine in mice. However, this effect depends partly on genetic factors, with some strains of mice showing little or no difference in amphetamine toxicity between the aggregated and isolated conditions (312). The toxicity of amphetamine in aggregated mice is greatly reduced if the animals have had 40 hr. of previous habituation to the same group of three in which they are placed after amphetamine administration (313). A study of the effects of several variables showed that amphetamine toxicity was increased by the stressors of elevated environmental temperature and forced activity as well as aggregation. Aggregation failed to increase toxicity under conditions in which motor activity was not stimulated (314).

A variety of other stressors have also been found to potentiate toxicity and pharmacological effects of compounds. Amphetamine toxicity was greater in mice after 4 weeks of chronic isolation stress, whether they were isolated or placed in a group after amphetamine injection (315). A similar result was reported after only 13 days of isolation, beginning at weaning (316). Isoproterenol toxicity was likewise found to be greater in rats after 13 weeks of isolation (317). The pentylenetetrazol seizure threshold in mice was lowered by restraint for a very brief (15-sec.) period immediately prior to the test (318). A subsequent study in the same laboratory showed similar effects on seizure threshold after more prolonged body immobilization (for 7.5 to 60 min.) and also after 20 presentations of inescapable electric shocks, at 1-min. intervals (319). In both studies, there was evidence for adaptation to the effects of more prolonged restraint. A study of the effects of three environmental temperatures on acute toxicity of a number of compounds showed the greatest toxicity at the hottest temperature (37°) for amphetamine and most of the other compounds tested, with the least degree of toxicity at 28° for the tranquilizers and at the coldest temperature (18°) for the stimulants (320). The lethal effects of scorpion and rattlesnake venom were potentiated by either cold (2°) or heat (35–38°) stress, with the greatest resistance being found at normal room temperature (321).

Contrary to these reports on the potentiation of drug effects in stressed animals, rats subjected to unilateral hindleg ligation showed shorter sleeping time after injection of hexobarbital, meprobamate, or zoxazolamine (322). This stressor was shown to lower the blood levels of hexobarbital, pentobarbital, and meprobamate (323). However, phenobarbital produced no significant difference in sleeping time (322) and blood levels (323) between the stressed and nonstressed animals. Shorter sleeping time was reported after injection of pentobarbital or a com-

TABLE I.—SUMMARY OF THE EFFECTS OF SEVERAL COMPOUNDS ON THE SPECIFIED BEHAVIORAL RESPONSE IN SEVERAL TYPES OF SITUATIONS

		Avoidance Response[b]		Avoidance Component of Conflict[b]		Stress Syndrome[b]	
	mg./Kg.[a]	Performance	Acquisition	Unavoidable Shock	Avoidable Shock	Alarm Reaction	Ulcers
CPZ	2	− −	− −	0	0	−	−
Reserpine	0.5	− −	−	− −	0	−	+
Chlordiazepoxide	10	−	+	− −	−	−	
Morphine	5	−	+	−	0	+	
Meprobamate	50	0	+	− −	− −	− −	−
Pentobarbital	5	0	+ +	− −	− −	−	−
Alcohol	1000	0	+	0	−	−	
Benactyzine	10	+	+	0	− −		
Scopolamine	0.5	+	−				− −
Dextroamphetamine	1	+	+ +	0	0	+ +	

[a] i.p. in rats. [b] +, increase; −, decrease; 0, unchanged; no entry, insufficient information.

bination of pentobarbital and CPZ, in rats which had been isolated for 4 weeks previously (315). The writhing response of mice to benzoquinone may be inhibited by electric shocks prior to the drug injection (324).

These studies, showing various ways in which a stressful situation influences the action of compounds, indicate that the physiological and endocrinological components of the stress syndrome interact with the administered compound. It would be useful to determine for each important drug whether its effects are potentiated, counteracted, or unaffected by stress, as an aid in determining appropriate doses during stress and normal conditions and also as an addition to scientific knowledge about the drug's mechanisms of action. The drug effects might also be influenced differentially by different intensities or types of stressor or at different stages of the stress syndrome. In contrast to the finding that severe stress potentiates the depressant effect of CPZ on behavior of rats (305–307), evidence has been reported (325) that the mild stress of exposure to a novel environment counteracts the depressant effect of CPZ on spontaneous motor activity of mice.

DISCUSSION

During stress, the physiological and behavioral alterations occur in an attempt to maintain or restore homeostasis. The marked increase in endocrine secretions and activation of the autonomic nervous system during the alarm reaction prepare the animal for violent fighting or flight. The energetic behavior may succeed in terminating the stressful situation and also discharges the excess energy potential, thus helping to restore the organism to its normal, homeostatic state. If the stressor continues, as in an inescapable situation, the best chance for survival is to conserve strength, with most of the physiological and behavioral responses returning to normal during the stage of resistance. The freezing, crouching reaction, often seen during prolonged stress, may also occur during the alarm reaction if the initial attempt to destroy or escape the stressor is unsuccessful. Violent, agitated behavior very quickly leads to exhaustion; the rigid, tense posture of crouching conserves energy and also keeps the animal alert to the environment and in a good posture for springing forward as soon as there is an opportunity for escape or attack. Another adaptive value of freezing in small animals is that a moving object is more likely to be seen by an enemy; furthermore, a predator is less likely to attack an animal that is immobile (326).

Summary of Drug Effects.—The findings reviewed in this paper may be classified as showing either a decrease (−), an increase (+), or no change (0) in the behavioral and physiological responses to stressors. Table I shows a classification of the effects of the compounds most commonly included in these studies for several of the most frequently used measures of behavior. The number of plus or minus symbols (one or two) indicates the degree of consistency with which the effect has been reported in the various studies. All species and routes of administration are included in the compilation of Table I, although the sample doses are specified as i.p. in rats. The absence of a symbol (+, −, or 0) indicates that the information is lacking. The behavioral tests of pain threshold and pain-induced aggression are not included here because almost all of the compounds have effects only at higher doses than those cited here. The data forming the basis for this table are rather meager and often inconsistent for most of these compounds; CPZ is the only one of these which has been

tested by more than one investigator for each of the six measures shown.

In spite of deficiencies in the available information, some meaningful patterns are apparent. The performance of an avoidance response is inhibited by several tranquilizing and general depressant drugs, unaffected by therapeutic doses of a muscle relaxant and hypnotic, and is increased by anticholinergic and adrenergic agents. Drug effects on acquisition of avoidance show a less consistent pattern, perhaps because drug effects on learning of a new response are more complex than drug effects on performance of a well-established response. Drug-produced decrements in avoidance performance cannot reasonably be attributed to a specific reduction of avoidance motivation, because the compound which most consistently inhibits avoidance (CPZ) has no effect in the approach-avoidance conflict situation, whereas the compounds which reduce avoidance of the shock in conflict tests (meprobamate, pentobarbital, and alcohol) have no effect on performance of avoidance and actually improve acquisition of an avoidance response. The two additional compounds which generally increase an animal's willingness to accept avoidable shocks, in a conflict situation where the food-rewarded responses are punished by shock (chlordiazepoxide and benactyzine), likewise improve acquisition of an avoidance response. Three compounds which reduce performance of avoidance (CPZ, reserpine, chlordiazepoxide) reduce physiological components of the alarm reaction, and dextroamphetamine increases both the behavioral and physiological responses, but the other compounds do not show much correspondence between behavioral and physiological effects.

These drug effects cannot be adequately explained in terms of general stimulation or depression. The first seven compounds listed in Table I might all be classified as depressants, but they show very different patterns of effects. A distinction between sympathetic and parasympathetic dominance may explain why the effects of reserpine, which depletes catecholamines, and of CPZ, a centrally acting α-adrenergic blocker, are generally opposite to the effects of the sympathomimetic agent, dextroamphetamine. Scopolamine and benactyzine, which reduce cholinergic stimulation, tend to resemble dextroamphetamine and differ from CPZ and reserpine in certain respects. The few studies on effects of cholinergic drugs, such as physostigmine, have shown profound decrements in avoidance. Five of the compounds listed in Table I (chlordiazepoxide, morphine, meprobamate, pentobarbital, and alcohol) do not have a marked preponderance of adrenergic or cholinergic effects. However, the balance or imbalance of the two divisions of the autonomic nervous system is a critical factor in the stress syndrome, as shown by the adrenergic stimulation characterizing the alarm reaction, followed by reciprocal parasympathetic stimulation which may lead to sudden death (273) or gastric ulceration. Some behavioral drug effects have been convincingly attributed to central sympathetic or parasympathetic stimulation. Avoidance is greatly enhanced by the combination of an adrenergic and anticholinergic compound (28); many other behavioral effects of drugs have been attributed to their central adrenergic or cholinergic activating or blocking effects.

The choice of drugs for protective or therapeutic effects may be expected to depend on certain features of the stress situation. Adrenergic or anticholinergic compounds may enhance and prolong the alarm reaction, helping the animal to destroy or escape the stressor. Termination of the stress situation is the purpose of the vigorous alarm reaction, and if successful this eliminates the need for adaptation to the stressor. However, in most cases the organism's own mechanisms provide sufficient adrenergic stimulation. Administration of certain compounds is likely to be disruptive, as indicated by the lethal effect of moderate doses of amphetamine in acute stress situations (19, 310). The most conspicuous behavioral effect of adrenergic or anticholinergic compounds is the persistence of unnecessary avoidance responses (203). Anticholinergics can be beneficial in counteracting the parasympathetic overstimulation which may cause sudden death (273) or gastric ulcers. However, therapeutic effects are found more often with tranquilizing drugs which prolong the stage of resistance. This might be due to the fact that in most experimental test situations the stressor is inescapable, so that survival is prolonged by physiological and behavioral adaptation rather than by increasing efforts to escape. In nature both escapable and inescapable stress situations occur, and the adrenergic and cholinergic systems apparently provide a mechanism for an appropriate response in either type of situation. Drugs influence simultaneously the behavioral and physiological responses to stress, but our knowledge of these effects is severely limited by the fact that drug effects on behavior have usually been tested in animals previously subjected to the stress situation repeatedly, for

TABLE II.—EFFECTIVE DOSE[a] OF CPZ AND MEPROBAMATE FOR INHIBITING AVOIDANCE PERFORMANCE OF RATS

	Lever Pressing		
	Continuous	Warning Signal	Pole-Jump
CPZ	1.1	3.0	3.5
Meprobamate	103.0	135.0	72.0
Ref.	(37, 45)	(45)	(92)

[a] mg./Kg. i.p.

many days or even weeks, whereas drug effects on physiological stress responses have usually been measured by restraint or some other acute stress procedure during a single session of several hours.

Variations in Test Procedures.—Each compound has a wide variety of effects in addition to the stimulant, depressant, adrenergic, or cholinergic action by which it is often classified. Differential effects may be analyzed by comparing drug effects in situations which differ in a particular specified feature. An important methodological aid for such comparisons is to measure effects of several doses in order to estimate the effective dose, usually defined as the dose which causes a definite change in the behavior of half of the animals (ED_{50}). This measure of the response to a drug is concise and may be standardized and used for comparing data obtained in different laboratories. Unfortunately, a wide range of variations in ED_{50} values has been reported by different investigators, even when using apparently the same procedures for the same compound, administered by the same route in the same species. Similar wide variation is found also in measurements of lethal dose (LD_{50}). Since the variations among laboratories which influence the toxicity or effectiveness of a compound would generally be expected to influence other compounds in the same way, the relationship among compounds in ED_{50} or LD_{50} might be expected to show more consistent results than the levels found for each compound singly. Table II compares the effective doses of CPZ with meprobamate for inhibiting avoidance in three different situations. A CPZ effect was found at a much lower dose in the chronic, continuous avoidance situation than when the extra stimulation of a warning signal was provided, and the pole-jumping response, which was least compatible with a crouching response, was least readily affected by this compound. Meprobamate affected avoidance only at doses which caused marked skeletal muscle relaxation; with this compound the warning signal had very little stimulating effect, and the pole-jumping response was most readily impaired, presumably because it required the highest degree of muscular coordination. These differential situations indicate some specific drug actions which may be identified; a great deal more could be learned from large-scale studies in which a number of different compounds are tested in several different situations, using the same species and route of administration. Unfortunately, such information is apparently very scarce at the present time.

The experimental findings reviewed in this paper permit some additional conclusions about interactions between drug effects and behavioral test situations. Generally, a lever-pressing avoidance response was most susceptible to drug effects, whereas avoidance by jumping on a pole or platform was most resistant to drug effects, with a running response being intermediate in this respect. The lever-pressing response was generally the most difficult to train, whereas the jumping response was usually the quickest way to escape the electrified grid and was readily learned. In the lever-pressing situation, it is probable that the usual procedure of training the animals to a stable level of avoiding over a long period of time somewhat counteracted the tendency to be more readily affected by the compounds. CPZ appeared to block avoidance at lower doses in rodents than in cats; this species difference might be interpreted as showing that the crouching reaction, which is potentiated by this compound, is a stronger response tendency in rodents. However, reserpine showed no such species difference, suggesting a different mechanism for the action of this compound in decreasing avoidance. A much greater decrement in a locomotor avoidance was caused by CPZ when the animal was required to select the correct one of two exits rather than being able to avoid by either route (213); perhaps the process of choice or decision potentiates the inhibitory crouching tendency. In an approach-avoidance conflict, alcohol has been shown to decrease the avoidance response in a runway (183, 184) but not in a lever-pressing test (175, 177), perhaps because the drug causes greater muscular interference with the manipulative than with the locomotor response. Some substantial differences in drug effects have resulted from seemingly minor procedural variations. Studies which isolate and experimentally manipulate the situational features influencing drug effects may contribute valuable information about drugs and behavior through the measurement of their interactions.

Recommendations for Experimenters.—Effective research in behavioral pharmacology requires testing several doses of each drug, to

obtain an ED_{50}. In order for this measure to be reliable it should be determined over a wide time range after administration, on a sizable number of animals. The value of an experiment is greatly increased by the use of several drugs in the same test situation and by a comparison of several measures of performance and several related procedures. These requirements can only be fulfilled by large-scale studies. It is often possible to use the same animals in testing different time intervals, doses, and compounds. This use of each animal as its own control reduces the number of animals needed, increases the sensitivity of the statistical comparisons, and also saves time in preliminary training. However, these advantages can only be obtained if drug effects are tested on performance of an already learned response, rather than on the process of acquisition or extinction. Also, the experimenter must be alert to the possibility that the nondrug performance may change during repeated tests, and that a test under a particular drug condition may influence performance in the following test session. In addition, the possibility of cumulative drug effects and development of tolerance should be taken into consideration.

Experimenters are encouraged to select test techniques which have already been used in a number of previous drug studies, such as the lever-pressing, shuttle-box, and pole-jumping avoidance. Most of the meaningful conclusions in this review have been based on results reported with the most frequently used techniques. If novel procedures are used, the data are greatly increased in value when the investigator also obtains comparable data from a related, commonly used technique. Some of the most novel techniques, such as immobility as an avoidance response (77) and lever-pressing escape from cold temperature (142), are potentially valuable methods which deserve and need a great many more studies to establish a pattern of drug effects under these experimental conditions. The characteristics of the test situation to be used may depend on the purposes. Performance that is difficult to acquire, such as a lever-pressing avoidance response, may have the advantage of being a measure of learned rather than innate behavior that is readily altered by drugs. On the other hand, a pole-jumping avoidance response has the advantage of being easier to train, and its similarity to natural behavior tendencies may be advantageous for certain clinical applications.

It is to be hoped that the findings on drug effects in animals will lead to useful clinical applications. This is a necessary and challenging task, with many complex factors to be taken into account in applying results to a different species under varied conditions. However, the experimenter should select techniques which are simple and yield readily understandable results. The complex, multiple-conflict situation tested in a series of studies with a jumping-stand discrimination (205–209) does not permit isolation and identification of the determinants of behavior. Even though the drugs may be used to alleviate multiple conflicts and complex neuroses, the preclinical tests should measure simple, prototype components of the naturalistic situation. A preliminary step is to obtain more data and gain better understanding of the drug effects in animal test situations. Some glib assumptions, such as the belief that CPZ decreases avoidance performance because of reducing fear of the shock, have been based on an inadequate amount of data. At present we still do not fully understand the crouching response pattern and the situations and drugs which influence it. The collection of further data will greatly increase the validity and usefulness of theories about drug effects on animal behavior and thus provide a firm basis for clinical applications. It would be easy to deplore the shortcomings of behavioral pharmacology research to date, but it is more constructive to emphasize its recency, with nearly all of the studies having been published since 1953. It is inevitable that such a new field of scientific knowledge is largely characterized by diverse methods, conflicting findings, and small-scale studies. Already the research seems to show improvement in scope and methods as well as a rapid increase in number of published studies. We can expect very rapid advance in the next few years.

The stress syndrome includes both behavioral and physiological components, which should be measured concurrently, such as by testing effects of drugs on behavior and also on brain amines, blood pressure, and other physiological measures in the same situation. Most of the behavioral tests currently being used to investigate centrally acting compounds involve a stress reaction to the animal. Cardiovascular, endocrine, and a variety of biochemical changes most likely occur; however, the quantitative changes will vary from animal to animal and will also depend upon the intensity of the stressor involved. Effects of the psychotropic compounds on animal behavior may in reality be effects on stress-induced alteration in one or more of the biological systems within the organism. The behavioral and physiological components of the stress syndrome interact with each other and with experimentally administered

compounds. The inclusion of both behavioral and physiological measures adds a new element of difficulty to pharmacological studies, but the value of the additional information may be expected to outweigh the disadvantage of the extra work required.

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Thermodynamics of Chelation by Tetracyclines

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Thermodynamic data were determined for the complexation of five tetracycline analogs with cupric ion. Free energy values were calculated from data previously published by the authors. Enthalpy determinations were made in a Dewar calorimeter. Entropy values were determined from ΔG° and ΔH° at 25°. For the formation of 1:1 complexes, ΔH° was approximately −12 Kcal./mole and ΔS° was approximately −4 e.u. for the three chlor-analogs studied (4-epi-chlor-, demethylchlor-, and chlortetracycline), while a ΔH° of −7.6 Kcal./mole and a ΔS° of +10.2 e.u. was obtained for tetracycline. It is suggested that all of the analogs studied form inner sphere 1:1 complexes with cupric ion but that the chlorine at C-7 compels the three chlor-derivatives to undergo a more severe conformational change than tetracycline in order to reach a favorable configuration for chelation. All of the above analogs showed a small negative enthalpy change and a large positive entropy change for 2:1 complex formation. It is suggested that all four analogs form outer sphere or ion-pair complexes during 2:1 complexation.

IN A PREVIOUS paper (1), the authors calculated the thermodynamic dissociation and stoichiometric stability constants for five tetracycline analogs with cupric ion. In light of the experimental evidence presented, it was felt that all five analogs investigated, tetracycline·HCl, chlortetracycline·HCl, demethylchlortetracycline·HCl, 4-epi-chlortetracycline·HCl, and 4-epi-anhydrotetracycline·HCl, formed 2:1 (ligand–metal) complexes with cupric ions. The present study was undertaken to further elucidate the thermodynamic relations involved in this complexation.

In order to reach accurate conclusions regarding the nature of the forces operating within complexes during their formation in solution, it is necessary to know the energy changes accompanying the reactions in question. Stability constants are related directly only to the change in free energy.[1]

$$\Delta G^\circ = -2.303\ RT \log \beta_i \qquad \text{(Eq. 1)}$$

Where β_i is the over-all stability constant. The values for the change in free energy for the tetracycline chelations studied may be calculated from the data presented in *Reference 1*.

A knowledge of the entropy change during the complexation reaction allows the investigator to estimate the effects of certain steric factors which may be involved in complex bonding. In order to calculate the entropy of complexation, the enthalpy of complexation must also be determined as seen in Eq. 2.

$$\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T \qquad \text{(Eq. 2)}$$

The enthalpy of complexation was determined by direct calorimetry. Although this method requires more experimentation than the determination of enthalpy by the variation of stability constants with temperature, the accuracy gained in the calorimetric method justifies the work expended, especially in this study where the maximum heat evolved for any one measurement was only 0.5 cal. Rossotti (2) presents an enlightening discussion as to the accuracy of the two methods.

EXPERIMENTAL

Materials.—Tetracycline · HCl, chlortetracycline · HCl, demethylchlortetracycline · HCl, 4-epi-chlortetracycline · HCl, and 4-epi-anhydrotetracycline · HCl were donated by Lederle Laboratories. All solutions were prepared as described in *Reference 1*.

Calculation of Dissociation and Stability Constants.—Dissociation constants and concentration of tetracycline present were determined by the method of nonlogarithmic titration curves (1, 3).

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[1] The choice of the standard state is of great importance in determining ΔG°, ΔH°, and ΔS°. Many authors choose as the standard state a hypothetical 1 molal solution with the properties of an infinitely dilute solution. This choice of standard state eliminates the effect of ionic strength and the ambiguity which arises when comparing values with other work. However, this advantage is usually outweighed by the inaccuracy inherent in the extrapolation of data to values at zero ionic strength. In recent years, therefore, use has been made of a less hypothetical molar solution of a given ionic strength (μ = constant) at 25°. This choice of standard state was used in this work. However, it must be pointed out that all determinations, calculations, and comparisons of thermodynamic functions must be made with reference to the same standard state.

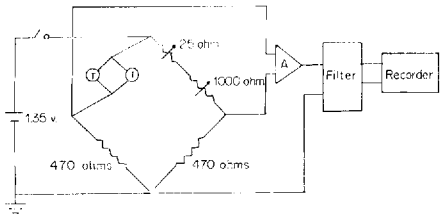

Fig. 1.—Circuit diagram for thermistor bridge. Key: T, 1000-ohm thermistor; A, amplifier.

Stability constants were determined as described in *Reference 1*. All determinations were carried out at an ionic strength of 0.01.

Calorimetry.—The design of the calorimeter was influenced by the desire to measure the heat of complexation for the same reactions from which the stability constants were determined. The work of Atkinson and Bauman (4, 5) was of special interest since they had used very dilute solutions. Therefore, modifications were made on a basic Wheatstone bridge in order to improve on the accuracy which they reported. A circuit diagram of the "thermometer circuit" is shown in Fig. 1. The bridge was driven by a Mallory 1.35-v. mercury battery (RM-12-R) which could be switched in and out of the circuit. Two arms of the bridge were fixed with matched 470-ohm, 0.5-w. carbon resistors. The temperature-sensing arm contained two 1000-ohm thermistors (Fenwall GB 31 P2) connected in parallel. The fourth arm contained two 10-turn wire wound potentiometers in series, the first a 1000-ohm potentiometer and the second a 25-ohm potentiometer. These potentiometers were used to zero the voltage output of the bridge at the beginning of a titration run. The voltage output of the bridge then goes to a model 1755 low-noise d.c. instrumentation amplifier[2] which is operated as a differential amplifier. The voltage output of the amplifier then passes through a model 7500 d.c. active filter[2] which attenuated all signal frequencies above 0.1 c.p.s. The voltage output was then fed into a Varian model G-10 graphic recorder.[3]

The calorimeter was constructed with a Dewar flask, based on the design proposed by Schlyter (6) as modified by Bauman (5), and was equipped with a thermostated buret for enthalpy titrations. The buret device was constructed from a microburet identical with the one utilized in the potentiometric procedures (1, 3). The entire device holds approximately 1.7 ml. of titrant, of which 1 ml. may be delivered to the solution. A micro-heater was utilized to calibrate the Dewar flask and its contents (7). The procedure was as follows.

The solutions to be titrated were placed in the Dewar flask, the pipet was filled with titrant, the calorimeter was closed and placed in a constant-temperature bath maintained at 25.00 ± 0.005°. The solution was either cooled or heated as was required to bring the temperature of the solution to 25°. At this time the bridge was balanced and a constant drift rate was established on the recorder. Heat was added by applying a voltage across the known resistance of the heater for a period of time which was electronically recorded (7). A steady drift rate is again obtained and the number of spaces between the two drift lines is measured. The heat sensitivity per space (C_s) can then be calculated from Eq. 3.

$$C_s = \frac{E^2 t}{(4.1840 \text{ joules/cal.})\, R\, (\Delta \text{ spaces})} \qquad \text{(Eq. 3)}$$

where C_s = calories per space,
E = voltage across heater,
R = heater resistance, 120.39 ohms,
t = heating time in sec.,
Δ spaces = number of spaces drift curve is displaced.

In the beginning of the calorimetric work, electrical calibrations were made before and after each addition of titrant. The average of the two calibrations was then taken as the C_s for that particular addition of titrant. However, when following this procedure, the time spent in joule calibrations in any one run was well over an hour, which was undesirable since epimerization takes place during the titration. Therefore, the average values of C_s for 49 calibrations with 158-ml. volume and 25 calibrations with 153-ml. volume were calculated.[4]

$C_s = (1.551 \pm 0.039) \times 10^{-2}$ cal./space for 158 ml.

$C_s = (1.504 \pm 0.037) \times 10^{-2}$ cal./space for 153 ml.

During the course of the work sample determinations of C_s were run to ascertain that the calculated values had not changed.

Most titrations were carried out with a total volume change of less than 0.6 ml.; therefore, it was felt that any change in C_s due to volume change would be imperceptible considering the size of the standard deviation. All joule calibrations were made on solutions with $\mu = 0.01$, so that it was assumed that C_s was the same no matter which tetracycline was being titrated.

To test the reliability of the calorimeter, the heat of neutralization of HCl by KOH was examined. The average of 27 determinations at 0.01 N HCl gave a value of 13.95 ± 0.46 Kcal./mole, which when corrected to infinite dilution (8) was 13.76 ± 0.46 Kcal./mole. Because the range was slightly higher than those values reported by calorimetry for very dilute solutions (9), all the parameters used in the determination were carefully rechecked. Since the heat of neutralization of hydroxide ions is used in the enthalpy determinations to be presented, it was decided to use the value obtained in this calorimeter, since any systematic error would then be carried through all of the calculations.

For each addition of titrant, the heat evolved, Q, in cal./L., is given by Eq. 4.

$$Q = \frac{C_s\,(\Delta \text{ spaces}) - (\text{heat of dilution of KOH titrant})}{\text{total vol., L.}} \qquad \text{(Eq. 4)}$$

The heat of dilution for 1 ml. of 0.4405 M KOH added to 158 ml. of ionic strength 0.01 was cal-

[2] California Electronic Mfg. Co., Inc., Alamo, Calif.
[3] Varian Associates, Palo Alto, Calif.

[4] Calibrations were necessary at both of these volumes, since all calorimetric determinations were carried out under identical conditions as the potentiometric determinations for the stability constants, which were carried out at both of these volumes.

culated from the values tabulated by Rossini (8) and found to be 7.3×10^{-2} cal./ml. of titrant.

The heat evolved in the titration of a solution of tetracycline·HCl and cupric ions is the sum of the heats evolved in the five reactions listed below.

$H_3T^+ + OH^- \rightleftharpoons H_2T + H_2O \quad \Delta H^\circ_1$ Reaction *1*

$H_2T + OH^- \rightleftharpoons HT^- + H_2O \quad \Delta H^\circ_2$ Reaction *2*

$HT^- + Cu^{++} \rightleftharpoons Cu(HT)^+ \quad \Delta H^\circ_3$ Reaction *3*

$HT^- + Cu(HT)^+ \rightleftharpoons Cu(HT)_2 \quad \Delta H^\circ_4$ Reaction *4*

$H^+ + OH^- \rightleftharpoons H_2O \quad \Delta H^\circ_5$ Reaction *5*

If $\Delta[\ ]$ is defined as the concentration initially minus the concentration after titrant (base) is added, and noting that multiplying the enthalpy for each reaction times the change in one of the reactants gives the heat evolved by this reaction, we can write Eq. 5.

$$Q = \Delta[H_3T]\,(\Delta H^\circ_1) - \{\Delta[HT^-] + \Delta[Cu(HT)^+] + \Delta[Cu(HT)_2]\}\,(\Delta H^\circ_2) - \{\Delta[Cu(HT)^+] + \Delta[Cu(HT)_2]\}\,(\Delta H^\circ_3) - \Delta[Cu(HT)_2]\,(\Delta H^\circ_4) + \Delta[H^+]\,(\Delta H^\circ_5) \quad \text{(Eq. 5)}$$

Before the values of interest in this work ΔH°_3 and ΔH°_4 can be calculated, the heats of reaction ΔH°_1 and ΔH°_2 must be determined. These heats can be found by measuring the heat of reaction for the titration of a pure tetracycline·HCl with base. This was done for each of the five tetracycline analogs studied here. The calorimeter was filled with a solution of the particular tetracycline hydrochloride. Simultaneously an exact duplicate of the solution in the calorimeter was titrated potentiometrically. To obtain a larger number of values for ΔH°_1 and ΔH°_2 the concentrations of the tetracycline analogs used in the titrations without metal were about 3 times as great as those used for the work reported in *Reference 1*. This procedure gave good values for all the tetracyclines except 4-epianhydro-, which is considerably less soluble than the other four derivatives.

The heat of reaction for Reactions *1* and *2* may be determined from Eq. 6.

$$Q = \Delta[H_3T^+]\,(\Delta H^\circ_1) - \Delta[HT^-]\,(\Delta H^\circ_2) + \Delta[H^+]\,(\Delta H^\circ_5) \quad \text{(Eq. 6)}$$

From the parallel potentiometric titration, $[H^+]$, $[H_3T^+]$, and $[HT^-]$ may be determined as described in *Reference 1*. Since the first and second dissociation constants for the tetracyclines are widely separated, $[HT^-]$ will be negligible at high hydrogen-ion concentrations and ΔH°_1 may be determined. At lower $[H^+]$, ΔH°_2 may be determined.

The Q values were calculated from the experimental data by use of Eq. 4. A sample determination for ΔH°_1 and ΔH°_2 is given in Table I for tetracycline·HCl. Due to overlap of the second and third dissociation constants, there are fewer determinations for ΔH°_2 and consequently these answers are not as accurate as those for ΔH°_1.

Once ΔH°_1 and ΔH°_2 are known, it is necessary to run two simultaneous titrations of tetracycline with metal—calorimetrically and potentiometrically.

TABLE I.—DETERMINATION OF ΔH°_1 AND ΔH°_2 FOR 153 ml. OF SOLUTION OF TETRACYCLINE·HCl, INITIAL CONCENTRATION (T°) = 14.32×10^{-4}, USING STOICHIOMETRIC DISSOCIATION CONSTANTS $K_1^c = 4.61 \times 10^{-4}$ AND $K_2^c = 2.48 \times 10^{-8}$

Total KOH Added, ml.	$[H^+]$	$[H_3T^+]$	$[HT^-]$	Q cal./L.	ΔH°_1 Kcal./mole	ΔH°_2 Kcal./mole
0.03	5.71×10^{-4}	7.91×10^{-4}	Negligible	...	...	...
0.11	4.84×10^{-4}	7.49×10^{-4}	Negligible	−3.22	−12.2	...
0.19	3.16×10^{-4}	5.84×10^{-4}	Negligible	−2.87	−12.5	...
0.27	2.136×10^{-4}	4.54×10^{-4}	Negligible	−2.89	−11.3	...
0.35	1.27×10^{-4}	3.10×10^{-4}	Negligible	−2.90	−11.7	...
0.47	2.38×10^{-5}	7.03×10^{-5}	1.41×10^{-5}	−4.21	−11.7	...
0.55	2.58×10^{-7}	7.30×10^{-7}	1.25×10^{-4}	...	...	...
0.63	7.07×10^{-8}	1.62×10^{-7}	3.72×10^{-4}	−1.42	...	−5.7
0.75	2.50×10^{-8}	Negligible	7.14×10^{-4}	−1.97	...	−5.7
					−11.9 Av.	−5.7 Av.

TABLE II.—DETERMINATION OF ΔH°_3 AND ΔH°_4 FOR 153 ml. OF SOLUTION OF TETRACYCLINE·HCl, INITIAL CONCENTRATION (T°) = 4.81×10^{-4} M, INITIAL CONCENTRATION OF ADDED HCl (A°) = 3.97×10^{-4}, INITIAL CONCENTRATION OF CUPRIC CHLORIDE (M°) = 2.45×10^{-4} M

Total KOH Added, ml.	$[H^+]$	$[H_3T^+]$	$[HT^-]$	$[Cu(HT)^+]$	$[Cu(HT)_2]$	Q cal./L.	ΔH°_3, Kcal./mole	ΔH°_4, Kcal./mole
0.0300	6.60×10^{-4}	2.27×10^{-4}	5.69×10^{-9}	9.53×10^{-5}	0	...	...	...
0.1047	4.88×10^{-4}	1.94×10^{-4}	9.32×10^{-9}	1.05×10^{-4}	0	−2.90	−5.5	...
0.1670	3.57×10^{-4}	1.59×10^{-4}	1.43×10^{-8}	1.17×10^{-4}	0	−2.40	−6.1	...
0.2334	2.33×10^{-4}	1.15×10^{-4}	2.42×10^{-8}	1.39×10^{-4}	0	−2.63	−10.8	...
0.2971	1.29×10^{-4}	6.75×10^{-5}	4.64×10^{-8}	1.72×10^{-4}	0	−2.38	−5.7	...
0.3340	7.47×10^{-5}	3.96×10^{-5}	8.14×10^{-8}	1.96×10^{-4}	0	−1.47	−9.8	...
0.3802	2.13×10^{-6}	1.05×10^{-5}	2.64×10^{-7}	2.44×10^{-4}	0	...	...	...
0.4095	3.81×10^{-6}	1.47×10^{-6}	1.16×10^{-6}	...	0.56×10^{-4}	−0.78	...	−1.9
0.4349	7.24×10^{-7}	1.73×10^{-7}	3.74×10^{-6}	...	1.24×10^{-4}	−0.57	...	−1.8
							−7.5 Av.	−1.8 Av.

TABLE III.—THERMODYNAMICS OF DEPROTONATION FOR TETRACYCLINE·HCl ANALOGS

$\Delta G^\circ_6 = -1.364 \log K_1$ $K_1 = [H_2T][H^+]/[H_3T^+]$
$\Delta H^\circ_6 = \Delta H^\circ_1 - \Delta H^\circ_5$

Analog	ΔG°_6 (Kcal./mole)	ΔH°_6 (Kcal./mole)	ΔS°_6 (e.u.)
Chlortetracycline·HCl	+4.46	+2.35	−7.1
Demethylchlortetracycline·HCl	+4.50	+2.35	−7.2
Tetracycline·HCl	+4.55	+2.05	−8.4
4-Epi-chlortetracycline·HCl	+4.90	+2.25	−8.9
4-Epi-anhydrotetracycline·HCl	+4.75	+1.95	−9.4

$\Delta G^\circ_7 = -1.364 \log K_2$ $K_2 = [HT^-][H^+]/[H_2T]$
$\Delta H^\circ_7 = \Delta H^\circ_2 - \Delta H^\circ_5$

Analog	ΔG°_7 (Kcal./mole)	ΔH°_7 (Kcal./mole)	ΔS°_7 (e.u.)
Chlortetracycline·HCl	+10.03	+6.75	−11.0
Demethylchlortetracycline·HCl	+9.79	+7.75	−7.5
Tetracycline·HCl	+10.51	+8.25	−7.6
4-Epi-chlortetracycline·HCl	+10.34	+7.65	−8.7

TABLE IV.—THERMODYNAMIC DATA FOR COMPLEXES OF TETRACYCLINE ANALOGS WITH CUPRIC IONS

Analog	ΔG°_3 (Kcal./mole)	ΔH°_3 (Kcal./mole)	ΔS°_3 (e.u.)
Chlortetracycline·HCl	−9.99	−11.7	−4.0
Demethylchlortetracycline·HCl	−10.71	−12.3	−5.4
Tetracycline·HCl	−10.64	−7.6	+10.2
4-Epi-chlortetracycline·HCl	−10.41	−11.5	−3.7

Analog	ΔG°_4 (Kcal./mole)	ΔH°_4 (Kcal./mole)	ΔS°_4 (e.u.)
Chlortetracycline·HCl	−6.95	−1.80	+17.3
Demethylchlortetracycline·HCl	−7.45	−2.60	+16.3
Tetracycline·HCl	−7.28	−1.80	+18.4
4-Epi-chlortetracycline·HCl	−6.92	−2.00	+16.5

Since the chelation steps of tetracycline with cupric ion are separated sufficiently, it was assumed that when $\bar{n} < 1$ only $Cu(HT)^+$ is formed and therefore, the $Cu(HT)_2$ terms may be dropped from Eq. 5. Using the calculations described in *Reference 1*, the concentrations of H^+, H_3T^+, HT^-, and $Cu(HT)^+$ may be calculated after each addition of base.

At $\bar{n} > 1$, $\Delta[Cu(HT)_2] = -\Delta[Cu(HT)^+]$ so that the only chelate term remaining in Eq. 5 will be $-\Delta[Cu(HT)_2]$ (ΔH°_4), and thus the heat of chelation for the second ligand may be calculated by determining $[H^+]$, $[H_3T^+]$, $[HT^-]$, and $[Cu(HT)_2]$ after each addition of titrant. A sample calculation of ΔH°_3 and ΔH°_4 is presented in Table II for tetracycline·HCl.

It should be noted that the ΔH°_3 and ΔH°_4 values show a wide range of individual points, yet when the averages of two different titrations of a particular analog were compared, the average values were very close. This was also noted in the joule calibrations. Since the calorimeter had no way to lose heat except by diffusion through the Dewar, it seemed that the calorimeter sometimes compensated by giving alternate low and high readings. Yet the average was a good measure of the actual heat evolved. Stability constants calculated from the data as presented in Table II showed good agreement with those values reported earlier (1).

RESULTS

As previously explained, the calorimetric work had to be carried out in two parts. Initially the heats of deprotonation for the first and second dissociating hydrogens were determined. The thermodynamic parameters of the first dissociating hydrogen are designated with a subscript *6*; the thermodynamic parameters of the second dissociating hydrogen are designated with a subscript *7*. The values for these parameters are presented in Table III. The values for K_1 and K_2 are the stoichiometric dissociation constants determined in the parallel potentiometric titrations (*vide supra*) and correspond to the thermodynamic dissociation constants reported previously (1). The values for ΔH°_1 and ΔH°_2 are determined experimentally as demonstrated in Table I. The value for ΔH°_5 is taken as −13.95 Kcal./mole as explained above.

The actual heat evolved in each determination of ΔH°_1 is about 0.5 cal., and the ΔH°_1 values calculated from these results are accurate to about ±5%. The ΔH°_1 values are approximately equivalent for all five tetracycline analogs, as is reflected in the agreement of the ΔH°_6 values in Table III. The epi-derivatives have ΔS°_6 values which are slightly more negative, indicating that the change in pK_1 (1) is an entropy effect as would be expected for a stereochemical change (compare chlortetracycline with 4-epi-chlortetracycline).

The actual heat evolved in each determination of ΔH°_2 is about 0.25 cal. Due to the overlap of pK_2 and pK_3 there are fewer determinations for ΔH°_2, and consequently, these values are not as accurate as the ΔH°_1 values. Due to the low solubility of the isoionic form of 4-epi-anhydrotetracycline, no accurate values of ΔH°_2 could be obtained for this analog.

Once ΔH°_1 and ΔH°_2 had been determined, tetracycline–metal titrations were run to find ΔH°_3, the heat of 1:1 chelation, and ΔH°_4, the heat of 2:1 chelation. These values are presented in Table IV. Particular notice should be directed toward the values determined for tetracycline·HCl where the exothermic heat of chelation, ΔH°_3, is significantly less than those values determined for the chlor-derivatives. Since the ΔG°_3's for the four analogs are reasonably similar, the large difference in ΔH°_3 for tetracycline·HCl, appears as an entropy effect causing ΔS°_3 to be +10.5 for tetracycline and about −4 for the chlor-analogs.

Before attempting to explain this difference, let us take a further look at ΔH°_4, the heat of complexation for the second ligand. The actual heat given off for each addition of titrant in ΔH°_4 determinations was in many cases below 0.2 cal. Also the number of determinations for ΔH°_4 in each titration was limited to the lower pH ranges to

TABLE V.—THERMODYNAMIC DATA FOR THE COMBINED REACTION *2 + 3*

$$Cu^{++} + H_2T + OH^- \rightleftharpoons Cu(HT)^+ + H_2O$$
$$\Delta G^\circ_{2+3} = -1.364 (\log K_1 + \log \beta_1 - \log Kw)$$

Analog	ΔG°_{2+3} (Kcal./mole)	ΔH°_{2+3} (Kcal./mole)	ΔS°_{2+3} (e.u.)
Chlortetracycline·HCl	−18.90	−18.92	−0.01
Demethylchlortetracycline·HCl	−20.00	−18.70	+4.4
Tetracycline·HCl	−19.23	−13.3	+19.9
4-Epi-chlortetracycline·HCl	−19.11	−18.91	+0.7
4-Epi-anhydrotetracycline·HCl	−19.90	−14.0	+19.8

insure that the third dissociable hydrogen from the chelated tetracycline was not present to a significant extent. For 4-epi-chlortetracycline only one value of ΔH°_4 could be calculated per titration before precipitation began in the calorimeter. This could easily be seen on the recorder as irregular bursts of heating replaced a smooth drift curve. Even with all the errors inherent in measuring such a small heat change and the pyramiding of effects of inaccurate ΔH°_1 and ΔH°_2 values, it is possible to get some idea of ΔH°_4, at least qualitatively. The ΔH°_4 values listed in Table IV show a reasonable degree of consistency, as do the ΔS°_4 values. In this case all four derivatives, the chlor-analogs and tetracycline itself, show a high positive ΔS°_4.

As mentioned previously, ΔH°_1 is the most accurate enthalpy determined in this work. Therefore, to prove that the difference in ligational entropy was not just a factor of an incorrect ΔH°_2, it was decided to examine the entropy of the over-all Reaction *2 + 3*.

$$Cu^{++} + H_2T + OH^- \rightleftharpoons Cu(HT)^+ + H_2O$$
$$\Delta H^\circ_{2+3} \quad \text{Reaction } 2 + 3$$

$$\Delta G^\circ_{2+3} = -1.364 (\log K_1 + \log \beta_1 - \log K_w) \quad \text{(Eq. 7)}$$

The ΔH°_{2+3} can be calculated, using only one predetermined value, ΔH°_1. In addition, Reaction *2 + 3* is a good approximation to what is actually happening in solution, since below $\bar{n} = 1$ [*i.e.*, where $Cu(HT)^+$ is forming] the concentration of free ligand (HT^-) is negligible and the addition of titrant (OH^-) results in pH changes (Reaction *5*), neutralization of H_3T^+ (Reaction *1*), and the conversion of H_2T to $Cu(HT)^+$. Therefore,

$$\Delta H^\circ_{2+3} = \frac{Q - \Delta[H^+](\Delta H^\circ_2) - \Delta[H_3T^+](\Delta H^\circ_1)}{\Delta[Cu(HT)^+]} \quad \text{(Eq. 8)}$$

The thermodynamic values obtained from such a calculation are illustrated in Table V. Since ΔH°_{2+3} can be calculated without knowledge of ΔH°_2, this calculation may also be made for 4-epi-anhydrotetracycline. Calculating ΔS°_{2+3}, a value of +19.8 is obtained for 4-epi-anhydrotetracycline, +19.9 for tetracycline, and much lower values for the three chlor-analogs.

DISCUSSION

Rossotti (10) has suggested that from a simple viewpoint, the entropy change on complex formation would be expected to be negative (about −25 e.u.) due to the conversion of translational to vibrational and rotational entropy, accompanying the decrease in the number of particles in solution. However, the role of the solvent must also be estimated in rationalizing ligational entropy changes. Association of a metal ion with a charged ligand in aqueous solution will bring about a decrease in the numbers of ions, (partial) neutralization of electrical charge, attenuation of the remaining charge, and displacement of water from the hydration spheres of the reactant (10). The last term is a major factor and has brought about a differentiation between inner and outer sphere complexes. An inner sphere complex is formed when a ligand is able to replace a water molecule from the hydration sphere of the metal. An outer sphere complex does not replace water from the metal hydration sphere but is rather an ion pair complex. A less favorable entropy change will be expected from the formation of an outer sphere complex than from the corresponding inner sphere complex.

However, it should be noted that the magnitude of the enthalpy of complexation must also be considered, and that judgment concerning the sphere of complexation cannot be made strictly from an entropy viewpoint. Outer sphere complexes with Cl^- or SO_4^{--} as ligands usually show a slight *endothermic* heat of formation (11), whereas inner sphere complexes presuppose the formation of strong covalent bonds, which show large exothermic heats of formation. It should also be expected that a ligand of the size and complexity of tetracycline would show large conformational changes during chelation which would be reflected in the entropy changes.

Therefore, in considering what type of complexation takes place, no set rule for entropy values may be established, but rather an over-all view of all the processes listed above by Rossotti must be taken, with special emphasis for tetracycline on the conformational changes expected. On the basis of the high negative ΔH°_3 values obtained for the 1:1 complexes, it would seem that the two tetracycline analogs and the three chlortetracycline analogs all form inner sphere complexes with cupric ions. However, there is a significant difference between the ΔH°_3 values obtained for tetracycline and 4-epi-anhydrotetracycline[5] as opposed to the ΔH°_3 values for chlortetracycline, demethylchlortetracycline, and 4-epi-chlortetracycline. Since the ΔG°_3 values for tetracycline and the three chlor-derivatives are approximately the same, the difference in enthalpy is probably a function of the conformational changes required for tetracycline and the chlortetracyclines to reach a favorable complexing configuration.

As a system becomes more ordered, the entropy

[5] Although ΔH°_3 has not actually been determined for 4-epi-anhydrotetracycline, it seems reasonable to assume, on the basis of the data presented in Table V, that ΔH°_3 for 4-epi-anhydrotetracycline will be very close to ΔH°_3 for tetracycline.

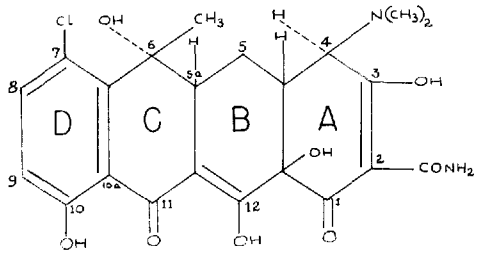

Fig. 2.—Configuration for chlortetracycline after Donohue *et al.* (12). Other authors (15) show the configuration for the enantiomorph.

of the system decreases. Therefore, the chlortetracycline analogs with negative $\Delta S°_3$ values seem to be required to undergo a more severe conformational change than tetracycline in order to reach a favorable configuration for chelation. From the data presented in Tables IV and V it would be expected that the requirement for a more severe conformational change would be dependent on the presence of the chlorine atom at carbon 7 (see Fig. 2). These ideas were tested by constructing framework molecular models[6] of the various tetracyclines using the bond lengths and configuration for chlortetracycline as determined by Donohue *et al.* (12) from X-ray data. The hydrogens on 6-methyl and 6-hydroxyl and the chlorine at carbon 7 were so constructed that the van der Waals radii for these atoms (13) were physically represented. From the model it is seen that the C ring can readily flip from a "boat" conformation where the 6-methyl is axial to the ring, to a "boat" conformation where the 6-methyl is equatorial to the C ring (while the 6-hydroxyl group goes from equatorial to axial positions) and that this flipping would be influenced greatly by the steric hindrance occurring between the chlorine at carbon 7 and either the 6-methyl or 6-hydroxyl.

On the basis of the data presented, it seems most likely that the first tetracycline ligand forms an inner sphere complex with cupric ions and that this complexation takes place with the formation of a chelate ring involving the cupric ion, oxygen 10, carbons 10, 10a, and 11, and oxygen 11, in which case it is necessary that all of the above atoms and ions lie in a plane (14). To accomplish this it is necessary that the C ring be essentially planar with the D ring, and in this case there would be considerable steric interaction between the chlorine atom and probably both the methyl and hydroxyl groups on carbon 6. Donohue *et al.* (12) point out that the Cl . . . CH_3 distance for this conformation is 2.99 Å. which is much less than 3.8 Å., the sum of the van der Waals radii. Obviously, if the chlorine at carbon 7 was not present as for tetracycline, the steric problem would be decreased and the entropy change would essentially depend on the replacement of water from the hydration spheres of copper.

In light of the above reasoning, it is hypothesized that the difference in the entropy of formation for 1:1 complexes of chlortetracycline and tetracycline is due to the strained steric requirement that chlortetracycline must undergo. If this hypothesis is true, it follows that there should be little difference between $\Delta S°_3$ for chlortetracycline and 4-epichlortetracycline, since the 4-dimethylammonium group would not be involved in complexation. This is found to be true in Table IV, and lends support to the idea that chelation takes place at oxygens 10 and 11.

It might seem that demethylchlortetracycline should have a $\Delta S°_3$ which is more positive than $\Delta S°_3$ for chlortetracycline, since demethylchlortetracycline has no 6-methyl group available for steric interaction with the chlorine. However, it seems reasonable to assume that for demethylchlortetracycline the 6-hydroxyl would generally be found axial to the C ring, since there would be much less steric interaction between the 6-hydrogen (equatorial) and the 7-chlorine. Therefore, it might be reasoned that it would be less favorable for the C ring in demethylchlortetracycline to become planar (bringing about steric interaction between chlorine and hydroxyl) than for the C ring in chlortetracycline, where there is no specially favored conformation for the hydroxyl (or methyl) group, and, thus, it could be hypothesized that a slightly more severe conformational change is required for demethylchlortetracycline than chlortetracycline in order to bring about complexation.[7]

In the case of 4-epi-anhydrotetracycline, it would be expected that the entropy of chelation would be similar to that of tetracycline (as is seen in Table V) for two reasons. First, there is no chlorine present at carbon 7 to cause steric interactions with the 6-methyl; and second, since chelation seems to occur at oxygens 10 and 11, entropy of chelation should not depend on whether the 4-dimethylammonium group is epimerized. In addition, for an anhydrotetracycline analog, the C ring is initially in a planar configuration due to the additional double bond at C_{5a}—C_6. Thus, it might be expected that all anhydrotetracyclines would have a $\Delta S°_3$ similar to tetracycline, regardless of whether a chlorine was present at carbon 7.

The results obtained for Reaction *4*, the formation of the 2:1 chelate from the 1:1 chelate and the free ligand, are generally similar for all four compounds, but considerably different from the results obtained for Reaction *3*. In the previous discussion it was hypothesized that the first ligand forms strong covalent bonds with the cupric ion. As a simple picture, we may consider a small cupric ion surrounded by the much larger tetracycline molecule, and although the C and D rings of the ligand are in a fixed position, the A and B rings and the bulky groups attached to them are still free to rotate. Now another large, negatively-charged tetracycline molecule complexes with the copper ion. It is very difficult to visualize a second tetracycline as being capable of approaching the cupric ion close enough to form a covalent ligand bond. This is confirmed by the low values obtained for $\Delta H°_4$ for all of the compounds studied, and thus it may reasonably be presumed that the second tetracycline forms an outer sphere or ion-pair complex.

[6] Prentice-Hall, Inc., Englewood Cliffs, N. J.

[7] An alternate hypothesis, suggested by the reviewer, proposes that the conformation in unchelated demethylchlortetracycline is the same as in chlortetracycline, perhaps due to hydrogen bonding to the 7-chlorine. Therefore, a similar conformational change occurs upon chelation leading to the observed similar entropy change. It should also be pointed out that the above explanations are only valid if chelation actually does take place at oxygens 10 and 11 as would be expected from the protonation scheme determined by Rigler *et al.* (16).

In this reaction $\Delta S°_4$ has a high value (see Table IV) for all of the analogs studied, both tetracycline and chlor-derivatives. This may be explained by the fact that when the second ligand attaches to the metal, the over-all charge of the complex becomes zero, and the number of ions in solution is decreased. This neutralization of charge and decrease in the number of ions is common to all the analogs studied and would be expected to give a positive entropy change. In addition, the conformational entropy changes between tetracycline and the chlor-analogs is not significant here, since little conformational change is needed to form an ion-pair complex bond.

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Ionization of Bases with Limited Solubility

Investigation of Substances with Local Anesthetic Activity

By IVO SETNIKAR

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Methods of obtaining straight-line representation of ionization of weak acids or bases were presented by Hofstee (2), by Benet and Goyan (3, 4), and by Leeson and Brown (5). The methods involve recalculations of the results and are strongly influenced by experimental errors (4). More immediate and easier to apply is the method proposed by Druckrey (6, 7), based on the use of a specially designed scale for the titrant, which yields a straight-line expression of the law of mass action. This method may also be adapted for expressing with a straight line the ionization process of weak acids or bases in which the unionized form is sparingly soluble, a fact which limits its availability for the ionization equilibrium.

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$$\frac{[BH^+]}{[B][H^+ \cdot H_2O]} = K' \quad \text{(Eq. 2)}$$

where K' is the apparent ionization constant, valid

for the particular system in which ionization takes place. Equation 2 describes with sufficient approximation the ionization equilibrium of a weak base, except at the boundaries of almost total ionization or unionization of the base. Without approximations the ionization equilibrium is expressed by Eq. 3, derived from Clark (8).

$$\frac{[A^-] - [H^+ \cdot H_2O] - [D^+] + [OH^-]}{([S] - [A^-] - [OH^-] + [H^+ \cdot H_2O] + [D^+]) \cdot [H^+ \cdot H_2O]} = K' \qquad \text{(Eq. 3)}$$

in which A^- and D^+ are the ions of strong acids and bases present in the solution and S is the total quantity of the base, *i.e.*,

$$[S] = [B] + [BH^+] \qquad \text{(Eq. 4)}$$

In the range of its validity Eq. 2, in logarithmic terms and substituting K_B' for $1/K'$, becomes

$$pH = pK_B' + \log \frac{[B]}{[BH^+]} \qquad \text{(Eq. 5)}$$

If B has a limited solubility, at a certain point of titration the excess of B precipitates and the concentration of the dissolved B remains constant during the rest of titration. When this phenomenon happens Eq. 5 describes the deionization process only up to the point at which B starts to precipitate. Above this point the deionization is described by:

$$pH = pK_B' + \log \frac{[C]}{[BH^+]} \qquad \text{(Eq. 6)}$$

where C is the concentration of dissolved B on saturation.

In the conditions described by Eq. 5, a straight line is obtained by plotting the pH on the ordinate and log $[B]/[BH^+]$ on the abscissa, or using charts in which the abscissa is graded to a special scale, corresponding to the values of log $[B]/[BH^+]$. Furthermore, it may be convenient to substitute $[B]/[BH^+]$ with the titrated fraction F, where F is given by $[B]/[S]$. Since $[B]$ is equal to $[A^-]$, *i.e.*, the titrating strong acid, F is also given by $[A^-]/[S]$.

Similarly, Eq. 6 is expressed by a straight line when the pH is plotted on the ordinate and log $1/[BH^+]$ on the abscissa, or using a chart in which the abscissa is graded to a special scale corresponding to the values of log $1/[BH^+]$. In this case too $1/[BH^+]$ may be substituted by the titrated fraction F. The correspondence of the logarithmic scales, with these special F scales is represented in Fig. 1. The LR scale of Fig. 1 (log $[B]/[BH^+]$) refers to Eq. 5. The L scale (log $1/[BH^+]$) refers to Eq. 6.

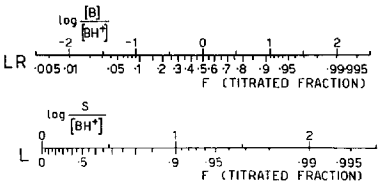

Fig. 1.—Scales of F (titrated fraction), which yield a straight-line relationship between F and pH for deionization processes of weak proton acceptors. Key: LR, scale equivalent to units of log $([B]/[BH^+])$ according to Eq. 5; L, scale equivalent to units of log $([S]/[BH^+])$ according to Eq. 6.

Equation 5 shows that $pK_B' = pH$ when $[B]/[BH^+] = 1$ or $F = 0.5$. Therefore the pK_B' can easily be found, either from the point at which $[B]/[BH^+] = 1$, or from any other point on the ionization line, since its slope, for monoprotic species, is the same on a pH *versus* LR diagram.

On the contrary Eq. 6, which may also be written as

$$pH = (pK_B' + \log C) + \log \frac{1}{[BH^+]} \qquad \text{(Eq. 7)}$$

does not yield the pK_B' value, unless C, the concentration of B at saturation, is determined.

EXPERIMENTAL

Investigated Substances.—The investigated substances were: procaine; lignocaine; cocaine; Rec 7-0518, *i.e.*, ketocaine or 2-(*N*-diisopropylaminoethoxy)-1-butyrophenone; Rec 7-0544, *i.e.*, 1-(*N*-diisopropylaminoethoxyphenyl)-butan-1-ol; and Rec 7-0591, *i.e.*, 2-(*N*-diisopropylaminoethoxy)-3-amino-1-butyrophenone. Rec 7-0518, Rec 7-0544, and Rec 7-0591 are three new local anesthetics described by Setnikar (9, 10).

The hydrochlorides of these substances were dissolved in CO_2-free glass-distilled water at a 0.1 and 0.01 M concentration and submitted to titration with 2 N and, respectively, 0.2 N carbonate-free NaOH.

Apparatus and Procedures.—The pH was measured with a Beckman Zeromatic model 96 meter, standardized against 0.05 M potassium hydrogen phthalate (pH = 4.0) and 0.01 M sodium borate (pH = 9.2). NaOH was added from a 3-ml. microburet, calibrated to 0.01 ml. Measurements were taken at 20.0° and at 37.0° on 50 ml. of the solutions of the local anesthetics under continuous and uniform agitation (magnetic stirrer). After each addition of NaOH, the pH was read when the meter had reached a stable value. The meter was read

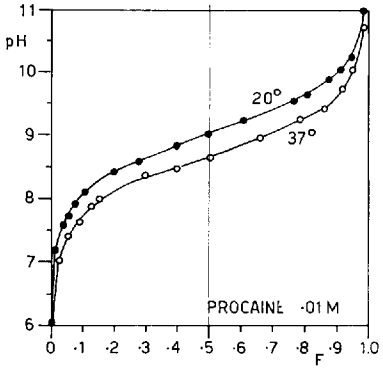

Fig. 2.—Ionization curve of 0.01 M procaine plotted by the conventional method. The titration was performed at 20.0° and at 37.0°, yielding two S-shaped deionization curves.

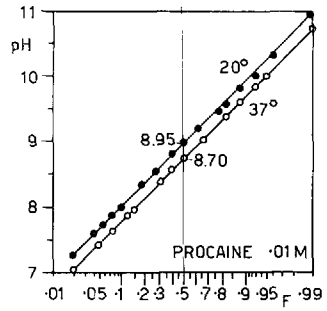

Fig. 3.—Procaine 0.01 *M*. Same data as in Fig. 2 plotted on a LR scale yielding straight-line relationships between pH and titrated fraction F. The pK_B' can be estimated from the whole titration and not only from the central data, as by the conventional method.

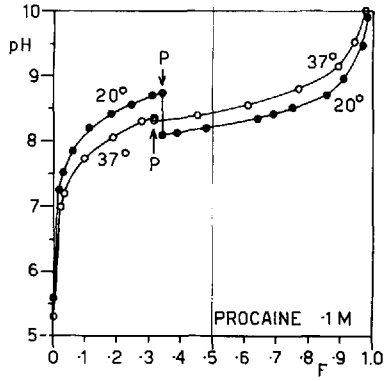

Fig. 4.—Ionization curve of 0.1 *M* procaine plotted by the conventional method. At the arrows, P, the undissociated base, starts to precipitate, markedly altering the deionization process. At this point the pH drops considerably, particularly on the curve obtained at 20°.

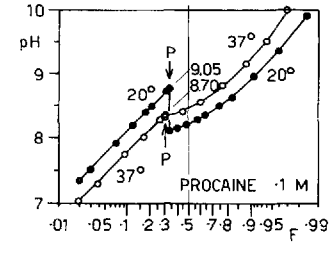

Fig. 5.—Procaine 0.1 *M*. Same data as in Fig. 4, but plotted on a LR scale for F. The initial straight-line part of the deionization process enables one to find the pK_B' values by extrapolation (9.05 at 20.0° and 8.70 at 37.0°). The data beyond the precipitation point are on a curved line, demonstrating a deviation from the theory described by Eq. 5.

10 min. after addition of the titrant when the titration was associated with precipitation.

The amounts of NaOH, corrected according to Parke and Davis (1), were expressed as fractions of the quantity necessary to titrate the whole base present in solution, and plotted on the charts *versus* the pH values.

Solubility of the Unionized Base.—Solutions of the hydrochlorides of the investigated substances, at a concentration of 0.1 *M* for procaine and lignocaine, and 0.01 *M* for the others, were deionized with a 5% excess of NaOH, filtered, or centrifuged, and the clear filtrate or supernatant acidified with HCl. These procedures were performed at 20° and at 37°. The concentrations of the substances in these acidified solutions were determined spectrophotometrically.

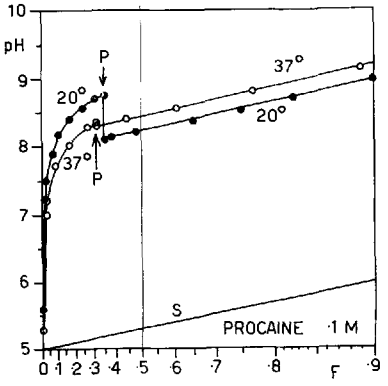

Fig. 6.—Procaine 0.1 *M*. Same data as in Figs. 4 and 5, but plotted on the L scale for F, which shows a straight-line deionization process after the precipitation point. Beyond this point the theory described by Eq. 6, therefore, applies. The line marked with S on the bottom of the figure shows the theoretical slope in these conditions.

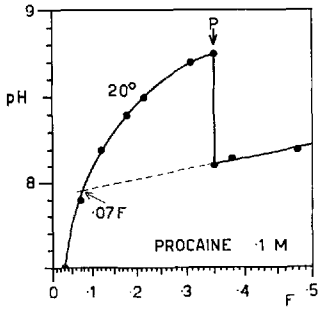

Fig. 7.—Detail of Fig. 6 showing a part of the deionization process at 20.0°. The arrow marks the point at 0.07 titration which corresponds to the maximum solubility of the unionized base in these conditions. This value is found by extrapolating the measurements after precipitation. From 0.07 F to 0.35 F, however, the base in unionized form remains still in solution, due to a phenomenon of hypersaturation. Phenomena of hypersaturation are often present, transiently as in this case or even during the whole titration, interfering with the evaluation of the maximum solubility and of the pK_B' values. They are not seen in back-titrations, *i.e.*, of the unionized base with a strong acid. In this case the dotted line is followed and the solution becomes clear only at the point in which the dotted line meets the first part of the titration curve.

RESULTS

The ionization curve for the tertiary amino group of 0.01 *M* procaine, plotted by the conventional method, is given in Fig. 2, yielding the well-known S-shaped curve. The same data plotted on the LR scale (*cf.* Fig. 1) yield a straight line with a slope of 45° (Fig. 3). In both instances the pK_B' value (second apparent ionization constant of procaine) is easily found.

The shape of the deionization curve of 0.1 *M* procaine is different, since between 0.3 and 0.4 F the unionized base precipitates and the deionization curve changes markedly. Particularly at 20.0° the pH drops by about 0.6. Then, proceeding in the titration, the pH raises again, first slowly, and then more rapidly, the final (right) part of the curve closely resembling the S-shaped deionization curve of soluble bases.

Wehr and Koelzer (11) noted that other bases with local anesthetic activity behaved in a similar complex way, but they offered no explanation.

The same data for 0.1 *M* procaine were plotted on a chart with the titrated fraction F expressed on the LR scale of Fig. 1. They yield a straight-line relationship between F and pH up to the precipitation point, *i.e.*, as far as the requirements of Eq. 5 are fulfilled (Fig. 5). Then the pattern departs markedly from the straight line and the theoretical pattern is approached again toward the end of titration.

By using a chart which yields a straight-line relationship between titrated fraction and pH when the requirements of Eq. 6 are fulfilled, it may be shown that, after precipitation, the theory described by Eq. 6 applies (Fig. 6) since the data are now on a straight line which has the theoretical slope.

The irregularities of the deionization curve of 0.1 *M* procaine shown by Fig. 4 are, therefore, related to the limited solubility of the unionized base and to the constant concentration of [B] in solution.

In the example given by 0.1 *M* procaine the solubility of B is limited but not very low. The estimation of pK_B' obtained in Fig. 5 by extrapolation of the straight-lined part of the deionization curve before precipitation may be considered reasonably precise, and so is the estimation of the maximum solubility of unionized procaine (Fig. 7).

For substances with a low solubility of the unionized base precipitation occurs after the addition of a very small amount of titrant and, due to the interference of transient hypersaturation phenomena, the evaluation of the pK_B' by extrapolation becomes rather arbitrary. An example of this is given by by the deionization curves obtained with 0.01 *M* and 0.1 *M* Rec 7-0518, which, in the unionized form, has a solubility lower than 1 mmole/L. (Figs. 8 and 10 and Table I). As shown by Figs. 9 and 11 the deionization of the Rec 7-0518 fits the theory of Eq. 6 throughout titration. In fact the relationship of the titrated fraction *versus* the pH on a chart with F on the L scale is straight-line and has the slope required by Eq. 6. Furthermore, the pH of the deionization lines of 0.1 *M* Rec 7-0518 is 1 unit higher than that of 0.01 *M* Rec 7-0518, due to the fact that log S and, therefore, also log $[BH^+]$ differ by one unit in the two conditions.

For substances with such properties both the pK_B' and the maximum solubility values may be estimated only with rough approximation. Un-

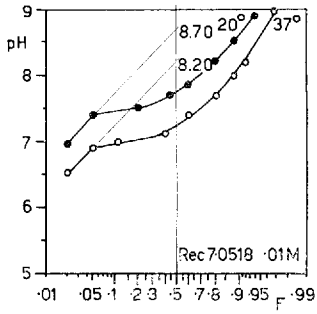

Fig. 8.—Deionization curve of 0.01 *M* Rec 7-0518 plotted on a LR scale for F. Owing to the very low solubility of the unionized form the process of precipitation starts at the very beginning of titration (in the range of 0.05 F). Taking account of the possible presence of hypersaturation phenomena, it becomes difficult to evaluate exactly the maximum solubility of Rec 7-0518. Since the calculation of the pK_B' value depends on the maximum solubility of the unionized base, the pK_B' can be estimated only with rough approximation. The figure shows how the pK_B' was estimated as 8.70 at 20.0° and as 8.20 at 37.0°.

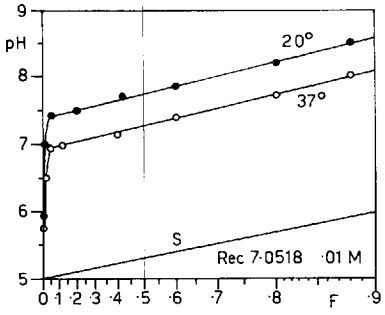

Fig. 9.—Rec 7-0518 0.01 *M*. Same data of Fig. 8 plotted on a L scale of F. After the precipitation point the theory described in Eq. 6 applies. At the bottom the theoretical slope (S) of the deionization process according to Eq. 6 is shown.

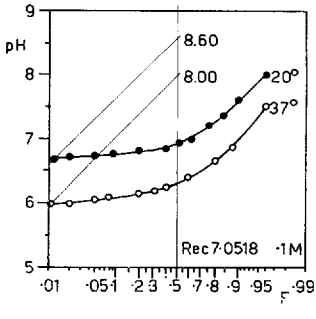

Fig. 10.—Deionization curve of 0.1 *M* Rec 7-0518 plotted on a LR scale for F. At this concentration it becomes still more difficult to evaluate the maximum solubility of the unionized form, since the precipitation obviously occurs at a lower titration point than for 0.01 *M* Rec 7-0518. Therefore, the calculation of the pK_B' value becomes still more approximate.

TABLE I.—APPARENT IONIZATION CONSTANTS OF THE INVESTIGATED BASES AND MAXIMUM SOLUBILITY OF THEIR UNIONIZED FORM AT 20.0° AND AT 37.0°[a]

		pKB′		Maximum Solubility, mmoles: From the Deionization Curves		Maximum Solubility, mmoles: Spectrophotometrically Determined			
		20°	37°	20°	37°	20°		37°	
Procaine (II)	0.01 *M*	8.95	8.70	S	S	...	..	...	..
	0.1 *M*	9.05	8.70	7	32	...	..	...	..
	BE	9.0	8.7	7	32	12	H	14	H
Lignocaine (II)	0.01 *M*	8.20 D	7.70 D	S	S	...	..	...	..
	0.1 *M*	8.00	7.70	18	16	...	..	...	..
	BE	8.1	7.7	18	16	16	..	15	..
Cocaine	0.01 *M*	8.78	8.35	2	2.5	...	..	...	..
	0.1 *M*	8.76	8.40	2	2.5	...	..	...	..
	BE	8.8	8.4	2	2.5	2	..	2	..
Rec 7-0518	0.01 *M*	8.70	8.20	0.5	0.5	...	..	...	..
	0.1 *M*	8.60	8.00	?	?	...	..	...	..
	BE	8.7	8.2	0.5	0.5	0.4		0.5	..
Rec 7-0544	0.01 *M*	9.10	8.55	0.4	0.4	...	..	...	..
	0.1 *M*	8.92	8.40	?	?	...	..	...	..
	BE	9.1	8.5	0.4	0.4	0.8	..	0.5	..
Rec 7-0591 (I)	0.01 *M*	4.60 D	4.32 D	S	S	...	..	...	..
	0.1 *M*	4.60 D	4.34 D	S	S	...	..	...	..
	BE	4.6	4.3	S	S	...	..	...	..
Rec 7-0591 (II)	0.01 *M*	9.40	9.40	0.8	0.2	...	..	...	..
	0.1 *M*	?	?	?	?	...	..	...	..
	BE	9.4	9.4	0.8	0.2	0.5	..	0.7	..

[a] BE = best estimate, based on the most reliable values; D = obtained directly, without extrapolation; S = soluble at the investigated concentration; procaine II = second deionization of procaine; lignocaine II = second deionization of lignocaine; Rec 7-0591 I = first deionization of Rec 7-0591; Rec 7-0591 II = second deionization of Rec 7-0591; H = a substantial amount of procaine (about 20%) hydrolyzes at the high pH values of some steps of the experimental conditions.

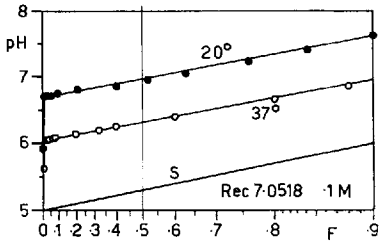

Fig. 11.—Rec 7-0518 0.1 *M*. Same data of Fig. 10 plotted on a L scale for F, in order to check the theory of Eq. 6. Besides by the straight-line alignment and by the slope of the data, the theory of Eq. 6 is verified also because the lines of Fig. 9 (Rec 7-0518 0.1 *M*) are higher by 1 pH unit than the corresponding lines of Fig. 11, as required by Eq. 6, since log S differs by 1 in the two conditions. Figure 9 becomes thus a right-hand continuation of Fig. 11.

fortunately, interference by hypersaturation phenomena and other technical difficulties make the estimation of maximal solubility by other methods problematic too. Therefore, the pK_B' values and the maximum solubility values of bases with a very small solubility of the unionized form, obtained by the described methods, must anyway be considered as very rough estimates.

The comments on Rec 7-0518 apply also to Rec 7-0544, since the solubility of its unionized form is also very low.

Rec 7-0591 has two basic radicals which ionize: one is the 3-amino group, with a pK_B' value of 4.6 at 20° and of 4.3 at 37°, and the other is the tertiary amino group in the *N*-diisopropylaminoethoxy chain, whose chemical-physical features are similar to those of the same radical in Rec 7-0518.

The results obtained with the investigated substances are summarized in Table I which leads to the following grouping.

(*a*) Substances soluble enough in the unionized form to remain in solution during the whole titration when the titration is performed on solutions with a concentration approximating that of the pharmaceutical solutions and in the range of pharmacological activity. This category includes procaine and lignocaine at 0.01 *M* concentration and Rec 7-0591 with regard to their properties during the first deionization process. The theory of Eq. 5 applies and the pH *versus* log $[B]/[BH^+]$ is a straight-lined relationship (*cf.* Fig. 3 of 0.01 *M* procaine). The pK_B' values can be determined directly.

(*b*) Substances with a more limited solubility of the unionized base. The theory of Eq. 5 applies until full saturation of the solution with the unionized base. Then the unionized form starts to precipitate and the theory described by Eq. 6 applies. This group includes 0.1 *M* procaine (Figs. 4–6), 0.1 *M* lignocaine, and 0.01 *M* and 0.1 *M* cocaine.

Maximum solubility of the unionized base and pK_B' values must be determined indirectly but still may be estimated with good approximation. Phenomena of hypersaturation are often present and can be demonstrated by plotting the data on an abscissa with a LR or L scale of Fig. 1. By the linearization so obtained it becomes possible to determine the actual concentration of soluble B during the remaining part of titration, when the excess of the unionized form precipitates.

(*c*) Substances with a low solubility of the unionized base. This group includes Rec 7-0518, Rec 7-0544, and Rec 7-0591, the last with regard to its properties during the second deionization process,

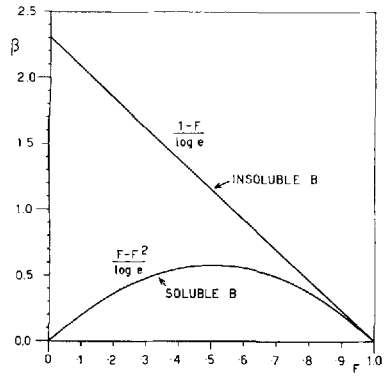

Fig. 12.—Buffering capacity β *versus* titrated fraction F of bases with insoluble unionized form or with soluble unionized form. The curves were calculated from the differential $\beta = dM/d\text{pH}$ and substituting for pH the right-hand member of Eq. 5 or, respectively, of Eq. 6. At a given concentration, the buffering capacity of bases with insoluble unionized form is much higher than that of bases with soluble unionized form. Furthermore, the maximum buffering capacity for the first type of bases is at the initial part of titration, whereas it is at the medial part of titration for the second type of bases.

Solubility of the unionized base and pK_B' values may be determined only with rough approximation.

DISCUSSION

Drop of pH Concomitant with Precipitation.—This behavior is frequently seen with substances whose unionized base is sparingly soluble, *e.g.*, 0.1 *M* procaine in Figs. 4–6. It is related to phenomena of hypersaturation of the unionized base, so that an excess of base provokes the precipitation of a part of [B] in Eq. 5, and the pH drops according to the new equilibrium described by Eq. 6. The maximum solubility of the unionized base, therefore, cannot always be deduced from the titrated fraction at the moment of precipitation, but must be calculated from the curve found after precipitation, extrapolating it in the direction of the first tract of the deionization curve, as exemplified in Fig. 7. This abrupt change of pH is never seen during the back-titration of the unionized base with a strong acid. In the back-titration the ionization curves follow the pattern of the dotted line of Fig. 7 and the solution becomes clear at the point in which the dotted line meets the first part of the ionization curve, which is then followed. This holds good for procaine and for all weak bases generally, which show the abrupt drop of pH during during deionization.

Buffering Capacity.—The tissue-tolerance for solutions with a pH different from that of the tissues depends partly on the buffering capacity of the solution, since possible damage to the tissues is related to the quantity of basic or acid radicals needed for re-equilibrating the pH. The buffering capacity is given by

$$\beta = \frac{d\,M}{d\,\text{pH}} \qquad \text{(Eq. 8)}$$

where M is the quantity of base or acid which provokes a change in pH.

For a base soluble in its unionized form, the buffering capacity β *versus* the titrated fraction F is given by the lower curve of Fig. 12 and is equal to:

$$\beta = \frac{F - F^2}{\log e} \qquad \text{(Eq. 9)}$$

The maximum value of β is at 0.5 titration, *i.e.*, when the pH = pK_B'.

A base which is insoluble in its unionized form has a much higher buffering capacity, equal to

$$\beta = \frac{1 - F}{\log e} \qquad \text{(Eq. 10)}$$

as shown by the higher curve of Fig. 12. The maximum value of β in this case is at zero titration. At a given concentration, a buffering system formed by a base with limited solubility of the unionized form is, therefore, more damaging for the tissues than a system formed by a base with a good solubility of the unionized form, when the pH differs from that of the tissues by the same degree. This explains the observation of Wehr and Koelzer (11) that low precipitation points on the pH or on the titration scale are correlated with low tolerance.

Effect of Temperature.—Conventionally pK_B' values are determined at 20° or at other temperatures close to room temperature.

As shown by Table I the pK_B' at 37° is usually lower, by a value up to 0.6, than the pK_B' at 20°. This phenomenon is related to the influence of temperature on the ionization constant of water which is 14.167 at 20.0° and 13.620 at 37°, with a drop of more than 0.5 unit.

The investigated bases have, therefore, a stronger proton-accepting property at 37° than at 20° and measurements taken at 20° or 25° can be misleading with regard to any inference on the tolerance of solutions for tissues at 37°.

It is interesting to note that for bases with a limited solubility of the unionized form an increase of the solubility of this form has an effect comparable to an increase of pK_B', *i.e.*, a shift toward a higher pH region of the whole deionization curve. The increase of temperature has, therefore, two opposite effects on the position of the deionization curve: due to the increase of the solubility of the unionized base, the deionization curve is shifted toward the top (*cf.* Eq. 6) and due to the decrease of the pK_B', the curve is shifted toward the bottom. The second effect seems usually to prevail over the first.

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Interaction of Di- and Tricarboxylic Acids with Glutaric Anhydride in Aqueous Solution

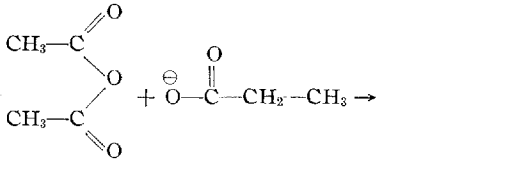
By JOSEPH R. ROBINSON, ARNOLD J. REPTA, and TAKERU HIGUCHI

Anionic forms of polycarboxylic acids such as succinic, citric, etc., interact reversibly and rapidly with glutaric anhydride in aqueous solution at room temperature to produce species which undergo subsequent hydrolysis. The species formed *in situ* from citrate was reacted with aniline and the resulting products isolated by column chromatography. Results of the chromatographic study suggested a mechanism based on the initial formation of a mixed anhydride which cleaved to produce an anhydride of the attacking anionic species. Spectrophotometric investigations have shown that the rate of formation and subsequent hydrolysis of the anhydride are dependent upon the pH and the buffer concentration. These reactive species are presumed to be formed to a varying extent in any formulation containing citrate, particularly under autoclaving conditions.

MANY BIOCHEMICAL systems as well as pharmaceutical formulations contain polycarboxylic acids. Citric acid, tartaric acid, malic acid, aconitic acid, and succinic acid are examples of food acids which occur naturally and are also often used in pharmaceutical preparations. In an earlier report (1) it was shown that these acids exist in solution in equilibrium with their corresponding cyclic acid anhydride forms which are capable of reacting with any nucleophilic species present. The present communication is concerned with results of studies designed to determine the rate and mechanism of transference of this anhydride character in a mixture containing two of these polycarboxylic acid species. Specifically, the interaction of citrate species with glutaric anhydride was investigated.

In aqueous solution, since water is a weak but an effective nucleophile, acid anhydrides undergo relatively rapid hydrolysis with half lives of the order of minutes. In the presence of other more potent electron donors, which are often constituents of frequently used pharmaceutical buffers, other reactions may take place preferentially. Thus, it has been suggested that acetic anhydride reacts with propionate ions with subsequent formation of a mixed anhydride (2).

Scheme I as written would be expected to be largely irreversible in the presence of a large excess of the attacking carboxylate species. If an analogous reaction takes place between a cyclic anhydride and a polycarboxylic acid species, on the other hand, an equilibrium system such as



Scheme I

that shown in Scheme II may be expected to take place (3).[1]

Formation of citric anhydride probably results from intramolecular attack from the neighboring carboxyl group of the citrate moiety on a carbonyl carbon in the mixed anhydride. The mixed anhydride may also revert back to glutaric anhydride by the same mechanism and is probably present only in relatively low concentration.

The present investigation was designed to determine whether the postulated reactions would occur and to establish the individual rates and products of the system. This was done by observing spectrometric changes in the reacting systems and by chromatographic analysis and identification of the end products.

RESULTS AND OBSERVATIONS

Spectrophotometric Studies.—Glutaric anhydride has a characteristic ultraviolet absorbance spectrum that can be utilized to follow its hydrolysis in aqueous solution. When citric acid is employed as a buffer at a pH where substantial quantities of the di-ionized species are present, a change in ultraviolet absorbance is observed. This interaction can be conveniently followed since the reactants, intermediate species, and the final products apparently

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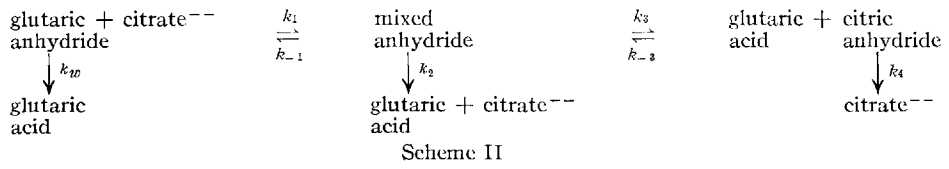
[1] Higuchi *et al.* (3) have also shown that acyl exchange of this type takes place with great facility. Their paper should be consulted for specific details.



Scheme II

possess sufficiently different ultraviolet molar absorptivities to permit observation of the various reactions. A typical absorbance change at 248 mμ with time for a system containing initially 7.05 × 10^{-3} moles of glutaric anhydride in 0.3 *M* citrate buffer at pH = 5.0 is shown in Fig. 1. There is a small increase in absorbance initially, followed by a logarithmic approach to an equilibrium value. This corresponds presumably to a G + B ⇌ C → D type relationship where species C possesses a slightly higher molar absorbance than the reactants or the final product. The experimental observation noted above is in agreement, at least in form, to the reactions implicit in Scheme II.

It is apparent from the proposed reaction scheme that there are two possible species which could correspond to species C, the mixed anhydride and citric anhydride. Similarly, the deterioration of the species could be attributable to decomposition of the mixed anhydride or hydrolysis of citric anhydride.

If one assumes $k_3 \gg k_1$, k_{-1}, k_2, k_2, and k_w, and that k_{-3} is negligible,[2] the concentration of species such as citric anhydride can be expressed as:

$$[CA] = \frac{k_1[B]\ [GA]_0}{k_1[B] + k_w - k_4} \times [e^{-k_4 t} - e^{-(k_1[B] + k_w)t}] \quad \text{(Eq. 1)}$$

where [CA] = citric anhydride, [GA] = glutaric anhydride, and [B] = citrate buffer. If the absorbance of a reacting system initially containing glutaric anhydride and citrate is followed at 248 mμ, a plot of log $[A_T - A_\infty]$ against time (where A_T is the absorbance at any time, t, and A_∞ is the limiting absorbance) will yield a straight line in the terminal phase, provided $(k_1[B] + k_w) \gg k_4$. This is because under such a circumstance $(A_T - A_\infty)$ will be expected to be essentially proportional to the concentration of citric anhydride when the total anhydride concentration becomes very small. The slope of the logarithmic plot would then be equal to $-k_4$. Extrapolation of this line will give an imaginary $(A_0' - A_\infty)$ value (which is proportional to [CA]) at time zero. A semilogarithmic plot of $[(A_T' - A_\infty) - (A_T - A_\infty)]$ against time will yield a linear relationship with a slope equal to $-(k_1[B] + k_w)$.

The rates of appearance {corresponding to $(k_1[B] + k_w)$} of the strongly absorbing intermediate species (citric anhydride?) were determined from plots of $(A_T' - A_T)$ for systems at several pH values and different buffer concentrations. Results are shown in Fig. 2. The data suggest that the initial reaction between the anhydride and citrate probably involves the di- and/or tri-ionized form of citric acid.

The apparent rates of loss of the active species (k_4) determined as outlined above are shown in Fig. 3 for several values of pH and various buffer concentrations. The pH range studied was limited to that where citrate behaved effectively as a buffer. Based on the proposed mechanism of relatively rapid formation of citric anhydride followed by a slower hydrolysis, the apparent pH dependency could be ascribed to a slower rate of formation of citric anhydride at lower pH values and buffer concentrations. The relationship presented above permitting separation of the individual rate constants requires that $(k_1[B] + k_w)$ be at least 4 to 5 times larger than k_4. Since in the lower pH range this minimum ratio in the reaction velocities is not apparently obtained, the limiting slopes do not reflect the true magnitude of k_4 in this range. The terminal slopes above pH = 4.5 are considered to reflect the true values of k_4 in that at these pH values, the rate of formation is considerably faster than the subsequent hydrolysis. In Fig. 3, above pH = 4.5, the lines connecting the experimental points extrapolate back to a common value of 11.5 × 10^{-3} sec.$^{-1}$. This is assumed to be the rate constant for hydrolysis of citric anhydride in water.

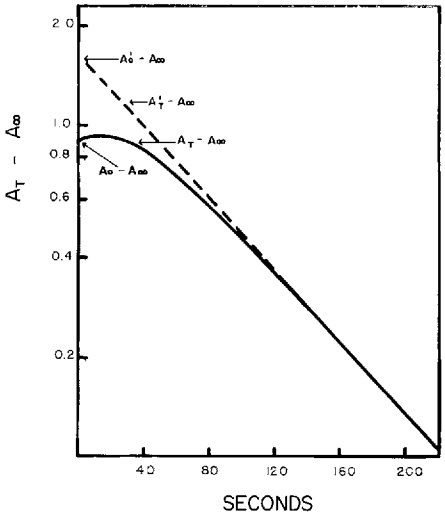


Fig. 1.—Semilogarithmic plot of absorbance change at 248 mμ for the system glutaric anhydride in citrate buffer. pH = 5.0, 0.3 *M* citrate; T = 25°.

The observed decrease in absorbance may also be attributed to slow disappearance of the mixed anhydride (to form citric anhydride) followed by rapid hydrolysis of citric anhydride. A mechanism such as this would require that the glutaric–citric mixed anhydride would be a relatively stable species which would not be expected based on the structure. Direct hydrolysis of the mixed anhydride is also a possibility, but again a stable mixed anhydride could be required. Further work is

[2] Pseudo first-order conditions were maintained and there was always at least a sixtyfold excess of citrate buffer.

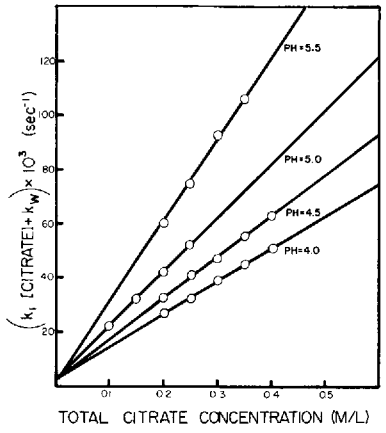


Fig. 2.—Rate of formation of the species formed from the glutaric–citric system at various pH values and different buffer concentrations.

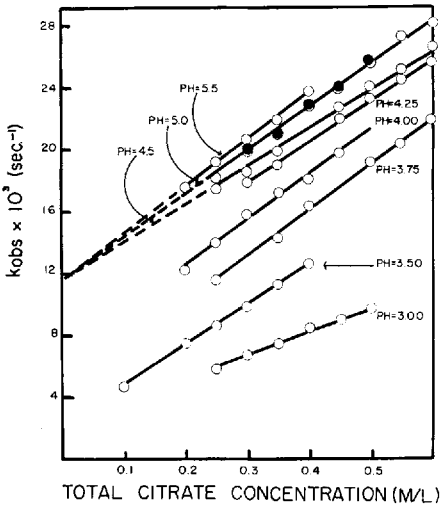


Fig. 3.—Observed rate of hydrolysis of the species formed from the interaction of glutaric anhydride in citrate buffer at various pH values and different buffer concentration. Key: ●, 1 *M* KCl; ○, H_2O.

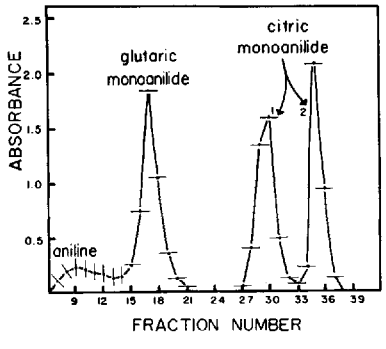


Fig. 4.—Typical chromatogram for the anilides formed by addition of aniline to the reaction mixture. The reaction was quenched with aniline after 30 sec. The eluate was extracted with 0.1 *N* NaOH and the alkaline solution analyzed spectrophotometrically at 241 mμ.

required to definitely establish which mechanism is correct, but intuitively the scheme involving rapid formation of citric anhydride followed by a relatively slow hydrolysis would seem the more likely.

Chromatographic Studies.—Although results of rate studies were strongly indicative of formation of citric anhydride species from mixtures of glutaric anhydride and citrate buffers, the data were not considered definite by themselves. Attempts were made to substantiate these findings by direct chemical examination of the reacting systems. The procedure adopted was isolation and quantification of the reaction products resulting from addition of aniline to the anhydride mixture, all anhydride species present presumably reacting extremely rapidly with the strong nucleophile aniline.

Separation of reaction products following addition of aniline to the reacting systems are evident in the chromatogram shown in Fig. 4. The particular run contained initially 0.3 *M* pH = 5 citrate buffer and 3.95×10^{-5} moles of glutaric anhydride with a trace of dioxane. The reaction was quenched with aniline after 30 sec. and separated by column partition chromatography and analyzed by ultraviolet spectrophotometry as described earlier (4). The double peak ascribed to citric monoanilide corresponds to the isomeric forms as previously suggested (4). Elemental analysis, equivalent weight, ultraviolet spectrum, and infrared spectrum of the compounds corresponding to peaks 1 and 2 were determined and found to be compatible with that of citric monoanilide.

Supportive evidence to corroborate the postulate that the two peaks correspond to the isomeric forms of citric monoanilide was obtained by hydrolyzing citranilic acid[3] and chromatographing the resultant products. Random hydrolysis of this citric acid imide will produce the two isomeric monoanilides of citric acid. Chromatographic separation of this mixture in the same manner as that employed for the anilides formed in the glutaric anhydride–citric acid mixture gave two peaks. The retention volume required for these two peaks was the same as that required for the two peaks in the glutaric–citric case.

It is conceivable that citric monoanilide could have formed through a lactone intermediate. However, previous work with the lactones of tartaric acid (5) has shown them to be unreactive toward aromatic amines under the conditions employed here, and therefore, this was ruled out as a possible pathway.

Citric monoanilide theoretically could have been formed through a mixed anhydride as well as citric anhydride. Previous work has shown (6) that addition of aniline to a mixed anhydride usually gave anilides of both compounds. Depending upon the nature of the mixed anhydride, the yield of one of the anilides may be favored over the other. When aniline was added to the reaction mixture where the pH of citrate buffer was 2.0, citric monoanilide could not be isolated.[4]

[3] Citranilic acid was prepared according to Higuchi *et al.* (4), m.p. 187–188° (literature values vary from 185–189°). Molecular weight determined by direct titration with sodium hydroxide was found to be 253 (calculated 249).

[4] At pH = 2.0, the fraction of aniline in the protonated form is quite high and therefore a large excess was employed. Isolation of glutaric monoanilide from the reaction mixture indicated that sufficient unprotonated aniline was present to react with anhydrides in the system.

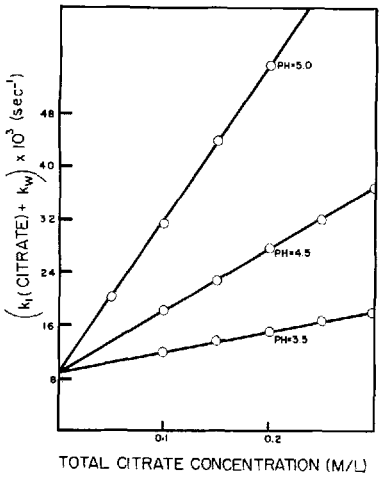


Fig. 5.—Disappearance of phthalic anhydride at 302 mμ as a function of pH and buffer concentration.

At a pH = 2.0, using 3.08 and 4.74 as the respective first and second ionization constants of citric acid (7), the monoionized and free acid would be present almost exclusively with very little of the di-ionized species present. If the monoionized citrate species can attack glutaric anhydride, and since citric monoanilide was not isolated, it is reasonable to assume that either the mixed anhydride is present in a very low concentration or that glutaric monoanilide is exclusively favored in this system.

An additional system that was tried was glutaric anhydride in a pH = 5.5 acetate buffer. Addition of aniline to this reaction mixture did not produce any acetanilide. This would indicate that this system, like the previous one, hydrolyzes *via* an unstable mixed anhydride or formation of glutaric monoanilide is exclusively favored. An alternative possibility is that acetate ion serves as a general base in this system.

To demonstrate the generality of the reaction, phthalic anhydride was used in place of glutaric anhydride. Phthalic anhydride hydrolysis was followed at 315 mμ in citrate buffer. Unlike the glutaric case, there was no apparent anomaly observed in the ultraviolet spectrum, indicating that if a new species was being formed in solution, it had less absorbance at this wavelength than the reactants. The rate of hydrolysis of phthalic anhydride as a function of pH and buffer concentration is shown in Fig. 5. Apparently this system, like glutaric anhydride, is sensitive to the di- and/or tri-ionized species of citric acid. Addition of aniline to the reaction mixture in a manner the same as before gave substantial quantities of citric monoanilide. This indicates that phthalic anhydride like glutaric anhydride is capable of reacting with citrate to form a species in solution which has anhydride properties.

DISCUSSION

Polycarboxylic acids are often used in combination as buffer components; in light of the findings of this study an exchange of anhydride character also presents a possible pathway for drug loss from solution. Equally important is the possibility that a myriad of products could result.

EXPERIMENTAL

Equipment and Reagents.—A Cary model 11 M.S. recording spectrophotometer was utilized for the spectrophotometric determinations. All pH measurements were made with a Beckman Zeromatic pH meter with an expanded scale.

Aniline was purified by repeated distillation and was stored under nitrogen prior to use. Dioxane was purified according to Vogel (8). Glutaric anhydride was recrystallized from ether until a m.p. of 56–57° was obtained. All other chemicals were of analytical or reagent grade.

Procedure for Kinetic Runs on Citric Acid–Glutaric Anhydride Reactions.—Citrate buffers of appropriate concentration and pH were prepared. One-hundred microliters of a 0.450 *M* glutaric anhydride in dioxane solution was mixed with 6 ml. of citrate buffer in a 2-cm. photometer cell. The reaction was followed spectrophotometrically at 248 mμ. All reaction solutions were equilibrated at 25 ± 0.1° prior to use. The concentration of dioxane used in all cases was determined to have a negligible effect on the rate constants.

Procedure for Kinetic Runs on Citric Acid–Phthalic Anhydride Reactions.—Citrate buffers of appropriate concentration and pH were prepared. Fifty microliters of a 0.0338 *M* phthalic anhydride in dioxane solution was mixed with 6 ml. of citrate buffer in a 2-cm. photometer cell. The reaction was followed spectrophotometrically at 302 mμ. All reaction solutions were equilibrated at 25 ± 0.1° prior to use.

Chromatographic Separation of the Reaction Mixture.—The chromatographic columns were prepared as outlined in a previous communication (4). The reaction mixture was prepared by adding 15 ml. of citrate buffer to 100 μl. of a 1.58 *M* glutaric anhydride in dioxane solution. The reaction was allowed to continue the requisite period of time and was quenched with 4 ml. of a 0.33 *M* aqueous aniline solution. The pH was adjusted to 3.13 with HCl and a 5-ml. sample was placed on the column with 5 Gm. of silicic acid. The eluting solutions consisted of 100 ml. each of chloroform, 1.5% butanol in chloroform, 10% butanol in chloroform, and 30% butanol in chloroform; each solution was saturated with the internal phase prior to use. The eluate was collected in 10-ml. fractions; the collecting vessel was rinsed with 10 ml. of chloroform and added to the 10-ml. fraction. Each sample was extracted with 20 ml. of 0.1 *N* NaOH.

Preparative Chromatography of the Reaction Mixture.—A chromatographic column with the following dimensions was employed: length = 60 cm., i.d. = 3.40 cm. Three-hundred grams of silicic acid was utilized as the support and 300 ml. of pH 3.13 phosphate buffer was employed as the internal phase. The eluting solutions consisted of 500 ml. each of chloroform, 1.5% butanol in chloroform, 5% butanol in chloroform, 15% butanol in chloroform, and 35% butanol in chloroform; each solution was saturated with the internal phase prior to use. The reaction mixture was prepared by mixing 100 ml. of 0.3 *M* pH = 5 citrate buffer

with 3.2 ml. of 1.58 *M* glutaric anhydride in dioxane solution. The reaction mixture was quenched at the end of 30 sec. with 16 ml. of a 0.33 *M* aqueous aniline solution. The pH of the resulting solution was adjusted to pH 3.13 with HCl and 30 ml. of this solution was placed on the column with 30 Gm. of silicic acid. The eluate was collected in 50-ml. fractions. The solvent was removed from each sample under reduced pressure and a mixture of chloroform–petroleum ether added to precipitate the compounds.

Characterization of Reaction Compounds.—*Glutaric Monoanilide.*—This was recrystallized from methanol–water, m.p. 129–130°. Molecular weight found by direct titration against standard NaOH was 205 (calculated 207). Known glutaric monoanilide was prepared by adding glutaric anhydride dissolved in dioxane to an aqueous solution of aniline. The sparingly soluble anilide precipitated and when recrystallized gave a m.p. 129–130°. Both known and unknown compounds gave identical infrared and ultraviolet absorption spectrums.

Citric Monoanilide.—1.—This was recrystallized from chloroform, m.p. 136–137°.

Anal.—Calcd. for C, 53.94; H, 4.87; N, 5.23. Found: C, 53.90; H, 4.94; N, 5.3.

Molecular weight determined by direct titration against NaOH was 269 (calculated 268). Infrared and ultraviolet spectra were characteristic of an anilide. NMR spectrum strongly suggested this to be the symmetrical isomer, 2-hydroxy-2-*N*-phenylcarbamide-1,3-propanedicarboxylic acid.

Citric Monoanilide—2.—This was recrystallized from chloroform, m.p. 127–128°. Molecular weight determined by direct titration against sodium hydroxide solution was 270 (calculated 268).

Anal.—Calcd. for C, 53.94; H, 4.87; N, 5.23. Found: C, 53.87; H, 4.85; N, 5.20.

Infrared and ultraviolet absorption spectra were characteristic of an anilide. NMR spectrum suggested this to be the unsymmetrical isomer, 2-hydroxy - 1 - *N* - phenylcarbamide - 2,3 - propanedicarboxylic acid.

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Interaction of Acetic Anhydride with Di- and Tricarboxylic Acids in Aqueous Solution

By ARNOLD J. REPTA, JOSEPH R. ROBINSON, and TAKERU HIGUCHI

Earlier studies have shown that cyclic anhydrides, such as succinic, glutaric, etc., interact with citrate ions in aqueous solution to form what appears to be a citric anhydride species. These interactions have been assumed to be highly reversible through intermediate formation of a mixed anhydride. The present studies were concerned with a comparable system which was expected to be essentially irreversible. Spectrophotometric investigations have shown that acetic anhydride apparently reacts with citrate in aqueous solution to form a new species which undergoes rapid hydrolysis. The rate of the initial reaction and the rate of the subsequent step appear to depend on the citrate concentration and pH. Results of chromatographic studies on products obtained by reaction with aniline at different phases of the reaction are presented.

IN AN EARLIER report (1), experimental evidence was presented which suggested that glutaric and presumably other cyclic anhydrides formed an equilibrium system in the presence of a large excess of citrate buffer in which the total anhydride concentration was distributed among glutaric anhydride, citric anhydride, and perhaps glutaric–citric anhydride. An attempt has been made in the present study to obtain a clearer picture of the reacting system by employing acetic anhydride to furnish the initial anhydride concentration; the noncyclic anhydride not being expected to participate effectively in any reversible process.

If the forward reaction pathway remains essen-

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If the forward reaction pathway remains essen-

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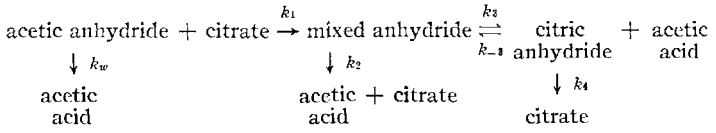

Scheme I

tially the same as in the cyclic anhydride case, a mixed acid anhydride species would be formed by interaction of acetic anhydride and citrate ion. The loss of the acetate in this step would, however, make formation of the mixed anhydride irreversible. The net effect of this would be to drive the reaction to the right. The proposed reaction is shown in Scheme I.[1]

In the present investigation the differences in the characteristics of this irreversible system from those of the previously studied reversible system have been explored.

RESULTS AND OBSERVATIONS

Spectrophotometric Studies.—Although acetic anhydride hydrolyzes rather rapidly in cold water ($t_{1/2} \cong 5$ min. at 25°), it appears to be capable of reacting with nucleophilic species such as citrate anions. When the hydrolysis is followed spectrophotometrically at 248 mμ in citrate buffer, at pH values where there is an appreciable quantity of di-ionized citrate, a species appears to be formed which has an ultraviolet absorptivity greater than the original anhydride. The absorbance–time profile observed for a system initially containing 1.08×10^{-3} M acetic anhydride in 0.3 M citrate buffer, pH = 5 and 25°, is shown in Fig. 1. The shape of the plot suggests that the reaction involves relatively rapid formation and subsequent hydrolysis of some intermediate species.

As shown previously (1), the concentration of citric anhydride can be expressed[2] as:

$$[CA] = \frac{k_1[B][AA]_0}{(k_1[B] + k_w - k_4)} [e^{-k_4 t} - e^{-(k_1[B] + k_w)t}]$$

If $(k_1[B] + k_w) \gg k_4$, a semilogarithmic plot of $(A_t - A_\infty)$ against time (where A_t is the absorbance at any time t and A_∞ is the limiting absorbance) will have a terminal slope equal to $-k_4$. Extrapolation of this terminal line to zero time will give a value $(A_0' - A_\infty)$ which corresponds to the imaginary absorbance of the new species at time zero. A semilogarithmic plot of $[(A_t' - A_\infty) - (A_t - A_\infty)]$, at various times, against time will yield a linear relationship with a slope equal to $-(k_1[B] + k_w)$.

The rates of appearance $(k_1[B] + k_w)$ of the more strongly absorbing species in the system are influenced by both hydrogen-ion concentration and buffer concentration as shown in Fig. 2. As is evident from the plots, the rate of formation appears to

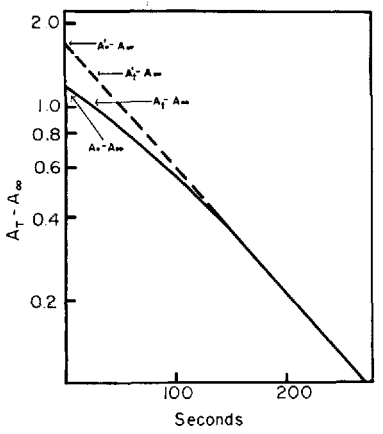

Fig. 1.—Semilogarithmic plot of absorbance change at 248 mμ for the system acetic anhydride in citrate buffer. pH = 5.0; T = 25°.

depend upon either the di- and/or the tri-ionized form of citric acid. It should be noted here that the apparent transfer of anhydride character was observed only at a pH = 3 or greater where the concentration of di-ionized citrate was appreciable.

The observed rates of loss (k_4) of the new species, found from the terminal slopes as described above are shown in Fig. 3 for various pH values and different buffer concentrations. From the proposed reaction Scheme I, if $k_4 \gg k_3$, one would be essentially observing the rate of loss of the mixed anhydride. Similarly, if $k_3 \gg k_4$, the observed hydrolysis would be that of citric anhydride. It is felt that the latter situation probably exists and that acetic anhydride reacts with the citrate buffer to produce citric anhydride which subsequently undergoes hydrolysis.

The apparent dependency upon hydrogen-ion concentration observed in Fig. 3 can be explained on the basis that if the rate of formation of citric anhydride is slow at low pH values and low buffer concentration, extrapolations of the terminal slope does not reflect the true hydrolytic rates for the disappearance of the species. It is suggested, therefore, that only above pH = 6 is the terminal slope representative of the true hydrolytic rate of citric anhydride.

The pH dependency is similar to that observed in the glutaric anhydride–citric acid case (1). The extrapolations of the higher pH values to zero buffer concentration would be expected to yield the uncatalyzed hydrolytic rate for citric anhydride. Since in both systems citric anhydride is apparently formed, the extrapolated rate value should be the same. The value of 11.5×10^{-3} sec.$^{-1}$ obtained in

[1] The reaction steps k_w, k_2, and k_4 are considered irreversible, since under the conditions employed here (25°, aqueous solution) the reverse reaction would be negligible (2).

[2] The assumptions involved in this derivation are the same as previously reported (1). The reaction step, k_3, is considered irreversible since at least a sixtyfold excess of buffer was employed at all times.

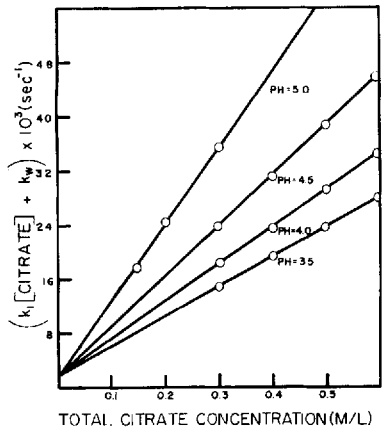

Fig. 2.—Rate of formation of the species formed from the acetic anhydride–citrate system at various pH values and different buffer concentrations.

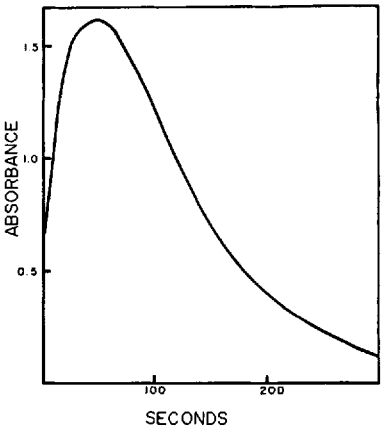

Fig. 4.—Absorbance-time profile for acetic anhydride, initial concentration 4.5×10^{-3} *M*, in 0.5 *M* phthalate buffer. The absorbance change was followed at 315 mμ. pH = 5.5; T = 25°.

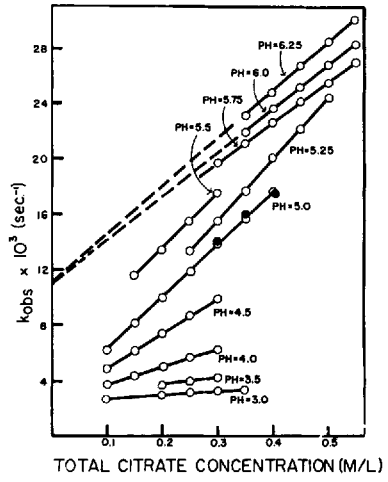

Fig. 3.—Observed rate of hydrolysis of the species formed from the interaction of acetic anhydride in citrate buffer at various pH values and different buffer concentrations. Key: ●, 1 *M* KCl; ○, H_2O.

this investigation is in good agreement with that reported in the earlier work (1).

Apparently the pH values, necessary to obtain a limiting maximum rate constant upon extrapolation to zero buffer at a given pH, are higher (pH = 6.0) for the present case than those found for the glutaric–citrate case (pH = 4.5). This dependence may be rationalized on the basis of steric effects. Since both glutaric and acetic anhydrides have approximately the same rate of hydrolysis in water, one cannot ascribe the observed phenomenon singly to the different sensitivities of the two species to nucleophilic attack.

As previously mentioned (1), the data presented can also be rationalized by an alternate pathway. This essentially requires that the rate-determining step corresponds to the decomposition of the mixed anhydride. A mechanism such as this requires that acetic–citric anhydride be a relatively stable species which seems unlikely. It should be pointed out that direct hydrolysis of the mixed anhydride is also possible, but the slow rate of hydrolysis observed would again require that the mixed anhydride be a relatively stable species.

The catalytic effect of citrate buffer on the hydrolytic rate of the assumed citric anhydride has been observed with other anhydrides. An example of this is the catalytic hydrolysis of acetic anhydride by acetate ions. The most acceptable theory for this catalysis is a general base effect, since nucleophilic attack would result in an identical anhydride (3). A general base effect might be a satisfactory explanation in the citrate case, but it should be pointed out that attack of citrate ions on citric anhydride would produce an intermediate citric–citric anhydride which could undergo hydrolysis at a faster rate than citric anhydride.

Supportive evidence for the formation of citric anhydride rather than the mixed anhydride was obtained by using phthalate as the buffer rather than citrate. Addition of acetic anhydride to phthalate buffer produces a species which can be followed at a wavelength where there is no interfering absorbance from the acetic anhydride. As shown in Fig. 4, a species forms and subsequently undergoes hydrolysis. Applying the same kinetic treatment as was described for the citric case allows for separation of the individual rate constants. Figure 5 shows the rate of disappearance of this species as a function of phthalate concentration and pH. The proposed mechanism for this interaction is relatively rapid formation of phthalic anhydride, followed by a somewhat slower hydrolysis. The apparent pH dependency observed here is explained on the same basis as the acetic anhydride–citrate case, *i.e.*, at low pH values and low buffer concentrations the rate of formation is slow, and, therefore, extrapolation of the terminal slope does not reflect the true hydrolytic rate. Above pH = 5.5 the limiting intercepts at zero buffer concentration value giving

an uncatalyzed velocity constant of 9.3×10^{-3} sec.$^{-1}$ which is in good agreement with reported work (4).

Because of the sparingly soluble nature of phthalic anhydride, it was possible to isolate it from the reaction mixture. Addition of excess acetic anhydride to the phthalate buffer gave a copious precipitate which redissolved upon standing. Isolation and characterization of this precipitate showed it to be phthalic anhydride, lending support to the hypothesis that the mixed anhydride has only a transitory existence in systems containing acetic anhydride and various carboxylic buffers.

Chromatographic Studies.—The presence of a citrate species possessing anhydride properties can be demonstrated by addition of aniline to the reaction mixture as has been previously noted (5). The anilides that are produced can be separated by chromatography as shown in Fig. 6. The typical chromatography run demonstrated in Fig. 6 was carried out using a 0.3 M, pH = 5 citrate buffer, and 8.0×10^{-5} moles of acetic anhydride; the reaction was quenched with aniline after 40 sec. The double peak corresponding to the two isomers of citric monoanilide is again in good agreement with earlier work (5). Verification that the peaks correspond to the two isomeric forms of citric monoanilide was carried out as previously described (1).

Determination of the Citric Anhydride-Time Profile by the Aniline Method.—The citric anhydride concentration in a mixture containing initially only acetic anhydride and citrate buffer can be estimated by quenching the reaction system with aniline and determining the concentration of the total citric monoanilide products. The anilides formed from samples drawn at different times during the course of the reaction were separated by column partition chromatography and the total yield of citric monoanilide determined. The results are shown in Fig. 7. The indicated concentration of citric anhydride as reflected by the total yield of citric monoanilide apparently increased initially with time. The data suggest that the hydrolysis of citric anhydride resulted in a decrease in the amount of the anilides formed in the terminal phase of the reaction. The yield of citric anilide was calculated based on a molar absorptivity for the citric monoanilide of 11,000.

It is apparent in this system that the anilide could have been formed from the mixed anhydride or from citric anhydride, although the spectrophotometric evidence presented supports citric anhydride rather than a mixed anhydride.

DISCUSSION

These studies indicate that, although the irreversible interactions of acetic anhydride with citrate

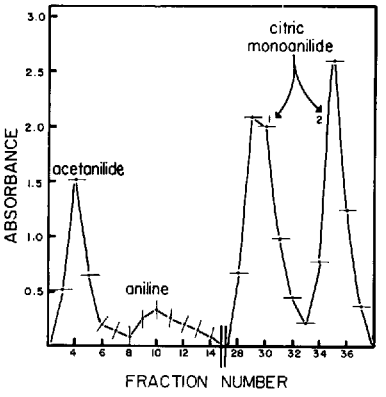

Fig. 6.—Typical chromatogram for the anilides formed by addition of aniline to the acetic anhydride–citrate system. The reaction was quenched with aniline after 40 sec. The eluate was extracted with 0.1 N NaOH and the alkaline solution analyzed spectrophotometrically at 241 mμ.

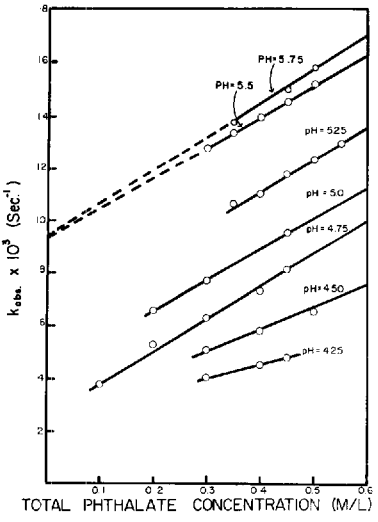

Fig. 5.—Observed rate of hydrolysis of the species formed from the interaction of acetic anhydride in phthalate buffer at various pH values and different buffer concentrations.

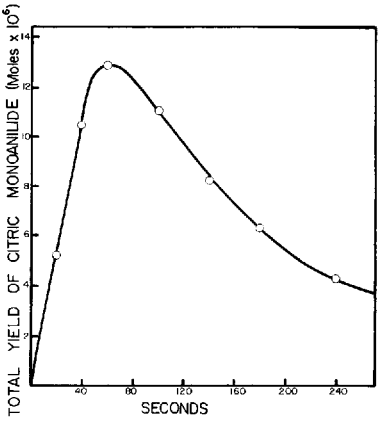

Fig. 7.—Total yield of citric monoanilide for the acetic anhydride–citrate system. The various points were obtained by withdrawing samples from the reaction mixture at various times and quenching the reaction with aniline. The total citric monoanilide present was then determined. pH = 5, 0.5 M.

differ markedly in character from the reversible reaction observed for the glutaric anhydride–citrate system, the apparent kinetic behaviors are surprisingly similar. The primary observation, aside from the irreversibility of the system, seems to be that the acetic anhydride reaction with citrate proceeds at a substantially slower second-order rate than that observed for the cyclic anhydride.

The experimental observations again strongly suggest intermediate formation of a reactive citric anhydride species. Although the compound was not isolated in these studies, the data suggest that acetic anhydride can be employed to produce substantial concentrations of citric anhydride which, in turn, can be conveniently employed for synthesis of citric acid derivatives in aqueous solutions.

EXPERIMENTAL

Reagents and Equipment.—Commercial acetic anhydride was purified by distillation. Dioxane was purified according to Vogel (6). Aniline was purified by repeated distillation and was stored under nitrogen prior to use. All other chemicals were of analytical or reagent grade.

All pH measurements and adjustments were made with a Beckman Zeromatic pH meter with an expanded scale.

Kinetic Procedure for the Reaction of Acetic Anhydride with Various Buffers.—Fifty microliters of a 1.08 *M* solution of acetic anhydride in dioxane was introduced together with 6 ml. of the buffer into a 2-cm. photometer cell. The reaction was allowed to proceed and was followed directly on a Cary model 11- M.S. recording spectrophotometer. All solutions were equilibrated at 25 ± 0.1° prior to use.

The dioxane concentration employed in these runs was determined to have a negligible effect on the rate constants.

Chromatographic Separation of Reaction Mixture.—The chromatographic columns were prepared as outlined in a previous communication (5). The reaction mixture was prepared by adding 15 ml. of the appropriate citrate buffer to 300 μl. of a 1.08 *M* acetic anhydride in dioxane solution. The reaction was allowed to proceed the requisite period of time and was quenched with 4 ml. of a 0.33 *M* aqueous aniline solution. The pH was adjusted to 3.13 with HCl and a 5-ml. sample was placed on the column with 5 Gm. of silicic acid. The eluting solutions have previously been described (1).

Blank determinations were made following the same procedure as above using (*a*) acetic acid, citrate buffer, and aniline solution, and (*b*) acetic anhydride, water, and aniline solution.

Preparative Chromatography of the Reaction Mixture.—A chromatographic column was prepared as previously reported (1). The reaction mixture was prepared by adding 100 ml. of pH = 5.0, 0.3 *M* citrate buffer to 4 ml. of a 1.08 *M* acetic anhydride in dioxane solution. At the end of 60 sec. the reaction mixture was quenched with 27 ml. of a 0.33 *M* aqueous aniline solution. The reaction mixture was placed on the column in the previously outlined manner and the eluate was collected and treated as reported (1).

Characterization of Compounds.—*Acetanilide.*—This was recrystallized from methanol, m.p. 114–115°. Commercial acetanilide was recrystallized from methanol and gave a m.p. 114–115°. A mixture of the known and unknown sample gave no depression of the melting point. In addition, both known and unknown samples gave identical infrared and ultraviolet absorption spectra.

Citric Monoanilide.—1.—Anal.—Calcd. for C, 53.94; H, 4.87; N, 5.23. Found: C, 54.09; H, 4.92; N, 5.22.

Molecular weight determined by direct titration against NaOH was found to be 271. (Calcd. 268.) Infrared and ultraviolet absorption spectra were characteristic of an anilide. The NMR spectrum strongly suggested this to be the symmetrical isomer, 2-hydroxy-2-*N*-phenylcarbamide-1,3-propanedicarboxylic acid.

Citric Monoanilide.—2.—This was recrystallized from chloroform, m.p. 127–128°. Molecular weight determined by direct titration against sodium hydroxide solution was 270. (Calcd. 268.)

Anal.—Calcd. for C, 53.95; H, 4.87; N, 5.23. Found: C, 53.98; H, 4.92: N, 5.19.

Infrared and ultraviolet absorption spectra were characteristic of an anilide. NMR spectrum suggested this to be the unsymmetrical isomer, 2-hydroxy-1 - *N* - phenylcarbamide - 2,3 - propanedicarboxylic acid.

Phthalic Anhydride.—This was recrystallized from chloroform, m.p. 130–131°. Commercial phthalic anhydride was recrystallized from chloroform and a mixture of the known and unknown compounds gave no depression of the melting point. Both known and unknown samples gave identical ultraviolet absorption spectra.

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Quantitative Studies of Urinary Excretion of Chlorpromazine Metabolites in Chronically-Dosed Psychiatric Patients

By A. G. BOLT, I. S. FORREST, and M. T. SERRA

A method was developed for the routine assay of conjugated and unconjugated chlorpromazine metabolites in human urine. The method could be adapted to excretion studies of related drugs, *e.g.*, other phenothiazines and imipramine derivatives. The unconjugated drug metabolites were extracted from alkaline urine into dichloromethane and assayed spectroscopically in 50 per cent sulfuric acid at 530 mμ. The conjugated chlorpromazine metabolites were determined after passing a sample of urine through an ion-exchange resin to remove the unconjugated metabolites and contaminating endogenous urinary constituents. The eluate was made up to 50 per cent sulfuric acid content, and drug metabolites estimated spectroscopically at 550 mμ. The total urinary drug excretion of 15 chronic mental patients receiving chlorpromazine in doses of 100 to 1400 mg. per day, varied from 21.1 to 70 per cent of the daily dose. The conjugated drug metabolites formed the major fraction of urinary metabolites. The ratio of conjugated to unconjugated drug metabolites ranged from 2.1 to 11.

ESSENTIALLY two groups of chlorpromazine metabolites are observed in human urine (1–6)—namely, (*a*) the unconjugated fraction consisting of unchanged drug, desmonomethylchlorpromazine, desdimethylchlorpromazine, their sulfoxides, and chlorpromazine-*N*-oxide; (*b*) the conjugated fraction containing the greater number and amount of urinary chlorpromazine metabolites (6, 7). The metabolites in this fraction are the *O*-glucuronides (and small amounts of ethereal sulfates) of the mono- and dihydroxy derivatives of chlorpromazine, their demethylated derivatives, and of the corresponding sulfoxides (6, 8). Trace amounts of other unknown conjugates may also be present.

The unconjugated metabolites have been consistently reported in the literature and represent from 5 to 10% of the daily dose. There are, however, discrepancies in the estimates in the major drug fraction containing the conjugated metabolites which may be due to the different methods used (4, 9, 10). Other methods reported in the literature involve chemical or enzymatic hydrolytic procedures which are time consuming and give low recoveries. The authors have previously described a method (11), using a strong cationic resin, which was less satisfactory than the present method. When the earlier procedure was proposed, hydroxlyated or methoxylated derivatives of chlorpromazine were not available as reference compounds for conjugated metabolites; in fact, these metabolites were later shown to be incompletely estimated.

Since significant inter-patient (7) and interspecies (12, 13) differences in urinary chlorpromazine metabolites have been noted, especially with regard to the large group of conjugated drug metabolites (7), simple and rapid procedures are needed for the separate estimation of the two groups of drug metabolites.

EXPERIMENTAL

Materials.[1]—Dichloromethane, ACS; 97% sulfuric acid, ACS; 0.1 *N* sulfuric acid solution; Sørensen's phosphate buffer (pH 6); 2 *N* sodium hydroxide solution; 2 *N* hydrochloric acid solution; 30% hydrogen peroxide solution; ion-exchange resin[2] IRC-50, analytical grade, 20–50 mesh; chlorpromazine HCl; 7-hydroxychlorpromazine; 7-methoxychlorpromazine HCl; chlorpromazine-5-oxide HCl.

Apparatus.—U.V. spectra were obtained using a Beckman DB recording spectrophotometer. The Beckman Zeromatic pH meter was used for pH measurements.

Determination of Unconjugated Drug Metabolites.—A 10-ml. aliquot of urine was adjusted to pH 9–9.5 and extracted with 3 × 10 ml. of dichloromethane (14). The extract was evaporated on a water bath under a stream of nitrogen, the residue was dissolved in 5 ml. of 0.1 *N* sulfuric acid solution, and the acid solution transferred to a 10-ml. volumetric flask. The flask was immersed in an ice bath and 5 ml. of concentrated sulfuric acid was added very slowly down the wall of the flask. The sulfuric acid mixture was heated for 15 min. at 65°, brought to room temperature, 1 drop of 0.1% H_2O_2 was added, stood for 15 min., and adjusted to 10 ml. with cold 50% sulfuric acid solution. The

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[2] Marketed as Amberlite IRC-50 by Rohm & Haas, Philadelphia, Pa.

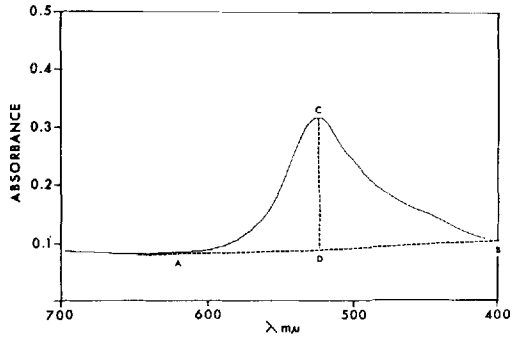


Fig. 1.—The spectrum of a typical dichloromethane urine extract after treatment with 50% sulfuric acid solution. Reading of absorbance at 530 mμ by the background cancellation method.

absorption spectrum of the acid solution was recorded between 400–700 mμ (see Fig. 1). Absorbance was measured at 530 mμ by a background cancellation technique as follows. A straight line AB was drawn between the points of minimum absorption on either side of the peak and a perpendicular line was drawn from the point of maximum absorbance, C, to intersect AB at D; the line CD was measured in absorbance units. A standard calibration curve was prepared from aqueous solutions of chlorpromazine sulfoxide hydrochloride. The absorbance of chlorpromazine sulfoxide after reaction with 50% sulfuric acid was proportional to concentrations up to 5×10^{-5} *M*.

Determination of Conjugated Drug Metabolites.—Chromatographic columns were prepared from the ion-exchange resin in the Na^+ form. One gram of resin was used for each ml. of urine, depending upon the quantity of metabolites in the urine.[3] The column was washed with a few ml. of Sørensen's pH 6 buffer which was discarded. From 0.5 to 2.0 ml. of urine[3] was then passed through the column, and the effluent was collected in a 10-ml. volumetric flask. The column was washed with pH 6 buffer to yield an effluent volume of 5 ml. The effluent was treated with concentrated sulfuric acid as described previously and maximum absorption read at 550 mμ by the background cancellation method. A standard calibration curve was prepared from aqueous solutions of 7-methoxychlorpromazine hydrochloride after treatment with 1 drop of 30% hydrogen peroxide (to form the corresponding sulfoxide). Absorption of the standard compound was read at 567 mμ after addition of concentrated sulfuric acid to give a final acid concentration of 50% by volume. Absorbance was proportional to concentration up to 5×10^{-5} *M* in 50% sulfuric acid.

Procedure.—Twenty-four hour urine collections from male chronic mental patients, receiving 100 to 1400 mg. of chlorpromazine hydrochloride per day, were assayed for conjugated and unconjugated chlorpromazine metabolites. Each determination was made in duplicate. At least two collections, about a week apart, were obtained from each patient. Additional collections were made in those cases in which urinary concentrations of chlorpromazine metabolites varied between samples. The patients were thoroughly supervised, both with regard to ingestion of medication and collection of 24-hr. specimens.

[3] An estimate of the quantity of urine to be used can be made according to a rapid urine color test (15); use 2 ml. of urine for a 1+ color reaction, 1 ml. for 2+, and 0.5 ml. for 3+ and 4+.

RESULTS

The results are summarized in Table I. Total urinary excretion of the combined groups of drug metabolites varied from 21.1 to 70%. Conjugated and unconjugated metabolites ranged from 16 to 51%, and 2.1 to 19% of the daily dose, respectively. All patients excreted much larger amounts of conjugated than unconjugated chlorpromazine metabolites; the ratio of the conjugated to the unconjugated drug metabolites varied from 2.1 to 11.

DISCUSSION

Method.—Since *O*-glucuronide derivatives of 7-hydroxychlorpromazine (II) were not available, 7-methoxychlorpromazine (I) was chosen as the reference compound for the determination of the conjugated metabolites, although the $\lambda_{max.}$ of 7-methoxychlorpromazine after reaction with 50% sulfuric acid was 567 mμ, compared with 550 mμ for the conjugated metabolites. 7-Methoxychlorpromazine was used for the following reasons.

(*a*) The absorptivity of 7-hydroxy- (the aglycone of 7-methoxy-) and 7-methoxy-chlorpromazines are nearly identical. (*b*) The conjugated drug metabolites consist mainly of *O*-glucuronides (II), in which the glucuronic acid residue is attached to the phenothiazine nucleus *via* an ether linkage (6) at the 7-position (16, 17). Thus, the structure of the chlorpromazine *O*-glucuronides is more closely related to that of 7-methoxychlorpromazine than to 7-hydroxychlorpromazine. (*c*) Under the experimental conditions 7-methoxychlorpromazine and the *O*-glucuronides of chlorpromazine reach maximum color development in 50% sulfuric acid solution faster than 7-hydroxychlorpromazine. (*d*) 7-Methoxychlorpromazine is more stable than 7-hydroxychlorpromazine and is more readily available.

In 50% sulfuric acid, the colored reaction products form more rapidly from the chlorpromazine and 7-methoxychlorpromazine sulfoxides than from the corresponding sulfides (11, 18), and therefore the sulfoxides were used as reference compounds.

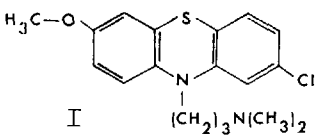
The present method was rapid and reproducible to 5%. Studies using thin-layer chromatography showed that all unconjugated drug metabolites were absorbed onto the exchange resin and that only conjugated drug derivatives remained in the effluents. These studies also showed that all unconjugated chlorpromazine metabolites were extracted into dichloromethane.

The present method determines approximately 95% of the chlorpromazine derivatives normally present in the patient's urine. However, the assay procedure does not estimate all the drug metabolites in urine. 7-Hydroxychlorpromazine, its demethylated derivatives, and corresponding sulfoxides, and the dihydroxychlorpromazine derivatives are not measured by the present procedure. 2-Chlorophenothiazine and its sulfoxide and other nonbasic (deaminated) metabolites are not completely extracted from urine into dichloromethane. However, only trace amounts of deaminated and hydroxylated metabolites are normally present in

TABLE I.—EXCRETION OF URINARY CHLORPROMAZINE METABOLITES IN 15 CHRONIC MENTAL PATIENTS ON CONTINUOUS DRUG THERAPY RANGING FROM 100 TO 1400 mg. PER DAY OF CHLORPROMAZINE

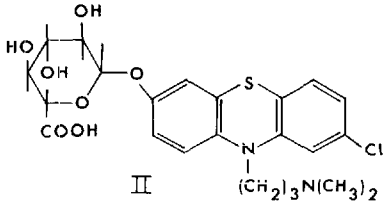
Patient	Dose, mg./24 hr.	Metabolites Excreted,[a] %			Conjugated/ Unconjugated
		Conjugated	Unconjugated	Total	
A	1400	25	3.6	28.6	7.0
		24	6.0	30.0	4.0
B	1200	37	8.2	45.2	4.7
		30	12.0	42.0	2.5
C	1200	28	4.3	32.3	6.6
		21	5.0	26.0	4.2
D	1200	32	3.6	35.6	8.9
		31	2.8	33.8	11.0
E	900	27	5.1	32.1	4.9
		26	4.1	30.1	6.3
F	900	26	3.2	29.2	8.1
		23	2.9	25.9	7.9
G	800	22	4.5	26.5	4.9
		21	5.5	26.5	3.8
H	750	29	5.5	34.5	5.3
		19	2.1	21.1	9.1
I	600	16	7.5	23.5	2.1
		22	6.4	28.4	3.4
J	600	44	10.1	54.1	4.4
		37	4.3	41.3	7.4
K	600	35	4.1	39.1	8.5
		34	4.6	38.6	7.5
L	300	51	19.0	70.0	2.7
		43	10.0	53.0	4.3
M	300	31	7.7	38.7	4.0
		31	9.3	40.3	3.2
N	300	33	3.8	36.8	8.7
		32	3.9	35.9	8.2
O	100	38	7.0	45.0	5.4
		38	8.0	46.0	4.8

[a] Average of two determinations calculated as percentage of the dose per 24-hr. as chlorpromazine hydrochloride.



I

7-Methoxychlorpromazine



II

Chlorpromazine (7)-*O*-Glucuronic Acid

urine. The authors have estimated that deaminated chlorpromazine metabolites represent less than 1% of the daily drug dose, and Goldenberg and Fishman (12) conclude that the unconjugated 7-hydroxy derivatives represent about 0.1% of the daily drug dose. However, the 7-hydroxy group of drug metabolites occur in somewhat larger amounts in patients (21) with chlorpromazine-induced skin pigmentation (20).

The procedures used to separate the chlorpromazine metabolites from urine do not exclude all the urinary constituents that give colored products in 50% sulfuric acid. However, the nonspecific background absorption of these interfering constituents was eliminated by the use of the background cancellation method previously described.

Other drugs derived from phenothiazine or imipramine produce colored radical ions in 50% sulfuric acid, and the biotransformation products derived from these drugs could presumably be measured by an assay procedure similar to the one reported here provided that appropriate reference compounds are available.

Results.—The excretion of chlorpromazine and its conjugated and unconjugated metabolites was not related to drug dose (100–1400 mg. per day). Thus, it was shown (Table I) that a patient receiving 100 mg. and another 1200 mg. chlorpromazine per day, both excreted 45% of the administered dose. These results differ markedly from those of Nadeau and Sobolewski (9), who reported earlier that the limits of urinary drug excretion were reached at a dose of 200 mg. per day.

In the present study total drug excretion varied from 21.1 to 70% of the administered dose, with an average excretion calculated at 37%. Previous data of Posner *et al.* (4), who had used methods similar to those of Nadeau and Sobolewski (9), showed recoveries of 7.2 to 24.7%. These authors also stated that there was a trend toward decreasing urinary excretion with increasing drug dose. Using an assay procedure employing biologically derived reference compounds, Huang *et al.* (10) recently studied seven patients receiving 300 to 1200 mg. of chlorpromazine per day, and recovered an average of 58% of the administered dose in urine. These recoveries were higher than those obtained in the present study, and the authors also observed a tendency toward decreased urinary excretion of

conjugated metabolites with increasing drug dose. The present study differs from earlier ones (4, 9, 10) by not showing any dose-excretion correlation, and by yielding per cent excretion values substantially higher than those of Posner *et al.*, considered less than comprehensive, but lower than those of Huang *et al.*, believed to include normal urinary contaminants.

The reported results (Table I) could not be expected to show a definite correlation between drug excretion and such clinical parameters as diagnosis, drug response, or mental status of the patients; this group of patients was selected only to establish the spread of urinary excretion over a large range of chronic doses. The population sample, though small, included members of the three major ethnic groups with the most diverse psychiatric diagnoses. However, preliminary data on the correlation between side effects of drug therapy and urinary drug metabolism were recently reported for the initial phases of drug administration (19) as well as for drug-induced skin hyperpigmentation (22, 23).

The method is currently being used to study the time required to reach a steady excretion rate, to determine the effect of simultaneously administered compounds on the biotransformations and urinary excretion of chlorpromazine, and to elucidate species differences in chlorpromazine metabolism.

When studying the urinary drug excretion in mental patients, a number of factors not readily controlled in a normal hospital setting may affect the reproducibility of the chemical results. They are variations in diet, and intake of food and liquids. Most patients were found to increase their urinary output by a factor of 2 or 3 under chronic administration; the volumes eventually became reasonably stable at this higher level. Large fluctuations in consecutive 24-hr. specimens tended to yield erratic results. Therefore, rigorous surveillance of controllable parameters like drug intake (preferably in liquid form) and of 24-hr. urine collections cannot be overemphasized.

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Solubility of Parabens in Syrup Vehicles

By ANTHONY N. PARUTA* and BHOGILAL B. SHETH

The solubilities of methyl, ethyl, propyl, butyl, and benzylparabens have been determined in sucrose vehicles of varying concentration. These syrup vehicles possess dielectric constants less than pure water, their respective dielectric constants decreasing with increasing sucrose concentration. The effect of both the concentration of sucrose added and the dielectric constant upon the solubility of the subject compounds is presented. The solubilities of these materials were seen to change to a relatively small degree with increasing sucrose concentration. Although these changes in solubility are minute, they would appear to be positive in character. The only definitive change noted was for benzylparaben where a relatively large change in solubility was noted.

THE GENERAL use and application of sucrose solutions of varying concentration for liquid pharmaceuticals is still widespread. In an effort to continue (1, 2) to determine the solvency characteristics of these media, the present study was undertaken. It was felt judicious that a study of a set of solutes of varying polar character be undertaken so that the effect of methyl, ethyl, etc., groups upon the solubility in common dissolution media could be studied. In this way, a tendency or characteristic effect of substituent groups could be delineated implying an effect that has been termed "solute polarity."

It has been found that relatively dramatic

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By ANTHONY N. PARUTA* and BHOGILAL B. SHETH

The solubilities of methyl, ethyl, propyl, butyl, and benzylparabens have been determined in sucrose vehicles of varying concentration. These syrup vehicles possess dielectric constants less than pure water, their respective dielectric constants decreasing with increasing sucrose concentration. The effect of both the concentration of sucrose added and the dielectric constant upon the solubility of the subject compounds is presented. The solubilities of these materials were seen to change to a relatively small degree with increasing sucrose concentration. Although these changes in solubility are minute, they would appear to be positive in character. The only definitive change noted was for benzylparaben where a relatively large change in solubility was noted.

THE GENERAL use and application of sucrose solutions of varying concentration for liquid pharmaceuticals is still widespread. In an effort to continue (1, 2) to determine the solvency characteristics of these media, the present study was undertaken. It was felt judicious that a study of a set of solutes of varying polar character be undertaken so that the effect of methyl, ethyl, etc., groups upon the solubility in common dissolution media could be studied. In this way, a tendency or characteristic effect of substituent groups could be delineated implying an effect that has been termed "solute polarity."

It has been found that relatively dramatic

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solubility effects occurred with the xanthine drugs, antipyrine, and several derivatives in syrup vehicles (2). In these cases, both increased and decreased solubility was noted with increasing sucrose concentration up to simple syrup. The parabens find wide use as preservatives in pharmaceutical liquid preparations as well as syrup vehicles, and it was felt desirable to determine their solubility in these vehicles. It was thought that if solubility effects, especially decreased solubility, occurred as extensively as with materials previously studied, the possibility of untoward effects such as preservative precipitation may occur.

EXPERIMENTAL

Materials.—The solutes used in this study were used directly as supplied by the manufacturer. The methyl and propylparabens were obtained from the Nepera Chemical Co. The ethyl and benzylparabens were obtained from Heyden Chemical Corp., lots 113 and 59, respectively. Butylparaben was obtained from Eastman Kodak, catalog No. EK 4574. Syrup vehicles were prepared from commercial granulated sugar and deionized or distilled water. Mixtures of absolute ethanol and either deionized or distilled water were used for dielectric constant calibration.

Equipment.—A WTW DK-06 multidekameter was used for dielectric constant determinations, a Bantam (mixed resin bed) demineralizer for producing deionized water. A Bausch & Lomb 505 spectrophotometer was used to determine sample absorbance and a water bath and attendent temperature controller was used for equilibration at 25°.

Dielectric Constant Determination.—The dielectric constants were determined by the use of a WTW multidekameter DK-06. Calibration curves were prepared from condenser readings and known dielectric constants (3) using ethanol–water mixtures. The dielectric constant values obtained for the syrup vehicles prepared gave excellent agreement with values of previous workers (4, 5). The accuracy of these determinations is about ±0.3 of a dielectric constant unit.

Solubility Determinations.—The protocol used in solubility determinations has been described previously (1, 2). Seventy-two hours was the time determined for equilibration at 25° ± 0.1°. All solubility runs were done at least four times, however, seven replicates were needed for the propyl and butylparabens in order to delineate the range of the magnitude of solubility for each given sucrose vehicle. Methylparaben caused some experimental difficulty since nonfilterable suspensions formed in syrup vehicles. It was found that allowing the methylparaben samples to lie quiescent in the water bath for several hours, clear samples were readily obtained.

RESULTS AND DISCUSSION

In Fig. 1, the solubility of methylparaben in mg./ml. *versus* both the concentration and the di-

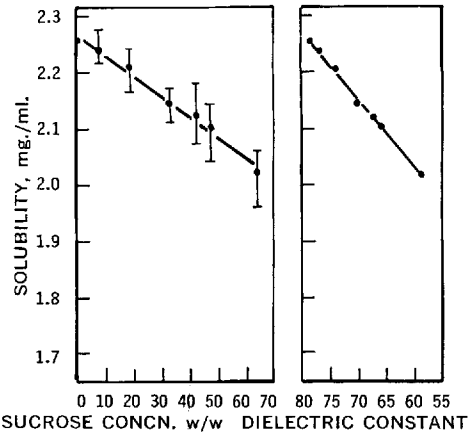

Fig. 1.—A plot of the solubility of methylparaben in mg./ml. at 25° as a function of sucrose concentration and dielectric constants.

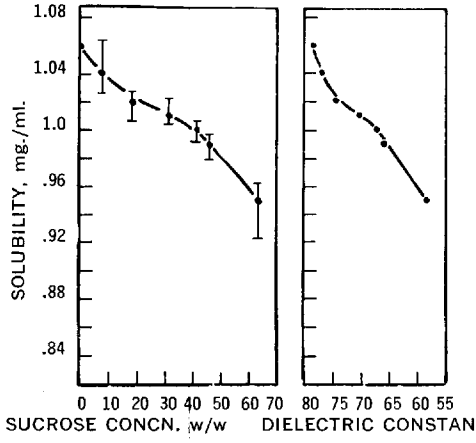

Fig. 2.—A plot of the solubility of ethylparaben in mg./ml. at 25° as a function of sucrose concentration and dielectric constants.

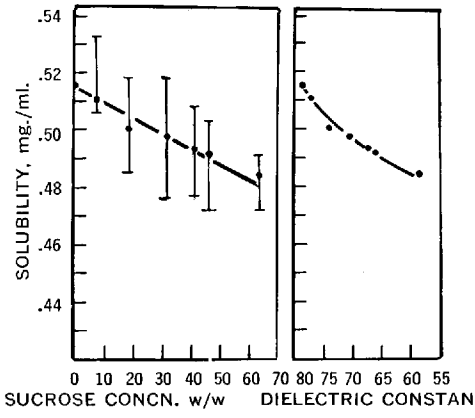

Fig. 3.—A plot of the solubility of propylparaben in mg./ml. at 25° as a function of sucrose concentration and dielectric constants.

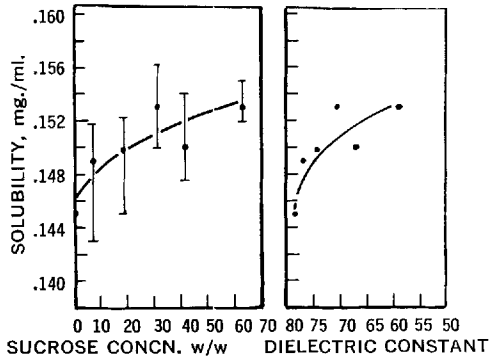

Fig. 4.—A plot of the solubility of butylparaben in mg./ml. at 25° as a function of sucrose concentration and dielectric constants.

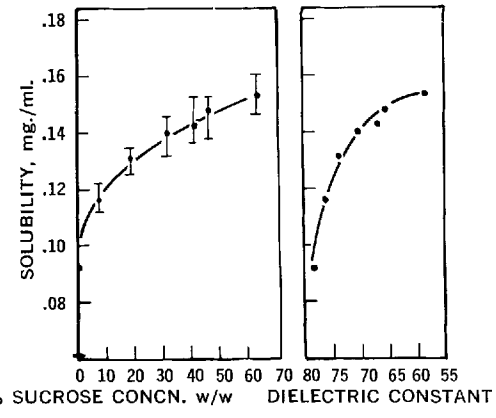

Fig. 5.—A plot of the solubility of benzylparaben in mg./ml. at 25° as a function of sucrose concentration and dielectric constants.

electric constant of the syrup vehicles is shown. The solubility is seen to decrease as a function of both increased sucrose concentration and decreased dielectric constant. The rate of change is approximately linear having values of about −0.1 mg./10% w/w sucrose and about −0.1 mg./10 dielectric constant units. The solubility of methylparaben in simple syrup relative to water has a value of about 0.9. The variation in solubility in mg./ml. in Fig. 1 and subsequent figures is shown as a vertical line through the point of the average value. The variation is shown as the highest and lowest solubility in mg./ml. over the number of runs for pure water or any particular syrup vehicle. The variation has only been shown on the sucrose concentration axis, since once the nature of the curve has been defined on this basis, the dielectric constant scale retains the same shape, accentuated to some degree due to the "squeezing-in" on a shorter x-axis scale. The latter is especially true in the case of ethylparaben shown in Fig. 2. Again the solubility in mg./ml. is plotted in the usual fashion. In this case, an approximately sigmoidally disposed and decreasing solubility curve is observed. The solubility ratio, the solubility in simple syrup relative to water has a value of about 0.9. Repetitive runs in the 20–40% w/w sucrose range showed small variation and it is felt that the curve shown is a true reflection of the solubility pattern. It should also be pointed out that the solubility curve on a dielectric constant basis accents the sigmoidal nature of this curve.

In Figs. 3 and 4, the solubility curves obtained for the propyl and butylparabens, plotted in the usual fashion, are shown. As can be seen from these plots, the variation of solubility over a number of runs is quite wide, and this is due to the very low solubility of these materials. It would seem that one can only simply describe these solubility curves in approximately quantitative terms. Thus, the solubility of propylparaben decreases

Table I.—Summary of the Average Solubility in mg./ml. for the Parabens in the Systems Noted

System	Solubility, mg./ml. Methylparaben	Ethylparaben	Propylparaben	Butylparaben	Benzylparaben
Water	2.26	1.06	0.516	0.145	0.092
7.5% w/w sucrose	2.24	1.04	0.510	0.149	0.116
18.5% w/w sucrose	2.21	1.02	0.500	0.150	0.131
31.5% w/w sucrose	2.15	1.01	0.497	0.153	0.140
41.5% w/w sucrose	2.12	1.00	0.493	0.150	0.143
46% w/w sucrose	2.11	0.99	0.491	...	0.149
63.5% w/w sucrose	2.02	0.95	0.484	0.153	0.153
Solubility ratio: mg./ml. syrup / mg./ml. water	0.89	0.90	0.94	1.06	1.7

Table II.—Summary of the Solubility Ratios (mg./ml. Syrup/mg./ml. Water) Found for the Paraben Derivatives

	Run, No. 1	2	3	4	5	6	7	Av.
Methylparaben	0.89	0.91	0.92	0.86	0.91	0.88	...	0.90
Ethylparaben	0.93	0.89	0.89	0.91	0.87	0.89	...	0.90
Propylparaben	0.90	0.93	0.95	0.96	0.97	0.93	0.93	0.94
Butylparaben	1.04	1.03	1.06	1.09	1.05	1.04	1.06	1.05
Benzylparaben	1.7	1.4	1.7	1.9	1.6	...	...	1.67

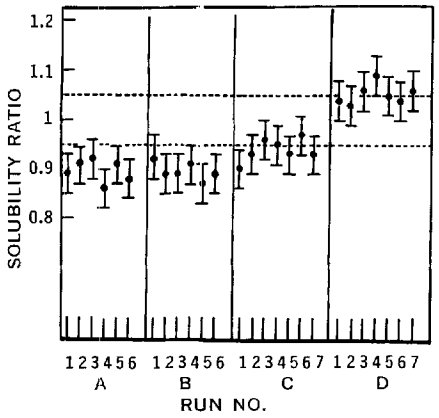

Fig. 6.—A plot of the solubility ratios for the *n*-alkyl parabens over repetitive runs including the determined variation. Key: A, methylparaben; B, ethylparaben; C, propylparaben; D, butylparaben.

slightly and has a solubility ratio of about 0.94. Butylparaben, on the other hand, increases slightly, the solubility ratio having a value of 1.06.

For both propyl and butylparaben, a nonlinear solubility curve has been drawn through the points of average value. Since the experimental values varied to such a large extent, the nature of the curve is considered arbitrary.

In Fig. 5, the solubility of benzylparaben in mg./ml. is plotted in the usual manner. For this derivative, having very low solubility in water, the solubility is seen to increase to a rather large extent, the solubility ratio having a value of about 1.7.

The average solubility in mg./ml. for each of the parabens studied in the syrup vehicles noted is given in Table I.

Although the solubility effects in syrup vehicles for the parabens are small, there is good consistency in the solubility ratios over a set of multiple runs. The value for independent solubility ratios for the number of runs stated is given in Table II.

The dielectric constants of the syrup vehicles were known, and it would be desirable to note any changes in this parameter for the saturated solutions of the parabens. The dielectric constants of the saturated solutions were measured at 25° on the DK-06 dekameter. As was found previously (2) no discernible patterns of change were found for the parabens. The dielectric constants for the saturated solutions, however, did follow the solubility curve and no sudden changes or aberrations in these values were noted for the range studied.

The solvency characteristics of sucrose vehicles can be viewed in two ways, the first being the decrease in the activity of water. With increasing sucrose concentration less water is available to interact with solute molecules, causing dissolution.

Second, the dielectric constants of syrup vehicles decrease with increasing sucrose concentration, implying decreased "polarity" of the dissolution media. The solubility of any given solute may then increase or decrease according to the mechanism or combination of mechanisms operative. The increase or decrease in solubility probably depends upon the nature of the solute and the original magnitude of the solubility in water.

It would be well to consider that a noted change in solubility should be outside or greater than 5%. In other words, it is presumed that a direction of change can only be classified as positively increased or decreased when all the data on repetitive runs fall outside this 5% limit.

In this regard, the solubility ratios for the *n*-alkyl parabens over repetitive runs (Table II) have been plotted in Fig. 6 using the largest variation observed for the solubility in simple syrup for each of the parabens.

The line unity represents 100% of solubility (of the value in water) and the dashed lines are the 5% limit described above. The vertical lines through the average data points represent the determined variation.

From the spread of values for the methyl and ethylparabens, it would seem that the solubility of these materials is affected to a very slight degree, *i.e.*, about 5%. For propyl and butylparabens, the descriptive term of "essentially linear" would apply, especially in view of the wide variation of solubility in the vehicles studied.

The only definitive change observed in the magnitude of solubility was for the benzylparaben where an average increase of about 70% in solubility was observed.

These results are perhaps not completely unexpected since the solubility parameter values given by Martin (6) are quite close to one another. This would indicate that the asymptotic right-handed portion of the solubility distribution curve over a small portion of the dielectric range (syrup vehicles ϵ = 60–75) for the parabens are very close to one another. This would be especially true in solvent systems such as sucrose in water relative to a hydroalcoholic solvent system where it might be expected that discriminatory differences in solubility would occur.

In conclusion, the solubility effects noted with the parabens of general interest are slight or nonexistent in contrast to previously noted effects with other materials (2).

Since large solubility effects have been noted for the xanthines and antipyrine (2) and small or no effect was noted for the parabens, it is felt judicious to continue this type of work. In this regard, the solubility of acetanilide and several derivatives are presently being studied in syrup vehicles.

The parabens are an important class of materials in pharmacy. Future work on the dielectric solubility profiles of these materials is presently under way and the authors' results will be given in a future communication.

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Simple Method for Determination of $^{14}CO_2$ from Expired Air

By S. Y. YEH, JOHN H. CAVANAUGH*, and L. A. WOODS

Two simple procedures for preparation of homogeneous $^{14}CO_2$ samples for liquid scintillation counting are described. In one procedure (*A*), the carbon dioxide absorbing agent and the carbonate solution are placed in one tube. Carbon dioxide is released from the carbonate solution by sulfuric acid and absorbed by phenethylamine directly. Added $NaH^{14}CO_3$ (16–8000 dpm/ml.) is recovered nearly quantitatively in the amine phase. Similar results are obtained with another procedure (*B*), with which $^{14}CO_2$ is released from the carbonate solution and flushed with nitrogen into a counting vial containing absolute methanol and phenethylamine.

COMMON METHODS for measuring expired $^{14}CO_2$ involve trapping by passage of the expired air through sodium hydroxide solution and subsequent reprecipitation as $BaCO_3$. The radioactivity of the preciptated $BaCO_3$ can be determined either on planchets in a gas-flow counter or as suspensions in agents such as thixicin (1), or silica[1] (2) in a liquid scintillation spectrometer. Preparation of planchets or suspensions involves precipitation, filtration, drying and/or powdering, and weighing a portion of the powdered sample, all of which are time-consuming procedures.

Dilute sodium ^{14}C-carbonate solution has also been suspended in silica (3) and radioactivity quantitated in a liquid scintillation spectrometer. The latter procedure is limited to a highly radioactive carbonate solutions and is inadequate to measure low levels of radioactivity in carbonate solutions collected from drug metabolism studies.

Another common method for measuring $^{14}CO_2$ involves acidification of the carbonate solution, diffusion of the generated $^{14}CO_2$ to an organic amine such as hyamine, primine, ethanolamine, ethylene amine, and phenethylamine, and determination of radioactivity with a liquid scintillation spectrometer (4–8).

Phenethylamine is reported to be superior to other amines because it provides (*a*) high capacity for absorption of CO_2 and (*b*) minimal quenching (9). Carbon dioxide reacts with phenethylamine to form phenethyl ammonium phenethylcarbamate and with sufficient stoichiometric excess (about 3 moles of amine per mole of CO_2), the reaction is rapid (9).

To insure complete absorption of CO_2 by diffusion following acidification of the carbonate solution, a period of 4–24 hr. is required. The duration depends on (*a*) the diffusion apparatus and (*b*) the shaking of the reaction flasks (4–8). Twenty minutes were reported to be adequate if a special diffusion apparatus under reduced pressure (100 mm. Hg) was used. Carbon dioxide was reported to be quantitatively absorbed, but no data were presented (9).

Since all of the above procedures require complicated apparatus, extreme care, and much time, two simple procedures for preparation of homogeneous $^{14}CO_2$ containing samples for liquid scintillation counting have been developed. The details are described in this paper. The procedures require simple apparatus and are practical for the routine simultaneous handling of a large number of samples.

EXPERIMENTAL

Reagents.—All reagents are analytical reagent grade. (*a*) Alcohol–amine solution; *n*-amyl alcohol in freshly distilled phenethylamine 1:1 v/v. (*b*) 9 *N* sulfuric acid. (*c*) 5.3 *N* NaOH solution. (*d*) Standard $NaH^{14}CO_3$ solution ($^{14}CO_2$ was bubbled into 5.3 *N* NaOH solution). A series of solutions containing $NaH^{14}CO_3$ was prepared by diluting the $NaH^{14}CO_3$ solution with 5.3 *N* NaOH solution which contained 5% sodium bicarbonate. (*e*) Phosphor toluene; 300 mg. [1,4-bis-2(5-phenyloxazolyl)benzene (POPOP)], and 9 Gm. of 2,5-diphenyloxazole (PPO) per liter of toluene.

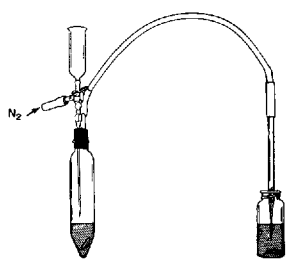

Fig. 1.—Apparatus for generation and trap for $^{14}CO_2$.

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[1] Marketed as Cab-O-Sil by the Cabot Corp., Boston, Mass.

TABLE I.—COMPARISON OF THE RECOVERY OF $^{14}CO_2$ BY FLUSHING WITH NITROGEN AND BY ABSORPTION OF PHENETHYLAMINE

$NaH^{14}CO_3$ Added dpm Mean ± S.D.	Expt., No.	Procedure *A* In Amine Phase dpm	%	In Aqueous Phase[c] c.p.m./0.2 ml.	Procedure *B* dpm	%
*809[a] ± 26	4		100			
*727 ± 17	10					
**457 ± 10	4					
**349 ± 11	4					
Blank	4			31.1 ± 0.5 (S.D.)	32.2 ± 2.6 (S.D.)	
39	2	39	100	31	38	97
77	2	77	100	30	78	101
154	2	157	102	32	166	108
308	2	317	103	33	327	106
616	2	647	105	31	657	107
1232	2	1283	104	33	1308	106
2463	2	2597	105	34	2632	107
4925	2	5154	105	35	5153	105
9850	2	10444	106	36	10613	108
19700[b]	2	20598	105	37		
		Mean ± S.D.	104 ± 2.2	33.2 ± 2.3		105 ± 3.4

[a] A 100-μl.* or 50-μl.** aliquot of a solution prepared by diluting the stock $NaH^{14}CO_3$ solution with 5.3 *N* NaOH solution was added directly to a counting vial. Because the inaccuracy of micropipets, the mean dpm (394 ± 48/50 μl.) obtained from four determinations using four different micropipets was used as standard. [b] An amount of 2.5 ml. of the diluted $NaH^{14}CO_3$ solution (see *Footnote a*) was used. [c] The radioactivity remaining in the aqueous phase is insignificant from identical blanks ($0.2 < P < 0.1$).

TABLE II.—RECOVERY OF $^{14}CO_2$ FROM SODIUM CARBONATE SOLUTION BY PROCEDURE *A*

$NaH^{14}CO_3$ Added, dpm	Expt., No.	Acid Added Eq. to NaOH, ml.	%	Radioactivity Recovered: Alcohol–Amino Phase vol., ml.	dpm Mean ± S.D.	%	Air Phase[c] Gross c.p.m.	Aqueous Phase vol., ml.	Gross c.p.m./ 0.25 ml.
238 ± 13[a]	7					100			
Blank	4						32.2 ± 2.6 (S.D.)		
5950[b]	4	1.5	100	11.5	5772 ± 363	97	33	2.5	39
5950	2	1.7	113	11.6	6092	102	32	2.5	38
5950	2	1.9	125	11.8	6052	102	31	2.5	34
5950	2	2.3	150	12.2	6044	102	33	2.3	34
5950	3	3.0	200	13.2	6063 ± 62	102	32	2.1	36
							32.2 ± 0.9(S.D.)		

[a] A 100-μl. aliquot of a solution prepared by diluting the stock $NaH^{14}CO_3$ solution with 5.3 *N* NaOH solution was added directly to a counting vial. The counting efficient of ^{14}C-toluene in the sample containing 100 μl. of $NaH^{14}CO_3$ solution is 56%. [b] An amount of 2.5 ml. of the diluted $NaH^{14}CO_3$ solution (see *Footnote a*) was used. [c] The air in tube was flushed with nitrogen and trapped in a counting vial containing the alcohol–amine and radioactivity determined. The counts obtained are not significantly different from identical blanks ($P < 0.5$).

Procedure A.—Exactly 2.5 ml. of the diluted $NaH^{14}CO_3$ solution is placed in a 43-ml. glass-stoppered centrifuge tube. Ten milliliters of the alcohol–amine solution is added to each tube. A 1.1 equivalent of sulfuric acid, 1.7 ml. of 9 *N* H_2SO_4, is slowly added *via* a buret to the centrifuge tube. The tube is cooled with ice water during the period of neutralization. Following neutralization, the stoppered tubes are shaken for 10 min. in an International shaker at 280–300 oscillations/min., and centrifuged at 1700 r.p.m. for 10 min. A 10-ml. aliquot of the organic layer, 2 ml. of absolute methanol, and 5 ml. of the phosphor toluene solution are pipeted into a clean counting vial and counted for three 10-min. periods in a Nuclear-Chicago series 720 liquid scintillation spectrometer. Samples are counted for three 10-min. periods to provide a 0.95 counting error of less than 2% for gross counts (10). Radioactivity in a given sample is considered significant when a value of 6 net c.p.m. or greater is obtained. The remaining alcohol–amine and aqueous solutions are drawn (with the aid of a propipet) into a 5-ml. graduated pipet. The volume of the alcohol–amine phase is estimated in the pipet after the organic and aqueous layers have separated.

Procedure B.—Exactly 2.5 ml. of the diluted $NaH^{14}CO_3$ solution is placed in a 43-ml. glass-stoppered centrifuge tube. Ten milliliters of absolute methanol in phenethylamine (1:1 v/v) solution is placed in a counting vial. The tube is stoppered with a rubber stopper which has previously been punctured with a No. 17 gauge needle and connected with a small Chieftain intravenous plastic tubing ($^1/_8 \times {}^1/_{32}$ in.).[2] The other end of the tubing is connected to a 6-in. glass tip,[3] in which a plug of glass wool is inserted. The tip of the Dispo-pette is immersed in the methanol–amine solution. The arrangement of the apparatus is shown in Fig. 1. Two milliliters of 9 *N* sulfuric acid is slowly added to the tube *via* a 5-ml. syringe while the solution is stirred with a magnetic stirrer. Following acidification, the generated CO_2 and the air in the tube is flushed 10 min. with about 0.5 lb./sq. in. pressure of nitrogen. The Dispo-pette and the magnetic stirring bar are rinsed with 1 ml. of absolute methanol each, and 5 ml. of the phosphor toluene is then added. The mixture is counted

[2] Chieftain intravenous tubing, American Hospital Supply Corp., Evanston, Ill.
[3] Dispo-pettes, Scientific Products, Evanston, Ill.

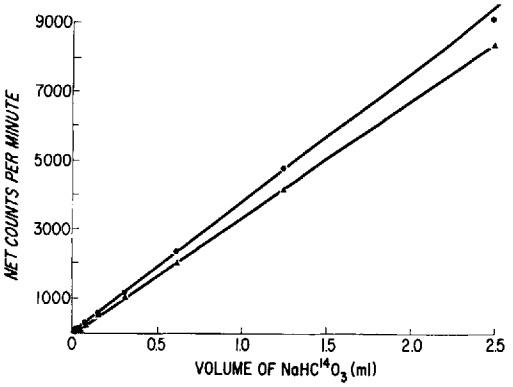

Fig. 2.—Plot of net c.p.m. *versus* volume of standard $NaH^{14}CO_3$ solution added. Key: ●, flushing with nitrogen; ▲, absorption by phenethylamine.

as described under *Procedure A*. The specific radioactivity of the standard $NaH^{14}CO_3$ is determined by first mixing a 100-μl. or a 50-μl. aliquot of the solution with two milliliters of absolute methanol, and then with 10 ml. of the alcohol–amine solution, and 5 ml. of the phosphor toluene.

All samples are corrected for quenching by addition of ^{14}C-toluene as an internal standard. Paired *t* tests were used to compare values obtained from samples and those of identical blanks.

RESULTS

The percentage recovery of $^{14}CO_2$ from sodium hydroxide–carbonate solution by procedure *A* is 104 ± 2.2 (S.D.) as shown in Table I. No significant radioactivity is found in the aqueous layer and in the air of the tube (Table II). A linear relationship is found between the mean of the net counts per minute (c.p.m.) obtained and the volume of $NaH^{14}CO_3$ solution added to the sample as shown in Fig. 2. The counting efficiency of ^{14}C-toluene in the sample containing 10 ml. of the alcohol–amine, 2 ml. of absolute methanol, and 5 ml. of phosphor toluene is approximately 60% of the ^{14}C-toluene in phosphor toluene alone.

The percentage recovery of $^{14}CO_2$ from sodium hydroxide–carbonate solution by procedure *B* is 105 ± 3.5 (S.D.) as shown in Table I. A linear relationship is found between the mean of the net c.p.m. obtained and the volume of $NaH^{14}CO_3$ in the sample as illustrated in Fig. 2. The counting efficiency of ^{14}C-toluene in the sample containing 10 ml. of the "trapping" methanol–amine solution, 2 ml. of absolute methanol, and 5 ml. of phosphor toluene is approximately 60% of the ^{14}C-toluene in phosphor toluene alone.

DISCUSSION

Procedure A.—The theory used in procedure *A* is based on the fact that dissociation of strong acids and strong bases is greater than that of weak bases. If sulfuric acid is mixed with a strong base such as sodium hydroxide–carbonate solution and a weak base such as phenethylamine, the sulfate ions will replace the hydroxide and carbonate ions in preference to reacting with the amine. Preliminary studies gave identical results whether sulfuric acid was added above or below the amine phase.

The result suggests that the affinity of sulfate ions for sodium ions is stronger than both the affinity of sulfate ions for amine ions and carbonate ions for sodium ions.

The higher recovery of $^{14}CO_2$ from known samples was presumably due to the inaccuracy of the micropipet used. The data indicate that $^{14}CO_2$ is recovered quantitatively from $NaH^{14}CO_3$ solutions by both procedures *A* and *B*.

Varying amounts of sulfuric acid (from 113 to 200% necessary to neutralize the NaOH solution) in studies on the recovery of $^{14}CO_2$ from sodium hydroxide–carbonate solution give identical results (Table II). The low recovery of $^{14}CO_2$ while the acid is just 100% equivalent (calculated) to the NaOH solution might have resulted because the actual strength of the acid was not sufficient (the acid and alkali solutions were not standardized by titration). Both the acid and alkali solutions were standardized for routine use. As progressively greater volumes of acid were used, there occurred an increase both in the volume (from 11.5 to 13.2 ml.) and the quenching of the amine–CO_2 phase.

The above procedure was also studied as to (*a*) the possible loss of $^{14}CO_2$ to air *via* diffusion through the alcohol–amine layer using the techniques of procedure *B*, and (*b*) the retention of radioactivity in the aqueous phase. The data shown in Table II indicate that there is no $^{14}CO_2$ loss to air [the air in the tube given 31–33 gross c.p.m. which is not significantly different from the counts obtained on identical blanks (32 gross c.p.m.) ($P < 0.5$)]. The radioactivity in the aqueous phase, determined by counting an 0.25-ml. aliquot with 10 ml. of the methanol–amine, 2 ml. of absolute methanol, and 5 ml. of phosphor toluene, is not significantly different from the identical blanks (Tables I and II, $0.2 < P < 0.1$).

Procedure B.—If one prefers to avoid shaking, centrifuging of the mixture, and transferring of the alcohol–amine-CO_2 to a counting vial in procedure *A*, procedure *B* is suitable. Varying the flushing time from 10 to 30 min. results in identical recovery of added $^{14}CO_2$ (Table III). It is believed that a shorter flushing period than 10 min. may be sufficient if a smaller flask is used.

Table III.—Effect of Flushing Time with Nitrogen on the Recovery of $^{14}CO_2$ Generated from Carbonate Solution

$NaH^{14}CO_3$ dpm Added Mean ± S.D.	Expt., No.	Flushing Time, min.	Recovery dpm Mean ± S.D.	%
175.1 ± 6.7[a]	3			100
4378[b]	4	2	4085 ± 115	93
4378	4	5	4312 ± 224	99
4378	4	10	4394 ± 229	100
4378	4	20	4522 ± 385	103
4378	3	30	4486 ± 56	103

[a] A 100-μl. aliquot of a solution prepared by diluting the stock $NaH^{14}CO_3$ solution with 5.3 *N* NaOH solution was added directly to a counting vial. The counting efficient of ^{14}C-toluene in the sample containing 100 μl. $NaH^{14}CO_3$ solution is 56%. [b] An amount of 2.5 ml. of the diluted $NaH^{14}CO_3$ (see *Footnote a*) solution was used.

SUMMARY

Two procedures have been used in this laboratory for measurement of the expired $^{14}CO_2$ of rats and mice, in various drug metabolism studies and have been found to give rapid, consistent results.

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(1) White, C. G., and Helf, S., *Nucleonics*, **14**, 46(1956).
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Some Physicochemical Properties of the Montmorillonites

By KEE-NENG WAI and GILBERT S. BANKER

The swelling, moisture sorption, particle size, cation-exchange, and drug adsorption properties of selected montmorillonites were studied. The swelling of montmorillonite was found to depend on both adsorptive and osmotic phenomena. The study of the sorption of alkaloidal drugs by the clay showed that brucine was sorbed by both adsorption and ion-exchange reactions, forming a monomolecular layer on the interior surface of the clay; methapyrilene and triethylamine were sorbed by an ion-exchange reaction; and niacinamide was neither adsorbed nor ion-exchanged.

THE HYDROUS magnesium aluminum silicate minerals, montmorillonites, because of their unusual properties and widespread occurrence have attracted the attention of workers in many fields of application. These clays are extremely plastic when moist, swell in the presence of water, and can be dispersed in water forming thixotropic gels. Bentonite U.S.P. is a mineral composed of 90% montmorillonite (1).

The applications of the montmorillonites in the pharmaceutical field have been explored extensively. Magnesium aluminum silicate[1] and bentonite, alone or in combinations with other common suspending agents, have been studied for their use as suspending agents (2–5). Guth *et al.* prepared different types of bentonites by saturating the clay with Na^+, K^+, Ca^{2+}, Mg^{2+}, and H^+. They used these various cation saturated bentonites to conduct a series of studies on the buffer capacity, cationic-exchange properties, and drug binding and release properties of bentonite (6–10). Many studies of montmorillonites as tablet disintegrants have been made, with the work of Feinstein and Bartilucci (11) being the most recent.

Although the montmorillonite class of clay minerals is widely used in the field of pharmacy, little has been reported in the pharmaceutical literature concerning their basic physical and chemical properties and the relation between such properties and pharmaceutical applications and utility. It was, therefore, the purpose of this work to conduct a pharmaceutically oriented study of the physicochemical properties of the montmorillonites, so that the various present applications and limitations or restrictions of the clays might be better understood and future applications more accurately determined.

EXPERIMENTAL

The three commercial montmorillonites studied in this work were magnesium aluminum silicate, magnesium aluminum silicate F, and WG.[2] These three types of clay will be referred to as clay I, II, and III, respectively, in the remainder of this paper.

Magnesium aluminum silicate has a composition of 61.1% silicone dioxide, 13.7% magnesium oxide, 9.3% aluminum oxide, 2.9% sodium oxide, 2.7% calcium oxide, and smaller amounts of titanium, ferric, and potassium oxides, 1.8% carbon dioxide, and 7.2% water of combination (12). The moisture content of the material will increase if exposed to moist atmospheres. The various grades of

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Abstracted from a thesis submitted by K. N. Wai to the Graduate School, Purdue University, in partial fulfillment of Doctor of Philosophy degree requirements.
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magnesium aluminum silicate have the same general composition of plain magnesium aluminum silicate, but differ primarily in mechanical treatments which have been employed to affect color, rehydration properties, aggregate particle size, and other gross properties of the various grades.

Magnesium aluminum silicate WG is a high viscosity grade of magnesium aluminum silicate in finer than 50-mesh powder form, which is easier to rehydrate than other grades (13). Magnesium aluminum silicate F is a microatomized (325 mesh) grade (13).

The magnesium aluminum silicates were selected for study from the montmorillonite class, based on their high relative purity and light color for clay minerals, small lot-to-lot variation, and the availability of grades of clay varying in aggregate particle size and other properties. The clays studied were all oven dried at 50° for 24 hr. prior to use to remove nonbound moisture which may have been sorbed by the materials during storage. The moisture content of the clays as supplied was between 6.2 and 7.5% and after drying was 3.4, 2.4, and 4.3% for clays I, II, and III, respectively. The starch contained 9.4% moisture before drying and 5.2% after. The physicochemical properties studied included swelling volume, moisture sorption, particle size, ion-exchange capacity, and drug binding.

Particle Size.—Electron microscopy[3] was used to determine the particle-size distribution of the clays.

Three samples of each of the three grades of clay were used for the electron micrographic analysis. One picture, at a magnification power of 4000×, was taken of each sample, and the negative films were used to prepare 8 × 10-in. photographs. The Martin's diameters (14) of all the particles were measured on the enlarged photographs. The cumulative per cent of the particles was plotted on a probability scale against the logarithm of the Martin's diameter of the particles. The geometric mean diameters and the geometric standard deviations of the weight distribution were obtained from the graphs.

Swelling Volume.—The swelling of the montmorillonites was studied in media containing sodium chloride in 0, 0.005, 0.010, 0.025, 0.050, 0.10, 0.25, 0.50, and 1.00 *N* concentrations. Clay samples, weighing 2.00 Gm. each, were placed in 100-ml. amber glass jars. Fifty milliliters of medium was added to each jar, and the jars were capped and shaken for 2 hr. on a mechanical shaker. The contents of each jar were then transferred to a 100-ml. graduated cylinder, and additional saline medium was used to wash the jar and fill the graduated cylinder to volume. After 12 hr. of undisturbed hydration in the cylinders, the volume occupied by each hydrated clay gel was measured. This gel height volume was called the swelling volume. Each swelling volume was calculated from the average of three replicate determinations. For the purpose of comparison, the swelling of starch U.S.P. in each medium was also measured.

Moisture Sorption.—Three constant relative humidities were obtained by placing saturated solutions of calcium sulfate, sodium nitrite, and magnesium chloride, respectively, in 9-in. evaporating dishes in separate chromatographic chambers sealed with glass sheets and stop-cock grease (15). The relative humidities in these chambers, measured by a Sedex hygrometer,[4] were 98, 66, and 35%, respectively. The temperature in the chambers was 26 ± 2°. The moisture absorption of the clay samples, as commercially supplied and as compressed disks, and starch U.S.P. in the form of powdered material was studied. Starch could not be compressed as a disk. The disks, 0.5 in. in diameter and 0.5 Gm. in weight, were compressed with a force of 10,000 lb. (50,000 p.s.i.) for 3 min. with a Carver press.[5] The moisture content of all samples was determined gravimetrically, initially, and after 7, 14, and 21 days of storage at each condition. The thickness and diameter of the disks were measured at the same time intervals and disk volumes were calculated.

Determination of the Exchangeable Cation of Montmorillonite with Radioactive Sodium.—A modified, inverse dilution technique was used to measure the amounts of exchangeable cation and free electrolyte in the clays. The experimental procedure was as follows.

(*a*) The ^{22}Na (10 μc.), in the form of a solution of NaCl in hydrochloric acid, was added to enough distilled water to make 1 L.

(*b*) The solution was divided into four 250-ml. portions in volumetric flasks. To these solutions was added enough sodium chloride to produce the equivalent of 0, 30, 60, and 100 mg. of Na_2O in 100 ml. of solution (plus the 1 μc. of ^{22}Na/100 ml.).

(*c*) The samples of the clays, weighing 1 Gm. each, were placed in 100-ml. dry-square bottles which had previously been soaked in dilute hydrochloric acid for 12 hr., washed, and dried.

(*d*) Fifty milliliters of the radioactive saline solutions were added to each bottle and the bottles capped and sealed with tape.[6] The bottles were placed in a mechanical shaker and agitated for 3 hr.

(*e*) The clays were separated from the media by ultracentrifugation,[7] with a force 100,000 times gravity.

(*f*) Three samples, each containing 2 ml. of the clear medium, were placed in 4-ml. sample vials. The radioactivity of each vial was measured with a crystal-well counter.[8]

Montmorillonite Reaction with Cationic Drugs.—Four cationic drugs were used to study the ion-exchange activity of the clays. They were methapyrilene hydrochloride, brucine sulfate, triethylamine hydrochloride, and niacinamide hydrochloride. The methapyrilene hydrochloride and brucine sulfate were analytical grade chemicals. The triethylamine and niacinamide hydrochlorides were prepared from the respective bases by reaction with hydrochloric acid followed by purification by recrystallization from alcohol, acetone, and ethyl acetate.

The alkaloidal salt was dissolved in alcohol U.S.P. The concentration of the alkaloid in the solution was adjusted so that the alkaloid in 50 ml. of solu-

[3] Philips EM 100, modified, electron microscope, North American Philips Co., Mount Vernon, N. Y.

[4] Sedex Inc., Boston, Mass.

[5] Fred S. Carver, Inc., New York, N. Y.

[6] Scotch Tape No. 33, Minnesota Mining & Manufacturing Co., St. Paul, Minn.

[7] Beckman Spinco Division, Stanford Industrial Park, Palo Alto, Calif.

[8] Packard Instrument Co., LaGrange, Ill.

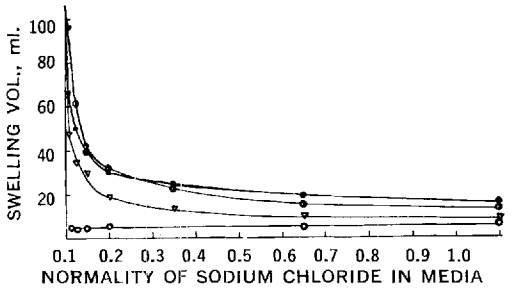

Fig. 1.—The swelling volumes of montmorillonites and starch in aqueous media containing various concentrations of sodium chloride. Key: ◑, clay I; ●, clay II; ▽, clay III; ○, starch.

tion would be completely removed by 1 to 3 Gm. of clay by the ion-exchange reaction. The solution was divided into ten 50-ml. fractions. To these fractions were added different quantities of clay II, varying from 1 to 8 Gm. The clay was removed from the solution by centrifugation after it had been shaken for 3 hr. with the solution. Ten milliliters of the clear alcoholic fraction was pipeted into an aluminum weighing pan and evaporated to dryness on a hot plate at 50°. The weight of residue was plotted against the weight of clays added.

RESULTS

Particle Size.—The particle size (geometric mean diameter) of the discrete clay particles of clays I, II, and III was 0.15, 0.11, and 0.12 μ, respectively, with a geometric standard deviation of 2.4 to 2.5 in each case. This does not reflect a significant difference between the discrete particle size of the various clay materials, although the aggregate particle size of each material as described earlier is quite different.

The surface area of each clay was also estimated using a gravimetric glycerol retention method (16). Based on an average of three replicate determinations for each clay, which were reproducible within $\pm 10\%$ for clay I and $\pm 5\%$ for clays II and III, the respective surface areas of the clays was 371, 339, and 300 M.²/Gm. This finding indicates that clay III, with the largest aggregate particle size (50 mesh), had the smallest available surface, and that microatomization of clay II also reduced its surface compared to clay I, according to the sorption procedure used.

Swelling Volume.—Figure 1 is a plot of the swelling volume of the three grades of clays and starch against the concentration of sodium chloride in the media. The swelling of the clays decreased as the concentration of electrolyte increased. This finding tended to substantiate the hypothesis of Norrish (17) that the second stage of swelling of a montmorillonite is essentially an osmotic one, since the increase in osmotic pressure of the medium caused a rapid decrease in the swelling of the clay. This could be a significant property when the clays are used as disintegrating agents for tablets and as suspending agents. In tablets containing large doses of strongly ionized chemicals, the chemicals might influence the swelling of the clay which, in turn, could decrease the disintegration rate; in suspensions, the strong electrolytes may affect the gel formation of the clays. The swelling of starch did not apparently depend on osmotic pressure and was

Table I.—Moisture Sorption of the Montmorillonites and Starch in Atmospheres of Various Relative Humidities

Material		Wt., Gm., and % Wt. Increase of Samples after Storage: Initial	7 Days	14 Days	21 Days
At 98% Relative Humidity					
Clay I	Wt.	2.007	2.470	2.572	2.528
	%	100	123.0	128.1	125.9
Clay II	Wt.	2.003	2.492	2.622	2.533
	%	100	124.4	130.9	126.5
Clay III	Wt.	1.972	2.439	2.517	2.451
	%	100	123.7	127.7	124.3
Starch	Wt.	5.704	6.382	6.532	6.416
	%	100	111.9	114.5	112.5
At 66% Relative Humidity					
Clay I	Wt.	2.058	2.235	2.240	2.236
	%	100	108.6	108.9	108.7
Clay II	Wt.	1.970	2.138	2.148	2.139
	%	100	108.5	109.0	108.6
Clay III	Wt.	2.018	2.182	2.185	2.180
	%	100	108.1	108.4	108.0
Starch	Wt.	5.548	5.691	5.754	5.702
	%	100	102.6	103.7	102.8
At 35% Relative Humidity					
Clay I	Wt.	2.030	2.090	2.089	2.088
	%	100	103.0	103.0	102.9
Clay II	Wt.	2.017	2.081	2.080	2.080
	%	100	103.2	103.2	103.2
Clay III	Wt.	2.053	2.118	2.115	2.115
	%	100	103.2	103.0	103.0
Starch	Wt.	5.547	5.584	5.597	5.546
	%	100	100.7	100.9	100.0

TABLE II.—SWELLING AND MOISTURE SORPTION OF COMPRESSED MONTMORILLONITES IN ATMOSPHERES OF VARIOUS RELATIVE HUMIDITIES

Material	Dimension	Initial	7 Days	14 Days	21 Days
		At 98% Relative Humidity			
Clay I	Vol., ml.	0.2484	0.4092	0.4192	0.3901
	Wt., Gm.	0.5008	0.6278	0.6412	0.6341
	Wt., %	100	125.4	128.0	126.6
Clay II	Vol., ml.	0.2503	0.3739	0.3829	0.3470
	Wt., Gm.	0.4927	0.6319	0.6464	0.6330
	Wt., %	100	128.5	131.2	128.5
Clay III	Vol., ml.	0.2473	0.4029	0.4253	0.3353
	Wt., Gm.	0.5157	0.6375	0.6605	0.6442
	Wt., %	100	123.6	128.1	124.9
		At 66% Relative Humidity			
Clay I	Vol., ml.	0.2460	0.3076	0.2885	0.2907
	Wt., Gm.	0.4964	0.5318	0.5318	0.5310
	Wt., %	100	107.1	107.1	107.0
Clay II	Vol., ml.	0.2490	0.2765	0.2725	0.2761
	Wt., Gm.	0.4915	0.5200	0.5205	0.5202
	Wt., %	100	105.8	105.9	105.9
Clay III	Vol., ml.	0.2457	0.2897	0.2847	0.2891
	Wt., Gm.	0.5101	0.5374	0.5383	0.5376
	Wt., %	100	105.4	105.5	105.4
		At 35% Relative Humidity			
Clay I	Vol., ml.	0.2449	0.2565	0.2575	0.2578
	Wt., Gm.	0.4886	0.4980	0.4979	0.4976
	Wt., %	100	101.9	101.9	101.8
Clay II	Vol., ml.	0.2458	0.2600	0.2505	0.2518
	Wt., Gm.	0.4907	0.4968	0.4923	0.4951
	Wt., %	100	101.2	100.3	100.9
Clay III	Vol., ml.	0.2473	0.2605	0.2598	0.2591
	Wt., Gm.	0.5110	0.5147	0.5146	0.5141
	Wt., %	100	100.7	100.7	100.6

TABLE III.—RADIOACTIVITY OF SODIUM CHLORIDE SOLUTIONS AFTER EQUILIBRATION WITH THE SODIUM IONS IN MONTMORILLONITE

Material		Na_2O Equivalent in 100 ml. of Soln., mg. 0	30	60	100
Clay I	c.p.m.	13,249	19,289	22,395	24,860
	%[a]	41.24	60.04	69.71	77.38
Clay II	c.p.m.	12,100	...	22,206	24,850
	%	37.66	...	69.12	77.35
Clay III	c.p.m.	16,839	...	24,040	25,898
	%	52.41	...	74.83	80.61

[a] Per cent of original activity (32,126 c.p.m.) after average background count (219 c.p.m.) was subtracted.

TABLE IV.—Na_2O EQUIVALENTS IN MONTMORILLONITES

Material	Free Electrolyte, Na_2O Equivalent, mg./Gm.	Exchangeable Electrolyte Na_2O Equivalent, mg./Gm.	Exchangeable Electrolyte Na^+ Equivalent mmole/Gm.
Clay I	13.08	18.63	0.601
Clay II	10.92	18.08	0.583
Clay III	17.83	16.19	0.523

not affected by the concentration of sodium chloride in the medium.

Moisture Sorption.—The hygroscopicity of a solid matrix has a direct effect on the stability of many active pharmaceutical ingredients. The hygroscopicity of clays may be influenced by their chemical composition, the amount and type of exchangeable cation present, the particle size, and the surface area of the clay sample.

The results of the moisture sorption study are summarized in Tables I and II. At the same relative humidity condition, all three grades of montmorillonite sorbed approximately the same amount of moisture, although, physically each clay represents a different state of aggregation of the colloidal particles—flake, powder, and fine powder. The aggregate condition of the clays did not appear to affect the moisture sorption of the clays as supplied or as compressed disks. The clays in original powder form and the clays which had been compressed with a pressure of 50,000 p.s.i. for 3 min. also sorbed approximately the same amount of moisture in a period of 7 days or longer. This shows that moisture penetrates the compressed clay rather rapidly. The montmorillonites were found to swell (Table II)

in a humid atmosphere, with the volume of swelling approximately equal to the volume of the water absorbed.

Determination of the Exchangeable Cation of Montmorillonite with Radioactive Sodium.—Consider the case of a clay sample suspended in distilled water containing a trace of $^{22}NaCl$. The free electrolyte in the clay sample dissolves freely in the medium while the exchangeable cations attach to the clay particles. The ^{22}Na, being chemically identical to natural sodium, exchanges indiscriminately with the exchangeable sodium and the sodium in the medium. At equilibrium the relationship in Eq. 1 would exist.

$$\frac{\text{free Na in clay}}{\text{total Na in clay}} = \frac{\text{radioactivity in soln. at equilibrium}}{\text{initial radioactivity}} = \text{free electrolyte in clay, \%} \quad \text{(Eq. 1)}$$

Consider another sample of clay suspended in saline solution containing a trace of $^{22}NaCl$ and x moles of NaCl. The free electrolyte in the clay sample dissolves freely in the medium and mixes with the sodium chloride originally in the medium. The ^{22}Na exchanges indiscriminately with the exchangeable sodium and the sodium in the medium. At equilibrium, the relationship in Eq. 2 is obtained.

$$\frac{\text{free Na} + x \text{ moles of Na}^+}{\text{total Na} + x \text{ moles of Na}^+} = \frac{\text{radioactivity in soln. at equilibrium}}{\text{initial radioactivity}} \quad \text{(Eq. 2)}$$

Solving Eqs. 1 and 2, one can estimate the amount of exchangeable sodium and the amount of soluble electrolyte in the clays.

Table III expresses the radioactivity, in terms of c.p.m. and percentage of initial activity, of 2 ml. of the solutions of radioactive sodium chloride which had been mixed and ion-exchanged with the clays according to the procedure previously outlined.

Utilizing the data in Table III, the free electrolyte and exchangeable electrolyte contents were calculated and are summarized in Table IV.

This experiment shows that the sodium in montmorillonite exists as exchangeable cation as well as free electrolyte. The presence of free electrolyte in the clays may be due to the presence of electrolyte in natural water which is used to wash the clays. Comparing the ion-exchange property and the swelling volume of the clays, it appears that the ion-exchange capacity of the clay is not the major factor that influences the swelling of the clay.

Considering that the cation-exchange capacity of clay II is 0.583 (Table IV) and that the surface area of the same clay is 339 M.²/Gm., the average distance between two ionized sites can be calculated to be about 10 Å. This is a reasonable estimation. It shows that the ion-exchange sites are distributed throughout the internal surface of the clay, and the cation-exchange activity takes place at the interior of the clay particles.

Montmorillonite Reaction with Cationic Drugs.—Figures 2 to 5 describe the sorption of the four drugs by montmorillonite. At first the drug concentration decreased very rapidly with the increased quantity

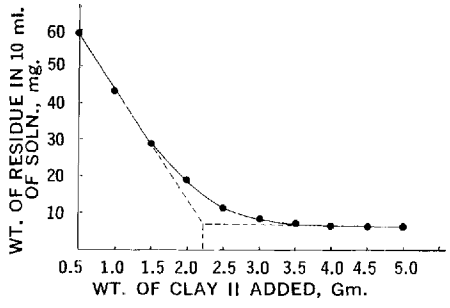

Fig. 2.—Sorption of methapyrilene by montmorillonite.

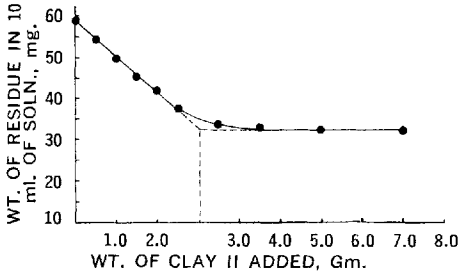

Fig. 4.—Sorption of triethylamine by montmorillonite.

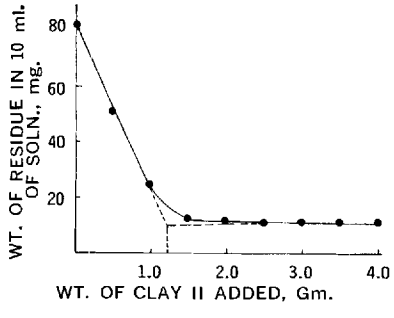

Fig. 3.—Sorption of brucine by montmorillonite.

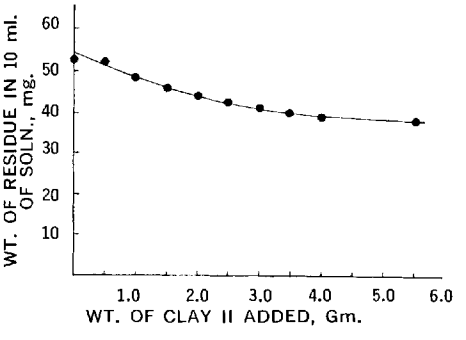

Fig. 5.—Sorption of niacinamide by montmorillonite.

TABLE V.—ABSORPTION OF ALKALOIDAL DRUGS BY CLAY II

	Methapyrilene HCl	Brucine Sulfate	Triethylamine HCl	Niacinamide HCl
Initial concn. of drugs, mg./ml.	5.89	8.11	4.90	5.45
Amt. of drug in 50 ml. of soln., meq.	0.989	0.915	1.780	1.718
Clay required to absorb all drug in 50 ml. of soln., Gm.	1.725	1.225	3.05	...
Ion-exchange capacity of clay, meq./Gm.	0.583	0.583	0.583	0.583
Drug exchanged with cation in clay, meq.	1.006	0.714	1.778	...
Drug adsorbed by clay, meq.	−0.017	0.201	0.002	...

of clay added. Finally, when all drug in the solution was removed by the clay, the weight of the residue-exchange product from the clay ceased to exchange with further addition of the clay. Each curve shows two distinct straight segments. The intersection at which the extensions of these two straight portions meet shows the weight of clay required to pick up all the drugs from the solution.

Table V illustrates the amounts of drug exchanged with the cations of the clay and the amount of drug adsorbed by the clay. The "initial concentrations of the drugs" and the amount of "clay required to absorb all of the drug in 50 ml. of solution" are obtained from Figs. 2–5. The "ion-exchange capacity of the clays" is obtained from the previous experiment. The "drug exchanged with the cation of the clay" is calculated by multiplying the "ion-exchange capacity" by "the amount of clay required to absorb all of the drug in 50 ml. of solution."

Table V shows that the clay quantitatively picks up methapyrilene and triethylamine by an ion-exchange reaction, and brucine partly by ion-exchange and partly by an adsorption reaction. Niacinamide was neither adsorbed by nor exchanged with the cations of the clay.

Brucine sulfate, being a large molecule, is picked up by the clay to an extent of 35% above the cation-exchange capacity of the clay; yet, triethylamine hydrochloride, being a small molecule, is picked up by the clay quantitatively with respect to the ion-exchange capacity of the clay. This is a further strong indication that the absorption of triethylamine by the clay is not a surface phenomenon of the clay. It is, instead, ion exchange.

Niacinamide, being an extremely weak acid, may dissolve in the alcohol in the form of niacinamide base, rather than the ionic form of its hydrochloride salt. Another possibility may be that the positive charge on the niacinamide cation may resonate and spread throughout the molecule, and weaken the charge of the cations, so that they cannot replace the original cations on the clay which are strongly charged. This may explain why niacinamide was not bound by the clay.

DISCUSSION AND CONCLUSIONS

The swelling of montmorillonite depends upon two main mechanisms—namely, adsorption and osmosis. The swelling by adsorption is illustrated by the adsorption of moisture, glycerol, and brucine by the clay. The swelling of montmorillonite by osmosis can be illustrated by the depression of the swelling volume of the clays by electrolytes.

The data on the measurements of specific surface area of the clays are the best proof of particle swelling of clays by adsorption. The glycerol and brucine actually penetrate into the interior of the clay particles. The adsorption of glycerol, moisture, and brucine to the internal surface of the clay particles results in swelling of the particles of the clay. Since the initial swelling of the clay is essentially by adsorption, the disintegrating activity of the clay on tablets may be hypothesized as primarily due to the adsorption swelling rather than osmotic swelling of the clay. If this hypothesis is true, then, many other clays which are good adsorbants may also be good disintegrating agents.

The cation-exchange activity of montmorillonite occurs at the internal surface of the clay particles. The physical entrapment of the organic molecules between the silica–alumina–silica layers may explain the strong and partial irreversible binding of organic molecules by clays. One can further theorize that the entrapment of cationic drugs in the interior of the clay particles may be applied to stabilize some unstable drugs and to produce sustained-release medications.

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Modification of Polyethylene Glycol Ointment U.S.P. XVI

By WINITA CHANDRANONDNAIWINIT and E. BLANCHE SOMMERS

The hydrophilic capacity of official polyethylene glycol ointment, modified by the addition of 0.5 per cent of dry unneutralized carboxypolymethylene compound, was increased 48 per cent. The relative effectiveness of the modified ointment as a vehicle for yellow mercuric oxide (1 per cent) and potassium penicillin G (1000 u./Gm.) using *Staphylococcus aureus* PS 81 as the test organism was determined. Antistaphylococcal activity was increased 23.3 and 31.3 per cent, respectively. The stability of penicillin in the modified and official bases was checked for 23 days. Antistaphylococcal activity declined to 18.8 and 13.5 per cent, respectively.

POLYETHYLENE glycol ointment U.S.P. XVI possesses such advantages as esthetic appearance, washability, inertness, and the ability to form an emollient base. However, its high degree of solubility precludes the addition of aqueous solutions much in excess of 5% of the total formula (1).

One of the criteria of an ideal ointment base is that of hydrophilic capacity. According to Beeler (2), various authors have described an ideal ointment base as being capable of holding at least 50% of water.

Due to the lack of this ability on the part of all of the polyethylene glycols, various additives have been tried with limited degree of success. For example, the addition of 5% of cetyl alcohol to the anhydrous base containing 47.5% each of polyethylene glycol (PEG) 4000[1] and polyethylene glycol 400 has been recommended (3). This addition increased the water absorption ability of the base 10%.

Since the official ointment in most respects is pharmaceutically and dermatologically acceptable except for its lack of water capacity, further study was prompted in regard to this characteristic. Different substances were tried in varying concentrations and carboxypolymethylene compound[2] proved to be the most satisfactory in regard to increasing the hydrophilic capacity of official polyethylene glycol ointment.

EXPERIMENTAL

Addition of Carboxypolymethylene Compound.—Polyethylene glycol ointment U.S.P. XVI was prepared and samples of the official ointment were modified by the addition of carboxypolymethylene compound in concentrations of 0.5, 0.75, 1, 2, and 5%. Aseptic techniques were observed and finished ointments were stored in closed jars at room temperature. The polymer was added according to the following methods.

Method 1.—One-half gram of unneutralized carboxypolymethylene compound reduced to a fine powder was thoroughly triturated into enough polyethylene glycol ointment to make 100 Gm. This process was repeated using 0.75, 1, 2, and 5 Gm. of the polymer.

Carboxypolymethylene compound owes much of its exceptional ability to thicken, suspend, and emulsify to its hydrophilic nature or affinity for water (4). It was used dry and unneutralized in an effort to increase its water absorption ability when present in the ointment base.

Method 2.—Method *1* was varied by adding the respective portions of unneutralized carboxypolymethylene compound to enough liquid polyethylene glycol 400 to make each portion weigh 60 Gm. The powder was first levigated with a small amount of the liquid glycol and successive portions of the latter were added until thorough incorporation was accomplished. Each 60-Gm. portion of the PEG 400 containing its respective concentration of the polymer was then heated with 40 Gm. of PEG 4000 on a water bath to 65°. The preparation was allowed to cool and was stirred until it congealed.

Method 3.—Carboxypolymethylene compound (0.5 Gm.) was neutralized with a solution of sodium hydroxide (10%). In order to avoid the presence of water to the greatest possible degree, the dry polymer was mixed with 10 ml. of the official ointment before it congealed. One milliliter of the sodium hydroxide solution was added and the mixture was allowed to stand for 30 min. It became very gelatinous, but readily liquefied when placed on a water bath, and it was then incorporated into the remaining liquid official ointment to make a total of 100 Gm.

The resulting preparation was darker and considerably stiffer than the official ointment. For this reason further modification of the official ointment by the addition of increased concentrations of the neutralized carboxypolymethylene compound was not attempted. Products of methods *1* and *2* were evaluated. The 0.5% formulation prepared by trituration of dry unneutralized carboxypolymethylene compound into the official ointment was

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[1] Marketed as Carbowax 4000 by Union Carbide Corp., New York, N. Y.

[2] Marketed as Carbopol 934 by B. F. Goodrich Chemical Co., Cleveland, Ohio.

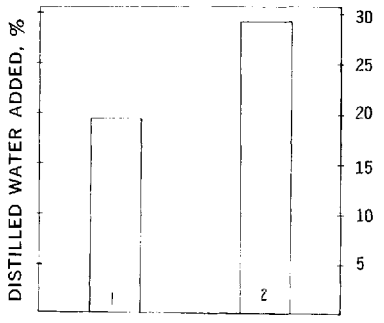

Fig. 1.—Comparison of absorption of water by PEG ointment U.S.P. (*1*); PEG ointment U.S.P. with 0.5% of dry unneutralized carboxypolymethylene compound (*2*).

selected as the most cosmetic in texture and appearance.

Hydrophilic Properties.—The official ointment and that modified with 0.5% of carboxypolymethylene compound were tested for hydrophilic capacity. Distilled water was added dropwise with trituration from a calibrated dropper to samples of each ointment until the point of flow was reached. Standardized point of flow was attempted in each instance by the delivery of a specified volume of the sample in a specified time from the same container.

After the initial hydration, the ointments thickened on standing. When this occurred, water was again added to the ointments in the same manner until they did not change consistency on standing for 30 days. Results of the initial hydration are shown in Fig. 1.

Relative consistency of the ointments was determined by the use of a rheostat connected to an electrically driven stirring device. The blades of the latter were submerged in the ointment under standardized conditions. The electrical power was slowly increased and the rheostat reading was taken at the first perceptible indication of the ointment's yield to the blades of the stirring device. Repeated experimental determinations gave values that differed within acceptable limits.

After the initial hydration, the samples of polyethylene glycol U.S.P. XVI containing an average of 19.6% of distilled water regained their original consistency in an average of 5.5 days on standing in tightly closed containers at room temperature. When compared to samples of the modified ointment containing the same amount of distilled water, the latter regained their original consistency in an average of 1 day. Distilled water was again added to the samples of the official ointment containing 19.6% of distilled water until each reached the point of flow. This time an average of 10.4% water/sample was required making a total of 30% of distilled water that was added. After standing 30 days, thickening occurred to a very slight degree.

The samples of the ointment modified by the addition of 0.5% of carboxypolymethylene compound, that contained an average of 29% water when initially hydrated to the point of flow, were stored in the same manner for 30 days. They thickened considerably but did not regain their original consistency.

Using fresh samples of the modified ointment, distilled water was added to each sample to the point of flow. This was repeated using the same samples until, on standing 30 days, these preparations did not thicken. A total of 34% water was required.

Evaluation as Vehicles for Yellow Mercuric Oxide.—The official and the modified ointments were tested to determine their relative effectiveness as bases for yellow mercuric oxide. One per cent of the latter was incorporated into samples of each

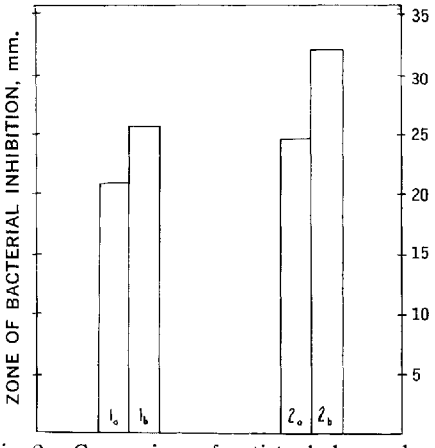

Fig. 2.—Comparison of antistaphylococcal activity. Key: 1_a, PEG ointment U.S.P. with mercuric oxide, 1%; 1_b, PEG dry unneutralized carboxypolymethylene compound ointment with mercuric oxide, 1%; 2_a, PEG ointment U.S.P. with potassium penicillin G, 1000 u./Gm.; 2_b, PEG dry unneutralized carboxypolymethylene compound ointment with potassium penicillin G, 1000 u./Gm.

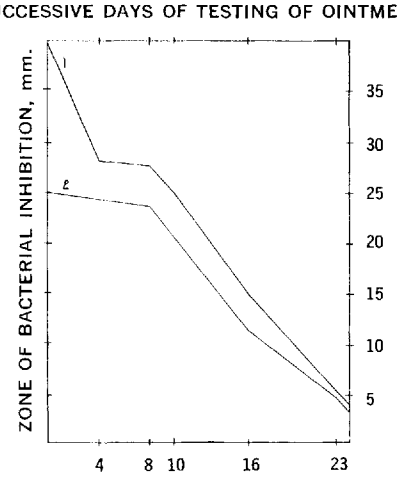

Fig. 3.—The rate of decline of potency of (*1*) PEG dry unneutralized carboxypolymethylene compound ointment with potassium penicillin G, 1000 u./Gm.; (*2*) PEG ointment U.S.P. with potassium penicillin G, 1000 u./Gm.

of the ointments by trituration until homogeneous composition was obtained. Aseptic techniques were observed.

A solution of nutrient agar in distilled water (2.3%) was prepared, autoclaved, and cooled to 45°. Each of 20-ml. samples of this solution was inoculated with 0.2 ml. of *S. aureus* PS 81 which had previously been incubated for 18 hr. at 37° in an aqueous solution of thioglycollate (1%). The 20-ml. samples were then poured into sterile Petri dishes and allowed to harden, after which 0.5 Gm. of the official ointment containing yellow mercuric oxide (1%) was placed in the center of the nutrient agar in each plate. This procedure was repeated using the modified ointment containing yellow mercuric oxide (1%).

These preparations were incubated at 37° and read after 48 hr. Bacterial inhibition zones were measured from the edge of the ointment to the edge of the zone of complete inhibition. Controls consisting of the nutrient agar, the official, and the modified ointments were prepared. They showed no zones of bacterial inhibition. Results are shown in Fig. 2.

Evaluation as Vehicles for Potassium Penicillin G.—Ointments of potassium penicillin G (1000 u./Gm.) were prepared using the official and the modified bases. The penicillin was incorporated into the bases by the same method used in the preparation of the mercuric oxide ointments.

Effectiveness of the two bases as vehicles for the penicillin was tested by the same method used for testing the yellow mercuric oxide ointments. Results are shown in Fig. 2.

Stability Studies.—The stability of potassium penicillin G in the official and in the modified bases was tested. The ointments, after preparation, were immediately stored in tightly closed containers at room temperature. Using *Staphylococcus* PS 81 as the test organism and the previously described media and techniques, the ointments were tested at 1, 4, 8, 10, 16, and 23-day intervals for potency of their contained penicillin. Results are shown in Fig. 3.

DISCUSSION AND CONCLUSIONS

Hydrophilic Capacity.—According to this investigation, the initial hydrophilic capacity of the official polyethylene glycol ointment (19.6%) is greater than that of 8–10% reported by the literature, and the addition of 0.5% of carboxypolymethylene compound increased this capacity to 29%.

However, a total of 30% water was added to the official ointment at successive times before it ceased to thicken when stored 30 days. When 29% water was initially added to the modified ointment, it thickened on standing, but did not regain the original consistency on standing for the same period of time. The addition of a total 34% of water was required before the modified base showed no change when stored for 30 days.

The changes that occurred in the consistency of the ointments on standing might be considered as thixotropic in nature. However, thixotropy is customarily demonstrated by a 2-phase system. Polyethylene glycol ointment is an organogel and thus exists as a 1-phase system (5).

It is important to note that total hydration (30%) of the official ointment and the initial hydration (29%) of the modified ointment were approximately the same. Also, the total hydration of the latter was 34%. It is probable that apparent increased hydration of the official ointment when carboxypolymethylene compound was added was predominately due to increased rate of hydration caused by the polymer.

Incomplete hydration (8–10%) could account for the observation by King and Sheffield (6) that "The polyethylene glycol ointments are not completely satisfactory cosmetically . . . ."

Evaluation of Bases for Yellow Mercuric Oxide and Potassium Penicillin G.—Polyethylene glycol ointment U. S. P. XVI and its modified form containing 0.5% carboxypolymethylene compound were suitable bases for mercuric oxide (1%) and potassium penicillin G (1000 u./Gm.). When the anti-infective agents were added to the official and the modified bases and the finished preparations were tested, both produced zones of bacterial inhibition; however, the modified base was superior to the official base.

Controls of the official ointment and of the modified ointment containing 0.5% carboxypolymethylene compound did not demonstrate anti-infective activity. The polymer did not possess anti-infective activity in this instance, but when it was added to the official ointment containing mercuric oxide or penicillin, the anti-infective activity of the ointment was increased. These results suggest that increased anti-infective activity that occurred when the polymer was present was not due to the polymer alone but to direct relationship of the polymer to the anti-infective agent.

Thus, the superiority in regard to anti-infective activity of the modified base when compared to the official base could be due to the ability of the polymer to act as an emulsifying agent and in this manner increase the ratio of anti-infective/bacteria exposure.

Stability of Potassium Penicillin G in the Official and Modified Bases.—The PEG's have useful roles as vehicles for penicillins (7). However, there is difference of opinion concerning the effect of these compounds on the penicillins. Some investigators (8, 9) have reported that the presence of these compounds hastens the decomposition of the antibiotic, while others (10) have found it to be remarkably stable in PEG bases. In studying this problem, Ferlanto and Clymer (11) found that penicillin stability varies with the vehicle and with the particular salt used.

Numerous references are found in the literature in regard to the stability of penicillin in polyethylene glycol bases. However, none of these was concerned with the effect of carboxypolymethylene compound when present as a component of the polyethylene glycol base. It was decided to determine that effect.

According to the data of this investigation, the potency of the penicillin in the official and the modified bases when stored at room temperature declined to 18.3 and 13.5% of their original activity at the end of 23 days. This decline was fairly gradual and consistent except for the rapid decline of the penicillin potency of the modified base during the first 4 days. On further testing, this occurred again and remains unexplained.

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Investigation of Factors Influencing Release of Solid Drug Dispersed in Inert Matrices II

Quantitation of Procedures

By SAURABH J. DESAI*, PARVINDER SINGH, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

Recently a number of factors governing the rate of release of drug from plastic matrices were investigated. This study showed that while the experimental results were generally in agreement with Higuchi's relationship, they were not always quantitative. The present paper describes a refined experimental procedure for quantitatively studying the various factors. Matrix porosities are determined in two ways so that available and inaccessible pores can be differentiated. Diffusion coefficients are independently determined. The matrix tortuosity can now be quantitatively determined independently of the solid drug release rate data. In addition to these experimental refinements, the limitations of the theory are reviewed and some useful modifications proposed.

A PREVIOUS communication (1) discussed preliminary results on the investigation of the factors influencing drug release from solid drugs dispersed in inert matrices. An attempt was made in that study to compare experimental release rate data to the Higuchi relationship (2). While it was found that qualitative and semiquantitative comparisons between theory and data could easily be made, considerable difficulty was generally encountered when a quantitative test of the theory with data was attempted.

It was believed that much of the difficulty was due to the porosity and the tortuosity of the matrix not being independent of the other variables and changing from experiment to experiment. For example, these studies (1) showed that a small amount of surfactant in the solvent phase could markedly increase the release rate from the polyethylene plastic matrix. It was shown that this was not an increased solubility effect, and therefore, must be related to the porosity or tortuosity factors.

It has now become apparent that, in order to clearly understand the basic mechanisms involved, a more systematic study must be undertaken. Wherever possible, each of the parameters in the theory should be quantitated independently and then incorporated into the theory to see whether the equation accurately predicts the rate. Then when discrepancies occur, real or apparent, physical interpretations that are meaningful may be assigned.

The purpose of this paper is to present details of methods, both theoretical and experimental, designed for the quantitative physical evaluation of the various factors involved in drug release from nondisintegrating matrices. It will be shown that these techniques should permit the unambiguous interpretation of release rate data in most instances.

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* Recipient of Eli Lilly Fellowship.

THEORY

The basic Higuchi relationship (2) for the rate of diffusional release of drug incorporated as solid drug in an insoluble matrix, from one surface of the matrix, is

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A PREVIOUS communication (1) discussed preliminary results on the investigation of the factors influencing drug release from solid drugs dispersed in inert matrices. An attempt was made in that study to compare experimental release rate data to the Higuchi relationship (2). While it was found that qualitative and semiquantitative comparisons between theory and data could easily be made, considerable difficulty was generally encountered when a quantitative test of the theory with data was attempted.

It was believed that much of the difficulty was due to the porosity and the tortuosity of the matrix not being independent of the other variables and changing from experiment to experiment. For example, these studies (1) showed that a small amount of surfactant in the solvent phase could markedly increase the release rate from the polyethylene plastic matrix. It was shown that this was not an increased solubility effect, and therefore, must be related to the porosity or tortuosity factors.

It has now become apparent that, in order to clearly understand the basic mechanisms involved, a more systematic study must be undertaken. Wherever possible, each of the parameters in the theory should be quantitated independently and then incorporated into the theory to see whether the equation accurately predicts the rate. Then when discrepancies occur, real or apparent, physical interpretations that are meaningful may be assigned.

The purpose of this paper is to present details of methods, both theoretical and experimental, designed for the quantitative physical evaluation of the various factors involved in drug release from nondisintegrating matrices. It will be shown that these techniques should permit the unambiguous interpretation of release rate data in most instances.

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THEORY

The basic Higuchi relationship (2) for the rate of diffusional release of drug incorporated as solid drug in an insoluble matrix, from one surface of the matrix, is

$$Q = \left\{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t\right\}^{1/2} \quad \text{(Eq. 1)}$$

where Q is the grams of drug released per unit area of surface at time, t, D is the diffusion coefficient of drug in the release medium, ϵ is the porosity of the matrix, C_s is the solubility of drug in the release medium, τ is the tortuosity of the matrix, and A is the concentration of drug in the tablet expressed as Gm./ml.

Some comments are now appropriate regarding Eq. 1. Most of these were pointed out in the original work (2).

The porosity, ϵ, refers to the volume fraction that is permeated by the solvent and available for diffusion in the already leached portion of the matrix. Therefore, in general,

$$\epsilon = \epsilon_d + \epsilon_{\text{air}} + \epsilon_{\text{other}} \quad \text{(Eq. 2)}$$

Here $\epsilon_d = \frac{A}{\rho_d}$ is the contribution to the porosity from the dissolved drug where ρ_d is the drug crystal density. The other two terms in Eq. 2 are the contributions to porosity from released air and from the leaching of other soluble additives in the mixture. As we shall see later, ϵ_{air} available for solvent penetration and drug diffusion, is very sensitive to the presence of surfactants with certain matrices.

The tortuosity factor, τ, corrects for the lengthened diffusional path caused by the necessary lateral excursions. In other words, it accounts for, or corrects for the additional distance a molecule must travel due to its circuitous path within the tablet. A straight channel will have a tortuosity of 1, whereas a spherical glass bead column will have a τ value of about 2 to 3.

It will be seen later that in some situations extremely large τ values ($\sim 10^3$ to 10^4) are encountered. In these cases the concept of the average porosity and the average tortuosity does not adequately describe physically the pathways and resistances for diffusion, and a more detailed consideration of the microscopic matrix permeability factors becomes desirable.

As was originally stressed (2), the model leading to Eq. 1 should fail when $\epsilon C_s \gtrsim 2A$. To derive a general analytical expression which includes the large ϵC_s cases appears to be extremely difficult because the pseudo steady-state assumption cannot be made. It appears safe to state that, as long as $2A$ is more than about 3 times greater than ϵC_s, the model should be quantitatively meaningful. The authors' initial quantitative studies of Eq. 1 will therefore be limited to those cases involving solutes of low to moderate solubilities.

Another limitation of Eq. 1 is that it does not explicitly account for the effects of the diffusional movement of the solvent or for the possibility that the solute diffusion coefficient may be concentration dependent in the diffusion barrier. Both of these factors could become important when C_s is moderate to large, say $\gtrsim$ 0.1 Gm./ml. The modified equation (see *Appendix* for the derivation) taking these factors into account may be written

$$Q = \left\{\frac{D'\epsilon C_s}{\tau} t\left[2A - 2\epsilon\int_0^{C_s}\frac{DC\,dC}{D'C_s - KC}\right]\right\}^{1/2} \quad \text{(Eq. 3)}$$

where D' is the effective (or apparent) diffusion coefficient that takes into account both of the effects mentioned above. As will be shown later D' may be conveniently determined by a single run in a conventional diffusion cell.

The term in Eq. 3 involving the integral has the same physical meaning as the $\frac{1}{2}\epsilon C_s$ term in Eq. 1. It represents, therefore, the solution holdup of solute in the leached matrix. This integral term has a value between $\frac{1}{2}\epsilon C_s$ and ϵC_s and may be approximately evaluated by methods discussed in the *Appendix*.

It should be pointed out that Eq. 3, like Eq. 1, breaks down when

$$\epsilon C_s \gtrsim 2A$$

However, it should extend the quantitative applicability of the theory to much larger C_s values than Eq. 1.

The effect of solute binding has not been included in Eq. 1. For the case in which binding to the matrix is linear, *i.e.*, constant partition coefficient, a modified equation may be derived by the same mathematical procedure used previously (2). One has in this case

$$Q = \left\{\frac{\epsilon DC_s}{\tau}[2A - C_s(\epsilon + K - K\epsilon)]t\right\}^{1/2} \quad \text{(Eq. 4)}$$

where $K = \dfrac{\text{(drug in matrix phase)}}{\text{(drug in solvent)}}$ at equilibrium.

Equation 4 assumes equilibrium binding and takes into account the same factors included in Eq. 1. The cases for time dependent binding or nonlinear binding would be much more difficult to handle mathematically.

It can be seen from Eq. 4 that unless the product, KC_s, is a significant fraction of A, the effect of binding should not be very important.

EXPERIMENTAL METHODS

Diffusion Coefficient.—The method used in these studies is, in principle, the same as that employed by McBain (3). Essentially, it involves measurement of the solute transfer rate through a sintered glass disk from one chamber to another.

The apparatus is shown schematically in Fig. 1. It consists of a porous sintered glass disk (E) mounted between two 150-ml. conical flasks (C) with side arms. One of the flasks is closed with a ground glass stopper, and the other with a special adapter (B). Stirring of the solution is achieved by using magnetic stirring bars (D). The entire apparatus is water jacketed (F) to maintain constant temperature.

The following procedure was followed. Before the beginning of each experiment, the glass disk was flushed with water to remove entrapped air. This was accomplished by filling one of the flasks with water and then applying pressure over it or by applying vacuum to the other flask. This step was an important one because it was assumed that identical conditions were maintained from one experiment to another.

Also, a few minutes before the experiment the drug solutions were heated in a flask to boiling and then cooled rapidly to within 10° of the temperature of the experiment. This step greatly helped to eliminate the development of gas bubbles in the solution chamber during the experiment.

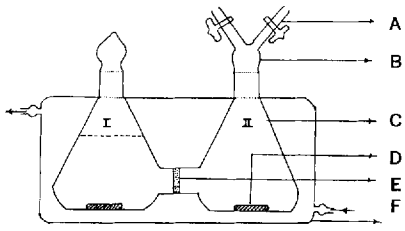

Fig. 1.—Schematic diagram of apparatus used to determine diffusion coefficients. Sintered glass disk diameter = 30 mm., thickness = 2.5 mm., and pore size = 4.0–5.5 μ. (See text for detailed description.)

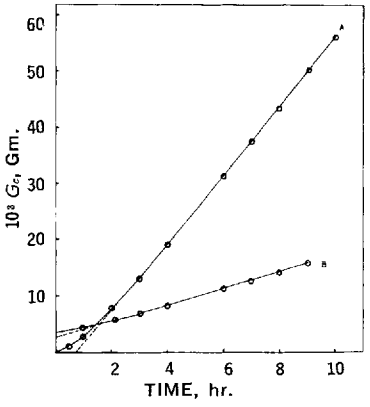

Fig. 2.—Typical diffusion runs with the apparatus. Curve A gives data for 6.8% sodium salicylate solution in flask II and water in flask I initially. Curve B gives data for saturated caffeine solution in flask II and water in flask I initially.

A measured amount of water was added to flask I, and simultaneously the drug solution was added to flask II keeping the levels of the liquids in the two flasks approximately the same. When the water addition to flask I was completed, flask II was quickly filled to the top and the adapter (B) was placed in position. More solution was added through one of the arms (A) keeping the stopcock of the other arm open for air displacement. Because the last traces of air were difficult to remove, the last few milliliters of solution were added by means of a fine-tipped pipet passed through the bore of the stopcock. After all of the visible air had been removed, the stoppers of the adapter were closed tightly and the magnetic stirring bars were started in both flasks. Samples were withdrawn at various time intervals for analysis.

The solution concentration in flask II was determined before and after each experiment. In most instances the changes, as expected, were negligible during the runs.

The cell constant, L, was determined using KCl solutions and the following relationship,

$$L = \frac{G_{KCl}}{D_{KCl}(C_2 - C_1)}$$

where C_2 and C_1 (with $C_1 \approx 0$) were the KCl concentrations in flasks II and I, respectively, D_{KCl} is the diffusion coefficient for KCl, and G_{KCl} is the KCl transport rate in the experiment.

A D_{KCl} value of 2.09×10^{-5} cm.^{2} sec.$^{-1}$ (4) was used with 0.10 M KCl solutions. G_{KCl} was determined by K^+ analysis using the Perkin-Elmer atomic absorption spectrophotometer model 303.

For the present apparatus it was found that at 30°

$$L = 2.45 \pm 0.10 \text{ cm.}$$

This value was also checked with benzoic acid solutions and the agreement was satisfactory using King's value for the diffusion coefficient for benzoic acid (5).

For the unknown solutes the diffusion coefficients were calculated from the data using the equation

$$D_e = \frac{G_e}{L\Delta C} \qquad \text{(Eq. 5)}$$

where G_e is the rate of solute transport and ΔC is the concentration difference between the two flasks.

It must now be pointed out that the D_e value obtained by means of experiment and Eq. 5 is the appropriate apparent diffusion coefficient to be used in either Eqs. 1 or 3 when $\Delta C = C_s$. This identity can easily be seen (Eq. 17a in *Appendix*) by examining the theory for the diffusion cell experiment in the same way as was done in the derivation of Eq. 3.

The direct use of the experimentally obtainable diffusion coefficient, D_e, in the theory for drug release from the matrix conveniently allows the absolute test of Eqs. 1 or 3 when ϵ and τ values are available from the measurements described later.

In Fig. 2 are given typical diffusion cell experimental data for two experiments. If the linear portions of the curves are extrapolated, it can be seen that in one curve (B) a positive intercept is obtained, while in the other curve (A) the intercept is negative. The magnitude of the intercept and whether it is positive or negative depends upon how the diffusion experiment is started. In the calculation of D_e the intercept is disregarded and only the straight line, steady-state portion of the data, is used.

Table I presents some D_e values determined by this method.

Solubility Determination.—An amount of drug, in excess of its reported solubility was placed in

Table I.—Diffusion Coefficients and Solubilities of Some Compounds Used in This Work

Drug	Solubility 10^2 Gm./ml.	Diff. Coeff. 10^6 cm.^{2}/sec.	Concn. of Soln. Used to Determine Diff. Coeff.
Sulfanilamide	1.08	12.9	1.08%[a]
Caffeine	2.50	6.3	2.50%[a]
Potassium acid phthalate	11.60	18.2	11.60%[a]
Sodium salicylate	65.00	23.1	65.00%[a]
Sodium salicylate	65.00	10.0	6.80%

[a] Saturated solutions.

TABLE II.—DATA INVOLVED IN THE DETERMINATION OF POROSITY FROM PHYSICAL MEASUREMENTS OF THE TABLET AND ITS COMPONENTS

Tablet Compn.	Wt. of Tablet, Gm.	I Vol. of Drug w/ρ	II Vol. of Plastic w/ρ	III Vol. of Tablet $\pi r^2 h$	IV Vol. of Air III − (I + II)	V Vol. of Air + Drug I + IV	ϵ V/III
5% Sodium salicylate	0.500	0.0159	0.5000	0.5820	0.0661	0.0820	0.113
10% Sodium salicylate	0.500	0.0318	0.4737	0.5625	0.0570	0.0888	0.158
20% Sodium salicylate	0.500	0.0637	0.4210	0.5340	0.0493	0.1130	0.211
20% Potassium acid phthalate	0.300	0.0368	0.2500	0.3310	0.0546	0.0914	0.275
20% Caffeine	0.300	0.0422	0.2500	0.3351	0.0429	0.0851	0.254
20% Sulfanilamide	0.300	0.0400	0.2500	0.3280	0.0380	0.0780	0.237

each of several 100-ml. volumetric flasks and 50 ml. of solvent was added. The flasks were shaken in a Burrell wrist action shaker for 24 hr. and immersed in a water bath maintained at 30°. These were then filtered with a Millipore filtering unit and the filtrate was analyzed spectrophotometrically. A rapid filtering process was adopted to prevent the precipitation of drug from the saturated solution during filtration. Solubility of the compounds investigated are reported in column 2 of Table I.

Drug-Matrix Partition Tendencies.—Where distribution of drug in the matrix was suspected, saturated solutions of the drug were shaken overnight with the matrix material. High slurry densities were generally employed to increase the sensitivity of this method for estimating K (Eq. 4).

Porosity.—In order to have a porosity value that could be reliably used in Eqs. 1 or 3, two independent methods were used to estimate this quantity. The first method involved calculating the maximum possible contribution to ϵ by air in the tablets. The resulting ϵ value would be the correct one to use in Eqs. 1 or 3 only if all of the air spaces were permeated by the solvent and became available during the drug release process.

From knowledge of the tablet volume, the densities of the drug and matrix material (and other additives, if any), and the weight percentages of all the components, these calculations were carried out. The tablet volumes were computed from tablet dimensions determined with a micrometer and the densities were determined with the Beckman air compression pycnometer.

Some typical data for polyethylene matrix–drug tablets are presented in Table II. The last column gives the porosities calculated by this procedure.

In the second method for estimating ϵ the tablets were completely leached of the solute, and the empty matrices were equilibrated with a dilute solution of a known concentration. The equilibration times depended upon the matrix permeabilities, but usually 1 to 2 weeks was adequate. These resaturated matrices were then exposed to fresh solvent after a brief rinse, and the total amount of solute released determined from the release time data (Figs. 3 and 4). These steps were carried out as described under *Tortuosity*.

Table III presents some of the data with the polyethylene plastic matrix. Columns 4 and 5 of Table

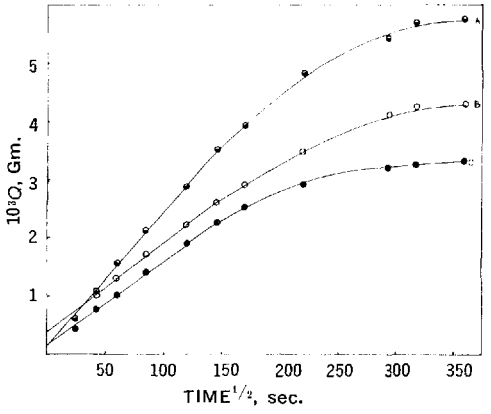

Fig. 3.—Solute release data from polyethylene plastic matrices used in the calculations of porosity and tortuosity. Solute release into 0.2% benzalkonium chloride of sodium salicylate from matrices equilibrated with 5% sodium salicylate solutions. Curves A, B, and C correspond to disks that originally contained 20, 10, and 5% solid sodium salicylate, respectively, and which were leached completely in 0.20% benzalkonium chloride solution.

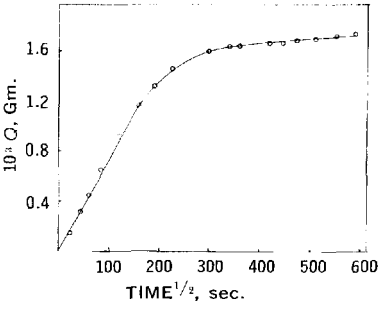

Fig. 4.—Release of caffeine into 0.10% dioctyl sodium sulfosuccinate (AOT) from a polyethylene plastic matrix which was equilibrated with a saturated caffeine solution. The matrix originally contained 20% solid caffeine which was leached completely in 0.10% dioctyl sodium sulfosuccinate (AOT) solution.

TABLE III.—POROSITY AND TORTUOSITY VALUES FOR SOME DRUG–POLYETHYLENE SYSTEMS DETERMINED BY RELEASE FROM SOLUTION SATURATED MATRIX METHOD

Tablet Compn.	Leached Out, mg.	Concn. of Saturated Soln., 10^2 Gm./ml.	ϵ by Liquid Leaching Method	ϵ by Physical Measurement	τ from Liquid Leaching
20% Caffeine in polyethylene	2.21	2.50	0.264	0.254	6.4
20% Sodium salicylate in polyethylene	7.09	5.12	0.258	0.211	2.9
10% Sodium salicylate in polyethylene	4.75	5.12	0.164	0.158	4.6
5% Sodium salicylate in polyethylene	3.92	5.12	0.130	0.113	4.8

III compare the two methods for determining ϵ. The agreement of this instance was very satisfactory.

As will be seen in a later communication, the agreement between the two procedures is not always so good. In these cases incomplete displacement of air appears to be the cause of the discrepancies.

It should be pointed out that good agreement between these two methods does not guarantee correctness of the ϵ value for the solid leaching process because air release may occur but only slowly during the solid release experiment. Good agreement between the two methods for determining ϵ does assure, however, a good reference point for evaluating the data by means of Eqs. 1 or 3.

Tortuosity.—The procedure for the determination of the tortuosity, τ, has been described previously (1). It is based upon the use of the following equation for the release of solute from one planar surface of a solution-saturated matrix

$$Q' = 2\epsilon C_0 \left(\frac{Dt}{\tau\pi}\right)^{1/2} \qquad \text{(Eq. 6)}$$

Here Q' is the amount of solute released per cm.^{2} at time, t, ϵ is, as before, the porosity defined by Eq. 2 and determined by the procedures described above, D is the diffusion coefficient obtainable by the experiment described earlier, and C_0 is the solution concentration.

Equation 6 was deduced for the present situation from the general equation for the release from a semi-infinite medium (6). Therefore, it would be quantitatively applicable for initial rates ($\simeq$ up to 30% release) only.

From the slopes of the initial linear portions of the Q' *versus* $t^{1/2}$ plots, τ may be calculated by means of Eq. 6 as

$$\tau = \frac{4\epsilon^2 C_0^2 D}{\pi\,(\text{slope})^2} \qquad \text{(Eq. 7)}$$

A typical plot is presented in Fig. 3 and some calculated τ values are given in the last column of Table III.

APPENDIX

Derivation of Eq. 3.—When steady-state diffusion takes place from a region of relatively high solute concentration toward essentially pure solvent, the movement of the solvent must be considered also in the problem. In the present problem when the solute is diffusing from $X = s$ to $X = 0$, the solvent, which is usually water in the authors' studies, will set up a concentration gradient, $\frac{dC_w}{dX}$ in the opposite direction and a tendency for solvent diffusion will be established.

However, the net movement of the solvent, G_w, must be zero, because at $X = s$ the solvent is not diffusing into the unleached region at steady state. Consequently, this must result in a bulk solution flow in the channels of velocity, v, from $X = s$ to $X = 0$. The important consequence of this bulk flow is that the solute transport rate is greater by $\frac{\epsilon}{\tau} vC$ than that given by Fickian diffusion alone. Mathematically we may write

$$0 = G_w = \frac{\epsilon}{\tau} D_w \frac{dC_w}{dX} + \frac{\epsilon}{\tau} vC_w \qquad \text{(Eq. 1}a\text{)}$$

and

$$G = \frac{\epsilon}{\tau} D \frac{dC}{dX} + \frac{\epsilon}{\tau} vC \qquad \text{(Eq. 2}a\text{)}$$

Here G is the solute transport rate, D_w is the diffusion coefficient of the solvent, C_w and C are, respectively, the solvent and the solute concentrations at X, and the other terms have already been defined.

Let us solve these equations assuming that both D and D_w are concentration dependent. The final results would then be more general and applicable even when appreciable viscosity changes are encountered or when significant solute–solute interactions occur.

Integrating Eq. 1a from $X = 0$ to $X = s$ one obtains

$$v \int_0^s dx = -\int_{C_w^0}^{C_w^s} D_w \frac{dC_w}{C_w}$$

whence,

$$vs = \int_{C_w^s}^{C_w^0} \frac{D_w dC_w}{C_w} = K \qquad \text{(Eq. 3}a\text{)}$$

where C_w^0 and C_w^s are the solvent concentrations at $X = 0$ and $X = s$, respectively. K is constant for a given solvent–solute pair at a given temperature when diffusion takes places from a saturated solution into pure solvent—the situation which is of most interest.

Equation 2*a* may now be solved after substituting for v from Eq. 3*a*. Integrating Eq. 2*a* from $X = 0$ to $X = s$ where $C = 0$ and $C = C_s$, one obtains

$$\int_0^s dX = \frac{\epsilon}{\tau}\int_0^{C_s} \frac{DdC}{G - \frac{\epsilon K}{\tau s}C} \qquad \text{(Eq. 4a)}$$

Since the left side of Eq. 4*a* becomes s, we have

$$s = \frac{\epsilon s}{\tau}\int_0^{C_s} \frac{DdC}{Gs - \frac{\epsilon}{\tau}KC} \qquad \text{(Eq. 5a)}$$

The s cancels, and

$$1 = \frac{\epsilon}{\tau}\int_0^{C_s} \frac{DdC}{Gs - \frac{\epsilon}{\tau}KC} \qquad \text{(Eq. 6a)}$$

For Eq. 6*a* to be true the product Gs must not be a function of C. Therefore,

$$Gs = K', \text{ a constant}$$

Now we may proceed as was done previously (2) for the derivation of Eq. 1. The amount of solute release from the matrix per unit area, Q, is

$$Q = As - M \qquad \text{(Eq. 7a)}$$

where M is the amount of drug in the pores from $X = 0$ to $X = s$ as solution. M then is given by

$$M = \epsilon\int_0^s CdX \qquad \text{(Eq. 8a)}$$

Since from Eq. 2*a*, dX is given by

$$dX = \frac{\epsilon D\,dC}{\tau\left(G - \frac{\epsilon K}{\tau s}C\right)} \qquad \text{(Eq. 9a)}$$

$$M = s\epsilon\int_0^{C_s} \frac{\epsilon DCdC}{\tau\left(sG - \frac{\epsilon K}{\tau}C\right)} \qquad \text{(Eq. 10a)}$$

Therefore,

$$Q = s\left\{A - \epsilon^2\int_0^{C_s} \frac{DCdC}{\tau\left(sG - \frac{\epsilon}{\tau}KC\right)}\right\} \qquad \text{(Eq. 11a)}$$

Differentiation of Eq. 11*a* with respect to time gives

$$\frac{dQ}{dt} = \left\{A - \epsilon^2\int_0^{C_s} \frac{DCdC}{\tau\left(sG - \frac{\epsilon}{\tau}KC\right)}\right\}\frac{ds}{dt} \qquad \text{(Eq. 12a)}$$

But

$$\frac{dQ}{dt} = G = \frac{K'}{s} \qquad \text{(Eq. 13a)}$$

Now Eqs. 12*a* and 13*a* may be solved by integrating from $t = 0$ to $t = t$ and from $s = 0$ to $s = s$ to give

$$Q = \left\{2K't\left[A - \frac{\epsilon^2}{\tau}\int_0^{C_s} \frac{DCdC}{K' - \frac{\epsilon}{\tau}KC}\right]\right\}^{1/2} \qquad \text{(Eq. 14a)}$$

If Eq. 14*a* is compared to Eq. 1, we find that K' may be associated with the factor $\frac{D\epsilon}{\tau}C_s$ of Eq. 1. Therefore, an effective (or an apparent) diffusion coefficient, D', may be defined as

$$D' = \frac{K'\tau}{\epsilon C_s} = \frac{Gs\tau}{\epsilon C_s} \qquad \text{(Eq. 15a)}$$

Therefore, Eq. 14*a* becomes

$$Q = \left\{\frac{D'\epsilon C_s}{\tau}t\left[2A - 2\epsilon\int_0^{C_s} \frac{DCdC}{D'C_s - KC}\right]\right\}^{1/2} \qquad \text{(Eq. 16a)}$$

Because Eq. 15*a* is general, this relation may be used to calculate D' from experimental data obtained with a conventional diffusion cell. Thus,

$$D' = \frac{G_c S_c \tau_c}{\epsilon_c C_s} \qquad \text{(Eq. 17a)}$$

where G_c is the measured solute transport rate per unit area of the barrier in a diffusion chamber with a diffusion barrier of thickness, S_c, porosity ϵ_c, and tortuosity, τ_c.

To accurately determine the value of the term involving the integral may be difficult unless accurate D *versus* C data are available. If such data are available and if ϵ and τ are known, then K should first be determined by means of Eq. 6*a* by a numerical integration procedure. Then, again by numerical integration, the integral term in Eq. 16*a* could be computed.

A reasonably accurate estimate of the integral term in Eq. 16*a* could frequently be made by employing a constant D value obtained from a diffusion experiment at low concentration.

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Investigation of Factors Influencing Release of Solid Drug Dispersed in Inert Matrices III

Quantitative Studies Involving the Polyethylene Plastic Matrix

By SAURABH J. DESAI*, PARVINDER SINGH, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

The procedure described in the previous paper has been applied to a number of cases. Release of sulfanilamide, caffeine, and potassium acid phthalate from polyethylene matrix disks into aqueous media were studied. The results show that in these cases the absolute tortuosity values are relatively small when all pores are accessible to the solvent. This situation is achieved, for example, when there is surfactant in the solution permitting adequate wetting of the channels. A direct correlation between surfactant activity and the rate of release has been found. Even when there was a relatively small amount of inaccessible pores, the tortuosities in these cases were found to be considerably greater. The results were obtained when surfactant was not incorporated in the release medium. It is proposed that these findings are consistent with an "encapsulated" drug particle model.

A PREVIOUS INVESTIGATION (1) showed that the Higuchi equation can be utilized to describe drug release from polyethylene matrices. The results of experiments which investigated the effects of drug type, concentration of drug in tablet, solvent media, etc., indicated that the above factors not only altered release rates directly as predicted by theory, but also indirectly by altering other parameters. Before a quantitative evaluation of the polyethylene system could be made, it was therefore necessary to establish experimental procedures to determine independently all parameters for the conditions of each study. In this way the interdependence of all parameters can be eliminated.

Experimental procedures to determine the diffusion coefficient, drug solubility, and matrix porosity were reported in a previous communication (2). These parameters can then be used in the equations which describe the release rates from matrices containing solid medicament and from matrices saturated with a solution of drug.

The release rate of drug imbedded in an insoluble matrix obeys the following equation:

$$Q = \sqrt{\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t} \qquad \text{(Eq. 1)}$$

where

D = diffusion coefficient of the drug in the permeating fluid,
ϵ = the porosity of the matrix,
τ = tortuosity of the matrix,
A = the total amount of drug present in the matrix per unit volume,
C_s = the solubility of the drug in the release medium,
Q = grams of drug released per unit area of surface.

If the same matrix is saturated with a solution, the release rates would obey the following relationship:

$$Q = 2\,C_0\epsilon\left(\frac{Dt}{\tau\pi}\right)^{1/2} \qquad \text{(Eq. 2)}$$

where C_0 = concentration of the solution in the matrix. The other terms have the same meaning as in Eq. 1.

Once the parameters of the matrix have been established, one can use Eq. 1 to determine the remaining parameter, tortuosity, from release data obtained using the matrix containing the solid drug. Equation 2 can also be used to calculate the tortuosity of the same matrix, but the release data obtained from the resaturated matrix must be used. If the tortuosities independently calculated by these methods agree well with each other, this would provide strong evidence that this method of attack can be used to quantitatively study the effect of experimental conditions on the release rates of drugs from inert matrices. Using this approach, this paper reports the effect of drug concentration, drug solubility, and other factors upon the parameters—tortuosity, porosity, and diffusion coefficient.

EXPERIMENTAL

The release rates from polyethylene matrices containing solid drug as well as solutions were studied using the techniques described previously (1). The parameters were evaluated as described in the subsequent paper (2).

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* Recipient of Eli Lilly Fellowship.

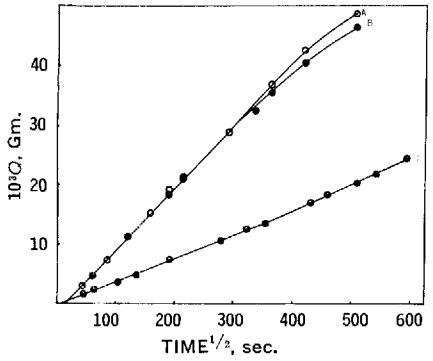

Fig. 1.—Release of potassium acid phthalate from polyethylene plastic matrices containing 20% potassium acid phthalate in various media. Key: A, 0.10% polysorbate 80 as the release medium; B, 0.10% HTAB; C, water.

RESULTS AND DISCUSSION

If a given matrix is studied by both the solid and saturated solution methods, it must be established that all parameters remain constant during the experiments. Since the addition of surfactant to the aqueous solvent media greatly increases the rate of drug release from polyethylene matrices, it had been postulated that all of the maximum possible matrix porosity is made available by the surfactant. This was verified by the fact that the porosity determined by the liquid leaching method (2) was equal to the combined porosity of drug and air. The latter was calculated by the physical measurement method (2). This was not true in nonsurfactant experiments. It was, therefore, decided to use surfactant solutions as the release medium to establish the quantitative applicability of the theory.

Quantitative Application of Theory.—Polyethylene matrices containing 20% sulfanilamide, caffeine, and potassium acid phthalate were studied. Both the solid and liquid leaching experiments were performed utilizing surfactant solutions. It should be pointed out that the compounds were selected on the basis of their suitability for establishing basic physicochemical points rather than their therapeutic value; similarly, the surfactants used were selected on the basis of their surfactant activity and system compatibility. All surfactant solutions used were above their critical micelle concentration to provide maximum surfactant activity.

The surfactant solution used in the sulfanilamide and caffeine studies was 0.1% dioctyl sodium sulfosuccinate[1] (AOT) but 0.1% hexadecyl trimethylammonium bromide solution (HTAB) was used with the potassium acid phthalate studies since the latter was not compatible with AOT.

The solid release curves of these systems are shown in Figs. 1, 2, and 3. Solid leaching curves in water are also included to show the magnitude of the surfactant effect which will be discussed later. Liquid leaching curves of the same matrices are shown in Figs. 4 and 5. To demonstrate the applicability of the solid and liquid leaching concepts, calculations of τ values using Eqs. 1 and 2, respectively, will be shown and compared.

[1] Marketed as Aerosol OT by the American Cyanamid Co., Wayne, N. J.

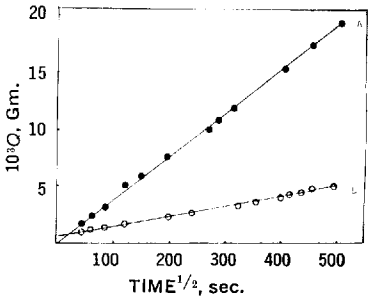

Fig. 2.—Release of caffeine from polyethylene matrices containing 20% drug into 0.10% AOT (A) and into water (B).

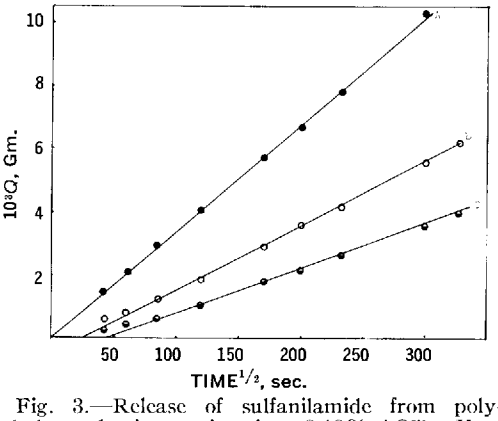

Fig. 3.—Release of sulfanilamide from polyethylene plastic matrices into 0.10% AOT. Key: A, 20% drug in matrix; B, 10% drug; C, 5% drug.

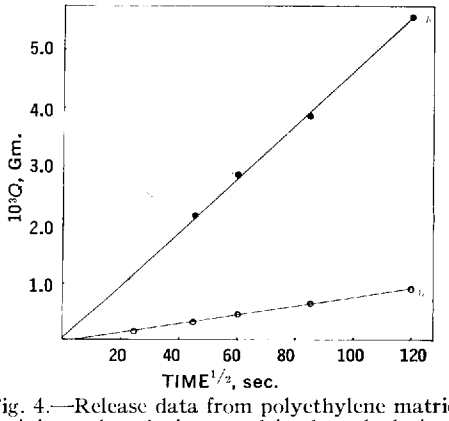

Fig. 4.—Release data from polyethylene matrices containing only solutions used in the calculation of tortuosity. Key: A, tablets originally contained 20% solid potassium acid phthalate which was released completely in 0.10% HTAB, was then equilibrated with a saturated potassium acid phthalate solution, and then released in 0.10% HTAB; B, same as A, except caffeine was used instead of potassium acid phthalate and 0.10% AOT was used instead of HTAB.

The following calculations using the data of a polyethylene matrix containing 20% sulfanilamide

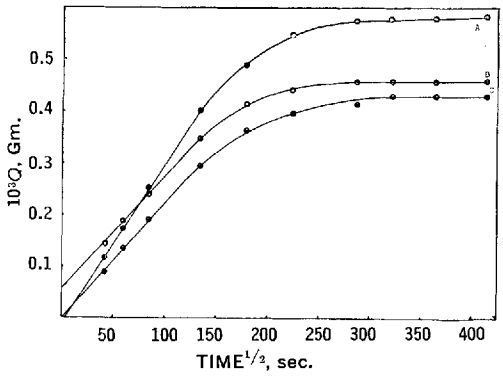

Fig. 5.—Solute release data from polyethylene plastic matrices used in the calculations of porosity and tortuosity. Key: A, tablets originally contained 20% sulfanilamide which was released completely in 0.10% AOT, was then equilibrated with a saturated sulfanilamide solution, and then released in 0.10% AOT; B, same as A, except tablets contained 10% drug; C, same as A, except tablets contained 5% drug.

in a surfactant medium will serve as an illustrative example.

weight of the tablet = 0.300 Gm.

volume of tablet = $\pi r^2 h$ = 0.328 ml.

$C_s = 1.08 \times 10^{-2}$ Gm./ml.

$C_0 = 0.95 \times 10^{-2}$ Gm./ml.

$$A = \frac{\text{wt. of drug}}{\text{vol. of tablet}} = \frac{0.060 \text{ Gm.}}{0.328 \text{ ml.}} = 0.182 \text{ Gm./ml.}$$

$D = 12.9 \times 10^{-6}$ cm.^{2}/sec.

$$\text{volume of plastic} = \frac{0.240}{0.960} = 0.250 \text{ ml.}$$

$$\text{volume of drug} = \frac{0.06}{1.500} = 0.040$$

volume of air = 0.328 − 0.290 = 0.038 ml.

$$\epsilon = \frac{0.038 + 0.04}{0.328} = 0.237$$

$Q/t^{1/2}$ = slope of solid leaching (Fig. 3) = 3.30×10^{-5} Gm./sec.$^{1/2}$

$Q/t^{1/2}$ = slope of liquid leaching (Fig. 5) = 0.303×10^{-5} Gm./sec.$^{1/2}$

Solving Eq. 1 for the value of τ and substituting the values of above parameters one obtains the following

$$\tau = \frac{D\epsilon(2A - \epsilon C_s)C_s}{(\text{slope})^2}$$

$$= \frac{(12.9 \times 10^{-6}) \times (0.237)\,(2 \times 0.182 - 0.237 \times 1.08 \times 10^{-2})(1.08 \times 10^{-2})}{(3.30 \times 10^{-5})^2}$$

$$= 10.7$$

and similarly Eq. 2 yields

$$\tau = \frac{4C_0^2\epsilon^2 D}{\pi\,(\text{slope})^2}$$

$$= \frac{4 \times (0.95 \times 10^{-2})^2 \times (0.237)^2 \times 12.9 \times 10^{-6}}{3.142 \times (0.303 \times 10^{-5})^2}$$

$$= 9.1$$

Comparison of the above values of τ shows excellent agreement and thereby indicates that the theory and the parameters employed are valid. Similar agreement was obtained with caffeine. A similar study with potassium acid phthalate showed a reasonably good agreement; the relatively higher τ value obtained for potassium acid phthalate by the solid leaching experiment may be due to the poorer wetting properties of HTAB compared to AOT. These results are summarized in Table I.

Examination of the ϵ due to air in Table I shows that it does not remain constant, although the same concentration of different drugs is used. This may be a reflection of the relative flow properties of the drug and plastic when under compression.

The above approach was also used to study polyethylene matrices containing 5 and 10% sulfanilamide. The curves are shown in Figs. 3 and 5, and the calculated parameters are listed in Table II.

Examination of Table II clearly shows that the ϵ of the matrix is not a direct function of the drug percentage. Although the porosity contribution from the drug increases with increasing concentration of drug, the porosity contribution from air is decreasing. In addition, there seems to be a slight increase in τ. Again, it should be noted that excellent agreement was shown between the tortuosities calculated from the solid leaching and solution leaching data.

These results explain why the release rates in an earlier study (1) did not follow the predicted rates assuming all parameters were constant and confirm the hypothesis that the porosity was not proportional to A.

Mechanism of Surfactant Action.—The surfactant seems to be instrumental in making available the maximum possible porosity. It was felt, therefore, that the effect should be investigated further. To eliminate the possibility that surfactant may

Table I.—Calculation of Tortuosity of Potassium Acid Phthalate, Sulfanilamide, and Caffeine in Polyethylene Matrices

Tablet Compn.	D 10^6 cm.^{2} sec.$^{-1}$	C_s 10^2 Gm. ml.$^{-1}$	Vol. of Tablet, ml.	ϵ Due to Air	ϵ Due to Air and Drug	A Gm. ml.$^{-1}$	C_0 10^2 Gm. ml.$^{-1}$	10^5Q Solid Leaching	10^5 Q Liquid Leaching	τ by Eq. 1	τ by Eq. 2
20% Potassium acid phthalate	18.2	11.60	0.331	0.165	0.276	0.181	9.30	10.2	4.36	18.5	8.0
20% Caffeine	6.3	2.50	0.264	0.128	0.254	0.179	2.50	3.8	0.71	8.2	6.4
20% Sulfanilamide	12.9	1.08	0.328	0.115	0.237	0.182	0.95	3.3	0.30	10.7	9.1

TABLE II.—POROSITY AND TORTUOSITY OF 5, 10, AND 20% SULFANILAMIDE IN POLYETHYLENE MATRICES USING SURFACTANT SOLUTION AS RELEASE MEDIUM

Tablet Compn.	ϵ Due to Air	ϵ Air Plus Drug	τ by Eq. 1	τ by Eq. 2
5% Sulfanilamide	0.125	0.154	8.5	6.7
10% Sulfanilamide	0.103	0.162	9.6	8.5
20% Sulfanilamide	0.115	0.237	10.7	9.1

solubilize some component of the plastic material, the polyethylene powder was shaken in the presence of excess of surfactant solution overnight. This slurry was then filtered and the polyethylene was washed with hot and cold water. Twenty per cent potassium acid phthalate tablets were made using this washed and then dried polyethylene powder. The release rates in water and surfactant were then studied; results were identical with the unwashed plastic indicating that the surfactant does not solubilize any of the plastic component.

The increase in release rates of drugs from a polyethylene matrix by surfactants seems to be a general effect. It has been shown that not only is this effect present with all the compounds studied but also that it is independent of the chemical nature of the surfactant. This is demonstrated by the identical release behavior of 20% potassium acid phthalate in polysorbate and HTAB solutions (Fig. 1).

The release rates of 20% sulfanilamide tablets were studied in several solution concentrations of AOT. The results obtained are presented in Fig. 6. In order to compare these results with the surfactant property of these solutions, plots of both the release rate and surface tension *versus* the surfactant concentration are shown together in Fig. 7. The data reveal an excellent correlation between the release rates and the surface tension of the release media. It was expected that the release rates would increase with increasing concentration of surfactant because of increased wetting power of surfactants with increased concentration in regions below the critical micelle concentration (CMC). It is interesting that the reported (3) CMC for AOT is 0.08% which is near the point in Fig. 7 where the plateau of the release rate curve begins.

If the above-proposed mechanism of surfactant action is true, then the presence of surfactant either within the tablet matrix or in the release medium could give essentially the same results. On the basis of the same principle, a drug with inherent surfactant activity may yield identical release rates in the presence and in the absence of a surfactant in the release medium.

Release rates of 20% potassium acid phthalate tablets containing 2% HTAB were studied in water. These results are plotted in Fig. 8 along with the results of 20% potassium acid phthalate determined in a solution of 0.1% HTAB. As expected, the release rates were about the same. The slightly higher release rate observed with the former case might be accounted for by small porosity differences due to the incorporation of the surfactant.

To study the second premise, hexadecylpyridinium

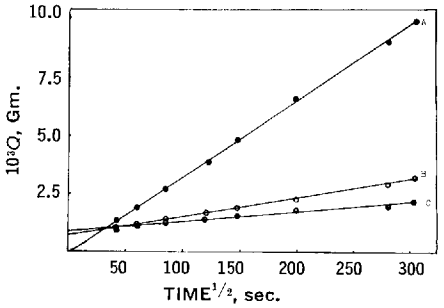

Fig. 6.—The effect of surfactant concentration of the release rate of sulfanilamide from a polyethylene plastic matrix containing 20% sulfanilamide. Key: A, 0.10, 0.50, and 1.0% AOT solutions; B, 0.05% AOT; C, 0.01% AOT.

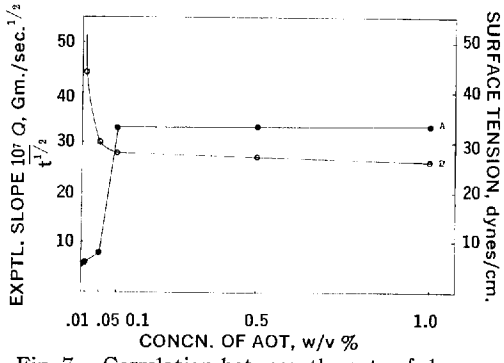

Fig. 7.—Correlation between the rate of drug release from matrix and the surface activity of the release medium. Key: A corresponds to experimental slope of the Q *vs.* $t^{1/2}$ plots (Fig. 6) for drug release from 20% sulfanilamide–polyethylene matrices at different surfactant (AOT) concentrations; B gives surface tension lowering as a function of the surfactant concentration.

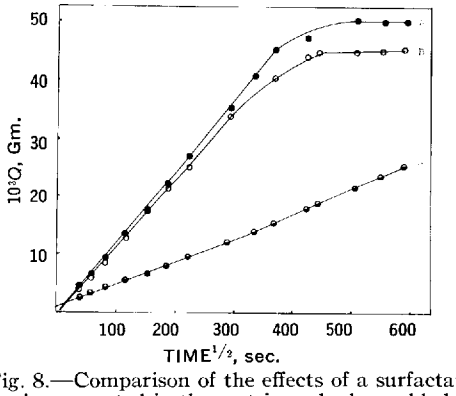

Fig. 8.—Comparison of the effects of a surfactant when incorporated in the matrix and when added in the release medium upon the release rate of potassium acid phthalate from polyethylene plastic matrices containing 20% potassium acid phthalate. Key: A, 20% potassium acid phthalate and 2% hexadecyltrimethylammonium bromide in the matrix with water as the release medium; B, only 20% potassium acid phthalate in the matrix and 0.10% surfactant in the release medium; C, same as B, except water is the release medium.

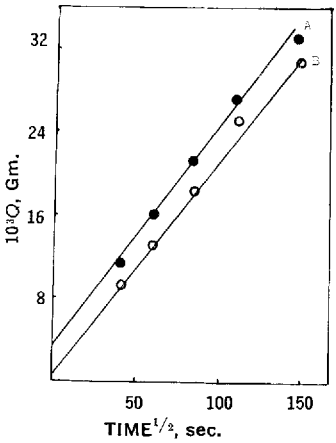

Fig. 9.—Release of hexadecylpyridinium chloride from polyethylene plastic matrices containing 20% hexadecylpyridinium chloride. Key: A, release medium contained 0.10% hexadecyltrimethylammonium bromide; B, water.

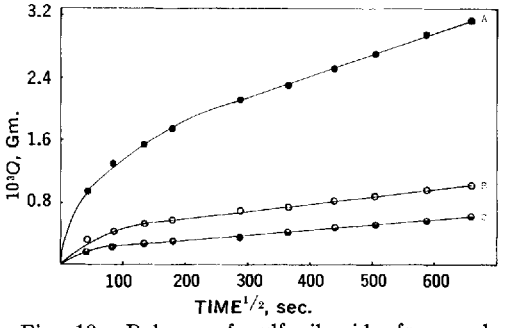

Fig. 10.—Release of sulfanilamide from polyethylene plastic matrices into water. Key: A, 20% drug in matrix; B, 10% drug; C, 5% drug.

TABLE III.—TORTUOSITY OF 5, 10, AND 20% SULFANILAMIDE IN POLYETHYLENE MATRICES USING SOLID RELEASE RATES IN WATER

Tablet Compn.	τ Using ϵ of Drug and Air	τ Using ϵ of Drug Only
5% Sulfanilamide	44,700	12,790
10% Sulfanilamide	4,700	1,700
20% Sulfanilamide	1,500	740

chloride was selected as the drug with inherent surfactant properties. Release rates were obtained in water and in 0.1% HTAB solution. The results are shown in Fig. 9. The release rates in both experiments were essentially the same as indicated by their slopes. The intercept in the data for the release in surfactant is probably due to the better initial wetting of the tablet surface when the surfactant was used in the release medium.

Nonsurfactant Release Rates.—As previously noted and illustrated in Figs. 1 and 2, the release rates in water are considerably slower than those obtained in surfactant solutions. Analysis of the parameters and the release rates strongly suggest that the wetting of all pores and the release of air from them are not achieved in nonsurfactant solutions. To further complicate matters, it appears that removal of air is slowly but continuously occurring as indicated by the slight curvature of several release rates observed in nonsurfactant solutions.

Attempts to apply Eq. 1 to both initial and limiting slopes of these curves yielded a very high value for the apparent tortuosity. Calculations were made assuming the two limiting cases—all air removed or no air removed. This seems to indicate that the air remaining in the tablets blocks pathways leading to drug particles and in this way effectively encapsulates them.

To further study this effect, the release rates of matrices containing 5, 10, and 20% sulfanilamide were studied in water. The results are shown in Fig. 10. The slopes in all these cases initially decreased with time and then exhibited a constant value. It is believed that the initially greater rate is due to the surface drug that is readily accessible to the solvent. The magnitudes observed are consistent with this assumption.

Using Eq. 1 and applying it to the constant slope portions of these curves, tortuosities were calculated using the two porosity values. The results of these calculations are shown in Table III.

The apparent tortuosities calculated in this manner are extremely high. These results suggest that drug is indeed effectively encapsulated by the polyethylene plastic. The increase in the calculated τ values with decreasing A is consistent with this type of model because the more dilute the internal (drug) phase the greater the likelihood of isolation of a drug particle in a sea of plastic. Then, if the wetting of the channels in the plastic itself is insufficient, the permeability of the media is largely determined by the low permeability of the plastic itself and not by a simple average of the void space and the plastic permeabilities.

It is believed that situations such as these may be best defined physically by the use of concepts other than the "average porosity" and the "average tortuosity" as are implied by Eq. 1. Preliminary theoretical studies based on the use of mixture relationship such as the Clausius-Mosotti and the Bruggeman equations (4) have yielded effective τ values of the order of magnitudes given in Table III when reasonable assumptions were made. A future communication will describe in detail comparisons of data with these theories for encapsulation.

SUMMARY

The interpretation of data based upon the quantitative determination of the parameters, ϵ, τ, and D, and their use in Eqs. 1 and 2 was shown to be valid for studies in which the porosity was known throughout the experiment. The results were excellent and showed that these methods can be quantitatively used to study the effect of various variables on the release rates of drugs from inert matrices.

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Investigation of Factors Influencing Release of Solid Drug Dispersed in Inert Matrices IV

Some Studies Involving the Polyvinyl Chloride Matrix

By SAURABH J. DESAI*, PARVINDER SINGH, ANTHONY P. SIMONELLI, and WILLIAM I. HIGUCHI

The mechanism of drug release from tablets made from mixtures of drug and polyvinyl chloride particles was investigated. Experimental data were evaluated by means of the Higuchi relationship for the process. The S-shaped nature of the amount released *versus* (time)$^{1/2}$ plots was studied, and it appears that this behavior was the result of the relatively slow release of air from the tablets. Matrix tortuosities ranging from about 1.5 to 3.0 were found for these polyvinyl chloride matrices.

IT HAS BEEN previously shown that polyvinyl chloride (PVC) matrices have exhibited peculiar behavior compared to those of polyethylene (1). The drug release from the PVC tablets showed S-shaped curves which are contrary to the predicted linear plots when the amount of release is plotted *versus* the square root of time. In addition, the PVC matrices did not show the surfactant effect observed with those of polyethylene. Finally, the PVC release rates were 4–6 times faster. These plots are shown in Fig. 1. Since this behavior may be representative of a general class of matrices, it was felt that this phenomenon should be thoroughly investigated.

Comparison of PVC and polyethylene powders reveals that the PVC powder has a much higher density (about 50%), a larger particle size (50–200 mesh as compared to smaller than 270 mesh), and a larger particle size distribution. This suggested that their respective matrices may also differ. The PVC powder should produce a more compact tablet due to its higher density and should be better bonded due to its wider particle-size distribution. Examination of their respective matrices shows that, although PVC is more compact, it exhibits poorer bonding. Polyethylene produces a tablet which has a very smooth surface, appearing as a fused solid, but PVC tablets seem rather friable and are easily repowdered by scraping.

EXPERIMENTAL

The release rates from PVC matrices containing solid drug as well as solutions were studied using the techniques and the evaluation of parameters described previously (1, 2). All studies were conducted using water as the release medium unless specified otherwise, and all percentages reported are on a w/w basis.

RESULTS AND DISCUSSION

The relatively poorer bonding characteristics of PVC may be partly responsible for its behavior. In an effort to improve its bonding characteristics, the effect of pressure was studied. Tablets containing 10% sodium salicylate were made at compressional forces of 2000-lb. increments between 4000 and 20,000 lb. Their release rates were independent of the compressional force. Figure 2 displays the curve obtained from the composite of all data and shows the range of random variations for each point. These results indicate that PVC is elastic throughout this range of compressional forces, and, therefore, its porosity should remain constant in these tablets. This was confirmed by the constancy of the dimensional measurements of all tablets.

Physical measurements of PVC tablets not only showed the presence of air, but also that the amount was considerably higher than in a similar matrix of polyethylene. The porosity of a 500-mg. 20% sodium salicylate tablet in PVC was 0.37, whereas the corresponding tablet made using a polyethylene matrix had a porosity of 0.22. The relatively large amount of air in the PVC tablet theoretically suggested that a large surfactant effect on the release rates might be observed. However, no such effects were found. This lack of surfactant effect implies that the surfactants used did not increase the wetting characteristics of PVC and that the removal of air was not facilitated. The discussion of this aspect will be deferred to a later portion of this paper.

The S-shaped curves exhibited by PVC tablets show that the slopes are not constant with time, but initially increase, then become constant, and then finally decrease with time. The beginning of the last phase corresponds to where essentially all of the solid drug in the matrix had just dissolved.

Several possible explanations were considered and investigated for the initial curvature of the S-shaped curves. One possible explanation may

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* Recipient of Eli Lilly Fellowship.

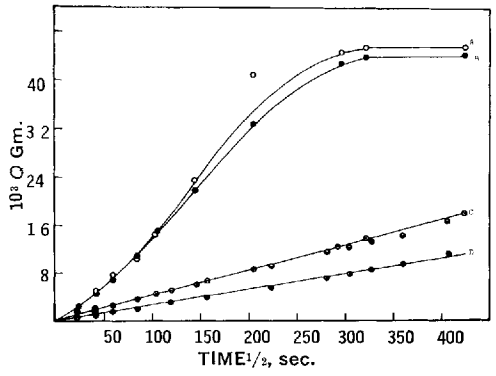

Fig. 1.—Effect of surfactants on sodium salicylate release rates. Key: A, 5% w/w sodium salicylate in PVC matrix using water as solvent; B, 5% w/w sodium salicylate in PVC matrix using 0.2% w/w benzalkonium chloride as a solvent; C, 5% w/w sodium salicylate in polyethylene matrix using the following solvents: ⊙, 0.2% benzalkonium chloride and ⊖, 0.2% sodium lauryl sulfate; D, 5% w/w sodium salicylate in polyethylene matrix using water as a solvent.

be the partitioning of drug between the PVC and the release medium. Theoretical considerations have shown (2) that the release dependence remains linear with respect to the square root of time if reversible drug partitioning should occur. If, however, the rate of partitioning is slow, then the linearity may be disturbed and the S-shaped curve may result. This possibility was ruled out on the basis of liquid leaching experiments using the same tablets which showed linear release plots. If partitioning was the cause of the S-shaped curve, the same result would be expected to occur in both solid and liquid leaching experiments.

This S-shaped curve can be due to any factor which governs the release rates not remaining constant as required, but changing as a function of time. A critical examination of all these factors clearly indicated that porosity would most likely be a variable. As an example, any swelling of the matrix due to the presence of water would alter the porosity, decreasing it with time. This possibility was investigated by accurately measuring the dimensions of tablets prior to and after soaking overnight in water. Blank PVC tablets and PVC tablets containing 10% sodium salicylate were used for these experiments. No change was noted in their dimensions due to the water exposure, and swelling was clearly eliminated as a factor in this system.

Another possibility for porosity variation may be due to a difference in the physical properties of the surface and interior portions of the matrix. This can be brought about by a difference in the flow properties of the matrix components during compression. If there is poor flowability, the effective pressure is not uniformly distributed throughout the matrix. As a result, the surface portion would be subjected to a greater pressure. This would produce smaller porosities and larger tortuosities at the surface and account for initial rates being slower.

If the above is true, it would predict that the release rate of a matrix whose surface was removed by scraping would yield a more rapid initial release of drug and would display linear behavior. For this purpose, matrices containing 10% sodium salicylate were compressed from the same mixture and then treated in the following manner. From one tablet about 6% w/w was scraped from the surface (surface to be exposed to the leaching action of the solvent) so that a uniform layer was removed and a new surface produced. Another had 12.5% w/w removed, and from a third 25% w/w was removed. As a control, one tablet utilized its original surface. Examination of Fig. 3 reveals that the release rates were not affected by the above treatment. This indicated that the physical properties of the surface and interior layers are not different, and that this is not an important factor in the experiments.

To further confirm this finding, the effect of lubri-

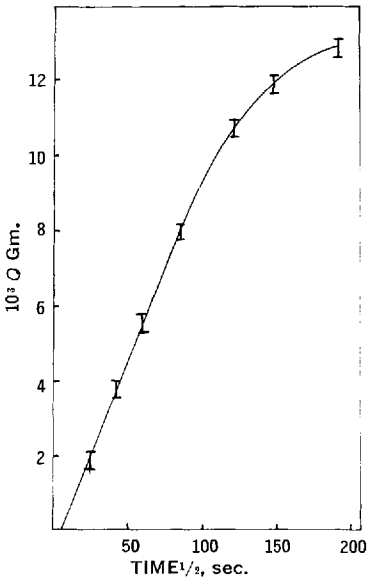

Fig. 2.—Effect of compressional force on release of 10% sodium salicylate–PVC tablets in water. Range of random spread of the data is indicated by the bars.

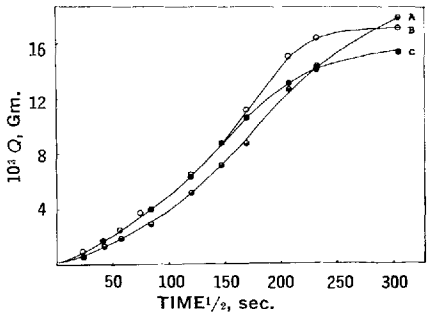

Fig. 3.—Effect of removal of the tablet surface layers on the release rates of 5% sodium salicylate tablets in PVC. Key: A, 6% w/w tablet surface removed; B, 12.5% w/w tablet surface removed; C, 25% w/w tablet surface removed.

cant addition to the matrix was studied. If the flowability of the PVC powder–drug mixture is not adequate, the addition of lubricant would improve flowability under compression and alter the matrix release profile. Figure 4 shows the release of 10% sodium salicylate matrices containing 3% talc or magnesium stearate. Comparison of these curves with the included plot of the matrix not containing any lubricant confirm the previous finding of sufficient flowability of the powder mixture under pressure.

It was finally reasoned that the initial incomplete removal of air from the matrix may be the cause of the S-shaped curves. This view was supported by the fact that a resaturated matrix yielded a linear plot. Such a matrix would have been exposed to solvent for a sufficient length of time to allow for the complete removal of air.

It is believed that the availability of air channels

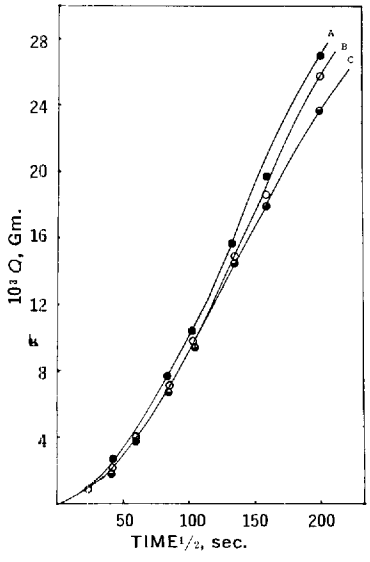

Fig. 4.—Effect of lubricant on the release of 10% sodium salicylate tablets in PVC. Key: A, 10% sodium salicylate; B, 10% sodium salicylate + 3% talc; C, 10% sodium salicylate + 3% magnesium stearate.

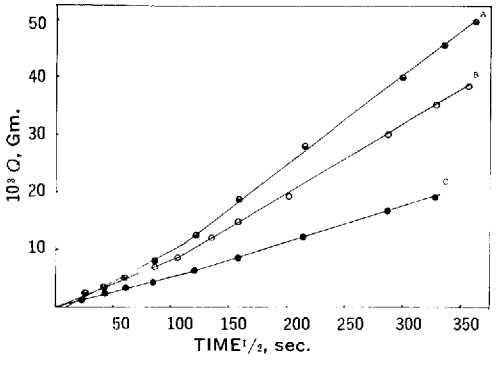

Fig. 5.—Release of 20% calcium benzoate (A), 20% caffeine (B), and 20% sulfanilamide (C) from PVC matrices into water.

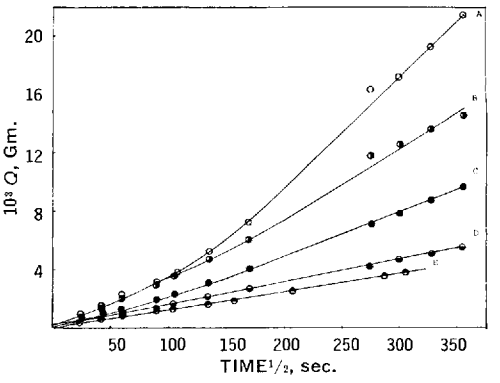

Fig. 6.—Effect of K_2HPO_4 concentration on release of 10% salicylic acid in PVC tablets. Key: A, 1.00 *M* K_2HPO_4; B, 0.50 *M* K_2HPO_4; C, 0.10 *M* K_2HPO_4; D, 0.01 *M* K_2HPO_4; E, water.

to the solvent depends on the rate of liquid boundary movement which should be a function of the drug solubility and would be expected to increase with an increase in solubility. If the drug is poorly soluble, the movement of the liquid boundary may be sufficiently slow to permit the prior removal of air. This would provide the linear plots predicted by theory. If the drug is very soluble, on the other hand, the drug solution boundary may move faster than the process of air removal, producing an increasing porosity with time and causing the observed curvature.

To test this concept, the release rates of sulfanilamide, caffeine, and calcium benzoate tablets were obtained and the corresponding data are shown in Fig. 5. The respective solubilities are 1.08×10^{-2}, 2.50×10^{-2}, and 3.08×10^{-2} Gm./ml., as compared to 65.0×10^{-2} Gm./ml. for sodium salicylate. In agreement with the previous discussion, the curves in Fig. 5 show the expected correlation between solubility and degree of curvature. As the solubility increased, nonlinearity also increased showing the most marked change for sodium salicylate.

This correlation can be shown in another way. The solubility of a drug can be varied by using different concentrations of buffer in the release medium. In this manner, the solubility of the drug can be varied and yet have the identical matrix properties operative in all cases.

The release rate of 10% salicylic acid dispersed in PVC was studied in different concentrations of K_2HPO_4 solutions. The results are given in Fig. 6, and they show that the release rates increase as the concentration of K_2HPO_4 is increased. In the presence of K_2HPO_4, salicylic acid in solution is converted to salicylate ion, the extent of the conversion being essentially proportional to the concentration of K_2HPO_4. Again, as the effective solubility increases, each plot displays increasing curvature in the release patterns. The rates are linear in water and at low K_2HPO_4 concentrations.

If the rate of air removal is the controlling factor, then the complete removal of air by vacuum before exposing the matrix to solvent should produce a linear square root of time dependence. To test

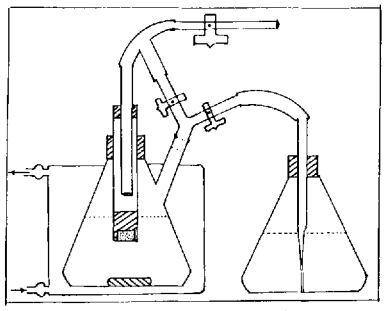

Fig. 7.—Schematic diagram of the apparatus used to study release rates after vacuum treatment.

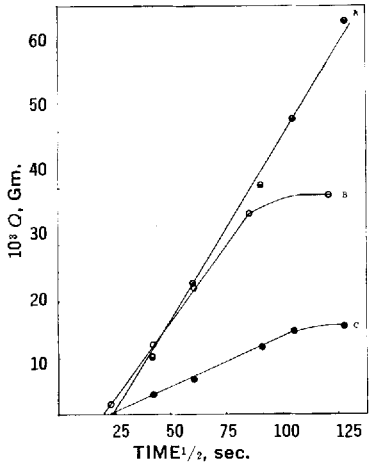

Fig. 8.—Release of 5, 10, and 20% sodium salicylate in PVC tablets into water after vacuum treatment. Key: A, 20% sodium salicylate; B, 10% sodium salicylate; C, 5% sodium salicylate.

this, a modified apparatus was made, and its schematic diagram is shown in Fig. 7. A tablet inserted in a glass tube as previously described (1) was mounted on a 125-ml. conical flask with a side arm. The flask was jacketed to maintain a constant temperature. The side arm was fitted with a Y-joint. One of the arms of the joint was connected to a vacuum pump and the other to a flask containing a measured amount of water. Vacuum was also applied to the top of the glass tube by means of a T-tube so as to maintain equal pressure on either side of the mounted tablet preventing any movement or damage of the tablet. Prior to the final application of vacuum, the connecting tube and stopcock bore from the water supply to the flask was completely flushed to remove the entrapped air. All connecting joints were sealed with "Apiezon" vacuum sealing material. A McLeod gauge was used to measure the pressure. A vacuum pressure of 0.9 mm. Hg was obtained before the water was allowed to flow into the sample flask. Immediately after the addition of water, the magnetic stirrer was started. The height of the tube containing the tablet had been previously adjusted to insure complete immersion of the tablet in water before the vacuum was released.

The results of the release of 5, 10, and 20% sodium salicylate tablets, obtained by the above vacuum procedure, are plotted in Fig. 8. The release rates of 20% sulfanilamide and 20% calcium benzoate tablets under similar conditions are shown in Fig. 9.

As predicted, all the release rates follow the square root of time dependence, strongly suggesting that the S-shaped curve is produced by the proposed mechanism. To further investigate this, liquid release rates of sulfanilamide and calcium benzoate tablets were carried out using procedures previously described (1, 2), and these results are plotted in Fig. 10. The quantitative evaluation of tortuosity by solid and liquid leaching procedures were made,

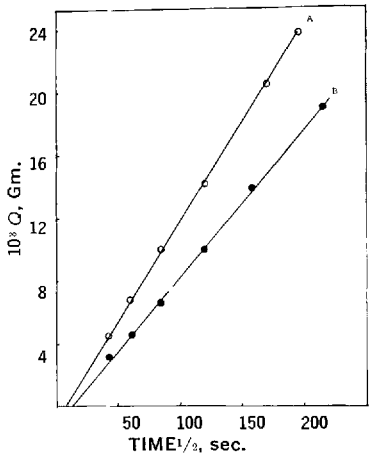

Fig. 9.—Release of 20% calcium benzoate (A) and 20% sulfanilamide (B) from PVC matrices into water, after vacuum treatment.

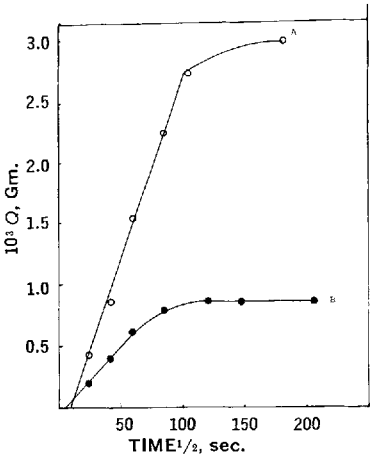

Fig. 10.—Release data from matrices saturated with drug solution. Key: A, tablet originally contained 20% calcium benzoate, which was completely leached (the matrix was equilibrated with saturated calcium benzoate solution prior to studying the liquid release); B, same as A, except sulfanilamide was used as the drug, and saturated sulfanilamide solution was used instead of calcium benzoate solution.

TABLE I.—CALCULATION OF TORTUOSITIES IN POLYVINYL CHLORIDE MATRICES CONTAINING SULFANILAMIDE AND CALCIUM BENZOATE. SOLID RELEASE STUDIED WITH VACUUM PROCEDURE

Tablet Compn.	D 10^6 cm.^{2} sec.$^{-1}$	C_s 10^2 Gm. ml.$^{-1}$	Vol. of Tablet, ml.	ϵ Due to Air and Drug	A Gm. ml.$^{-1}$	C_0 10^2 Gm. ml.$^{-1}$	$10^5 Q/t^{1/2}$ Solid Leaching	$10_5 Q/t^{1/2}$ Liquid Leaching	τ By Solid Leaching	τ By Liquid Leaching
20% Sulfanilamide	12.90	1.08	0.4311	0.390	0.2319	0.80	6.21	9.60	2.6	1.3
20% Calcium benzoate	9.20	3.08	0.4247	0.380	0.2354	3.28	8.5	11.3	3.2	2.0

TABLE II.—CALCULATION OF TORTUOSITY FOR 5, 10, AND 20% SODIUM SALICYLATE IN THE POLYVINYL CHLORIDE MATRIX. SOLID RELEASE RATES WERE OBTAINED USING VACUUM PROCEDURE

Tablet Compn.	D 10^6 cm.^{2} sec.$^{-1}$	Vol. of Tablet, ml.	I Vol. of Drug, ml.	II Vol. of Air, ml.	ϵ	III Wt. of Drug, Gm.	C_0 10^2 Gm. ml.$^{-1}$ $\frac{\text{III}}{\text{I} + \text{II}}$	Solid Release Rate $10^4 Q/t^{1/2}$	τ
5% Sodium salicylate	13.30	0.4385	0.0161	0.1093	0.286	0.025	0.200	1.79	1.7
10% Sodium salicylate	17.20	0.4294	0.0323	0.1006	0.309	0.050	0.376	4.34	1.6
20% Sodium salicylate	23.00	0.4189	0.0645	0.0908	0.371	0.100	0.644	6.55	3.8

and the results are summarized in Table I. The good agreement of the tortuosity values obtained by the two methods indicate that the total porosity is available when the vacuum procedure is followed.

The τ values were similarly calculated for the sodium salicylate systems. The calculated values for τ, however, were physically impossible as they were less than unity. Analysis of the situation revealed that when the vacuum procedure is used, the solid release equation should not be applicable. Under these conditions, the water is able to rapidly permeate all available channels. Since the dissolution rate of sodium salicylate is very rapid, the water which penetrated the matrix is rapidly saturated. If the amount of drug in the matrix, however, is not sufficient to produce a saturated solution, then all of the drug will be rapidly dissolved. As a result, a matrix containing no solid drug but only its solution will be produced. In these cases the release rates should follow the liquid release equation rather than that of the solid release.

The concentrations of the resultant solutions in the 5, 10, and 20% tablets were calculated on this basis assuming that all the volume of each tablet initially occupied by air and sodium salicylate became occupied by a solution containing all of the incorporated drug. The concentrations of the solutions in the matrix for 5, 10, and 20% tablets were calculated and found to be 0.20, 0.37, and 0.64 Gm./ml., respectively. In addition, τ values were calculated using the above solution concentrations in the liquid leaching equation and are listed in Table II. These values are in excellent agreement with those obtained from the sulfanilamide and calcium benzoate studies and provide strong support for the concepts developed here.

An interesting observation can be made by comparing the magnitude of the tortuosity values obtained for these different drugs in a PVC matrix with those that were obtained for the same drugs dispersed in a polyethylene matrix (3). It is seen that the tortuosity values obtained for the PVC matrix vary from about 1.5 to 4, whereas for the polyethylene matrix they vary from about 7 to 10. It has been shown (4) that the value of τ for a system composed of closely packed spheres is generally above 1.5 to 2.0. This indicates that the structure of a PVC matrix resembles that of a closely packed glass bead bed, and implies that PVC particles are elastic, and, therefore, they are not permanently distorted when compressed. The high τ values of the polyethylene matrix, on the other hand, indicate that its particles are plastic and are severely distorted when compressed.

APPENDIX

The equations used in the explanation of the rate process are:

$$Q = \frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s t^{1/2} \qquad \text{(Eq. 1)}$$

and

$$Q = 2\,C_0\epsilon\left(\frac{DT}{\pi\tau}\right)^{1/2} \qquad \text{(Eq. 2)}$$

where

Q = amount of drug released per unit area of the tablet surface exposed at time, t,
D = diffusion coefficient of the drug in release medium,
C_s = solubility of the drug in the release medium,
C_0 = concentration of the solution in the matrix,
ϵ = porosity of the matrix,
τ = tortuosity of the matrix.

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(4) Higuchi, W. I., and Higuchi, T., *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 598(1960).

Near-Infrared Spectroscopy of Amine Salts

By JOSEPH E. SINSHEIMER and ANNE M. KEUHNELIAN

The purpose of this investigation was to study the applicability of near-infrared spectroscopy to pharmaceutical analysis. Of particular interest was a model series of aliphatic amine hydrochlorides, and as typical pharmaceutical examples, the sympathomimetic amine salts were studied. Both solid state and chloroform solution spectra were obtained and their qualitative and quantitative applications were evaluated.

THE SPECTRAL characteristics of amines, especially aromatic amines, have been well established in the near-infrared region (1). However, while amine salts have been studied in the infrared (2–4), little has been established in the near-infrared region probably because of a lack of suitable solvents and sampling techniques.

Since the medicinal use of amines, especially as their amine salts, is extensive, it was felt that an investigation into the application of the near-infrared for the direct analysis of pharmaceutical amine salts would be of interest. This is particularly true in the case of aliphatic amine salts where the absence of ultraviolet absorption makes alternate spectral methods even more desirable. Molar absorptivity values in the near-infrared region are usually low even compared to the infrared region. However, measurements in the near-infrared can offer the advantage of the high resolution of the quartz optics employed. Use of cells and techniques more analogous to ultraviolet techniques than to those of use in the infrared also may be a potential advantage.

Objectives of the present study were the development of sampling techniques together with an investigation of the qualitative aspects, sensitivity, and conformity to Beer's law of near-infrared measurements of amine salts.

EXPERIMENTAL

Instrumental Parameters.—Beckman DK 2A: sensitivity, 50; time constant, 0.2; recording speed, 18 mμ/min.; wavelength expansion, 50 mμ/cm. Zeiss PMQ II with a M4Q III monochromator: amplification 5/1/1 and slit widths less than 0.08 mm. Both instruments were calibrated for wavelength accuracy against 1,2,4-trichlorobenzene (5).

Reagents.—Commercially available amines and sympathomimetic amine salts of production grade were used without further purification. Except where indicated as being commercially available, amine salts were prepared by adding 50 ml. of a saturated solution of HCl in anhydrous ether to 10 ml. of amine dissolved in 10 ml. of anhydrous ether. The resulting product was filtered, washed with three portions of 50 ml. of anhydrous ether, air dried, and stored in a desiccator over silica gel.

The reagent grade chloroform used as a solvent in spectrophotometric measurements was passed through a column of alumina to remove traces of water and ethanol. This process was followed by noting the absence of the strong ethanol peak at 2.90 μ. Chloroform treated in this manner was stored in a brown bottle for use within 5 days.

Solid State Spectra.—In general, from 25 to 40 mg. of amine salts taken directly from a desiccator were reduced to a powder in an agate mortar and pestle as quickly as possible to avoid the adsorption of moisture. The powders were transferred to a KBr die (Limit Corp. KB-01) and a 13-mm. disk was formed at 20,000 lb. for 5 min. Spectra were obtained on a DK-2A spectrophotometer from 2.80 to 1.05 μ or to as low a wavelength as dispersion permitted. A base line was established on the lowest absorbance range possible at 2.65 μ and with a slit width below 0.3 mm. by manual attenuation of the reference shutter.

Solution and Liquid Spectra.—(*a*) Absorption spectra of from 0.1 to 3% solutions of amine salt in chloroform were obtained on a DK-2A spectrophotometer from 2.35 to 2.10 μ. Near-infrared 1-cm. matching pair cells were used with chloroform in the reference cell.

(*b*) Absorption spectra for the parent amines were obtained from 2.65 to 1.05 μ in a 0.25-mm. short-path cell against silica as the reference.

(*c*) Percentage-transmittance spectra were obtained from 2.15 to 1.70 μ for 1% chloroform solutions of amine salts and 0.1% chloroform solution of the parent amines. In both series, spectra were obtained in 1-cm. matching pair near-infrared cells with chloroform in the reference cell. The 90 to 100% range of the DK-2A instrument was used.

(*d*) Quantitative measurements were made with the Zeiss spectrophotometer on 200 to 500 mg./10-ml. solutions of amine salts in chloroform. The major peaks of the recorded spectra were reconfirmed in the 2.33 to 2.10 μ range and the absorbance of these peaks determined. The validity of Beer's law was examined by determining the absorbances for 1 in 2, 1 in 5, and 1 in 10 dilutions of these initial solutions. Micro quantitative measurements were also made in a similar manner but with the use of the Zeiss microcell equipment (507425) with 1-cm. cylindrical MR 5 microcells of 0.2-ml. volume.

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TABLE I.—SOLID STATE SPECTRA OF MODEL AMINE HYDROCHLORIDES

Compd.	Absorption Bands, mμ
Ammonium chloride[a]	2633, 2342, 2195, 2075, 1950,[b] 1672, 1503
Primary amine salts	
Butylamine	2464,[b] 2426, 2411, 2335, 2307, 2252, 2170, 1719
tert-Butylamine[a]	2560,[b] 2514, 2451, 2384, 2339, 2306, 2269, 2223, 2192, 1691
Cyclohexylamine	2738, 2680, 2342, 2618, 2568, 2528, 2493, 2478, 2426, 2377, 2339, 2300, 2254, 2200, 1746,[b] 1706
Ethylamine[a]	2593, 2522, 2387, 2372, 2304, 2256, 2207, 2162, 1675, 1651
Isobutylamine	2603, 2556, 2401, 2334, 2307, 2265, 2217, 2170, 1687
Isopropylamine	2547, 2383, 2348, 2306, 2263, 2194, 1689
Methylamine[a,c]	2523, 2488, 2333, 2250, 2238, 2134, 1677
Propylamine	2550, 2496, 2458, 2405, 2329, 2292, 2262, 2138, 1726, 1689
Secondary amine salts	
Dibutylamine	2514, 2415, 2399, 2336, 2297, 2272, 2194,[b] 1759, 1719
Diethylamine	2638, 2603, 2558, 2515, 2462, 2411, 2380, 2353, 2297, 2257, 2197, 1726, 1681
Diisobutylamine	2620, 2556, 2508, 2450, 2409, 2312, 2269, 2200,[b] 1701
Dipropylamine	2711, 2596, 2344, 2453, 2422, 2399, 2337, 2304, 2274, 2307,[b] 1741, 1699
Piperidine	2703, 2668, 2646, 2603, 2568, 2556, 2498, 2478, 2435, 2415, 2347, 2332, 2304, 2285, 2266, 2200, 1747, 1721
Tertiary amine salts	
Tributylamine	2653,[b] 2563, 2513, 2450, 2407, 2252,[b] 2335, 2306, 2294, 2272, 1703
Triethylamine[d]	2663, 2633, 2581, 2474, 2453, 2399, 2346, 2307, 2295, 2260, 1759, 1693, 1681
Trimethylamine[c]	2492, 2448, 2346, 2257, 2096, 2014, 1804, 1671

[a] Solubility too limited to obtain spectra in chloroform in the 2.32 to 1.70-μ regions. [b] Partially resolved peak as a shoulder on a more intense peak. [c] Spectra influenced by the hygroscopic character of the salt. [d] There were no significant differences between the hydrochloride and hydrobromide salts.

RESULTS AND DISCUSSION

As is true in the infrared, carbon tetrachloride and carbon disulfide are also generally the most useful solvents in the near-infrared. However, neither solvent was of value in the present investigation either because of the interaction of the parent amines with these solvents (6, 7) or because of the lack of solubility of amine salts in the solvents.

Within their well-recognized limitations (3) solid state spectra appeared to be a promising approach to the authors' primary objective of the direct measurement of amine salts. Disks were prepared in the ratio of from 5 to 20 mg. of amine salt to 200 to 400 mg. of KCl. However, dispersion was great at either the high concentration or thick disks required for usable absorption. It was noted, however, that model amine salts could be pressed directly into satisfactory disks without addition of the halide salts. The spectra so obtained are summarized in Table I and a set of typical examples are shown in Fig. 1. It was also possible to form transparent disks directly with some sympathomimetic amine salts as summarized in Table II. In general, disks were formed under 10 tons force, although a few amine salts produced disks at even lower force. However, for the majority of compounds, it is reasonable to assume that a 20-ton system with a properly designed die for the efficient application of vacuum (8) would result in disks with less dispersion. In the present study, dispersion was partly compensated by the use of a KBr disk with high dispersion or by manual attenuation of the reference shutter. In either case, a slit width no greater than 0.3 mm. at 2.65 μ with a sensitivity of 50 was used. Under these parameters, there was no distortion of a test spectrum of didymium. Disks where held in the spectrophotometer in a pellet holder similar to the Beckman holder but with a threaded connection at the sample position to permit access to variable thickness disks. The crystalline form of the amine salt is important for disk formation. For example, 2-phenylethylamine hydrochloride in the form of platelets could not be compressed but in a microcrystalline form was compressible.

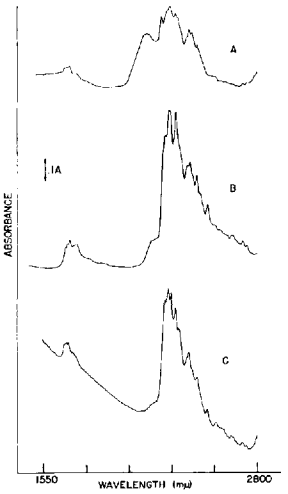

Fig. 1.—Solid state spectra. Key: A, butylamine hydrochloride; B, dibutylamine hydrochloride; C, tributylamine hydrochloride.

As can be noted in Fig. 1, the solid state spectra revealed a series of moderately strong absorbance peaks in the region from 2.55 to 2.15 μ which hold promise for quantitative measurements. Of the solvents and combination of solvents tested showing at least partial transparency (1) in the region of

TABLE II.—SOLID STATE SPECTRA OF SYMPATHOMIMETIC SALTS

Compd.	Absorption Bands, mμ
Aliphatic amine salts	
Cyclopentamine hydrochloride[a]	2571,[b] 2473, 2418, 2375, 2336, 2291, 2255, 1749, 1713
Isometheptane mucate	2656, 2526, 2496, 2451, 2394, 2326, 2294, 2267, 2248, 2150, 1716
Methylhexaneamine hydrochloride[a]	2444, 2407,[b] 2380, 2347, 2307, 2156, 1768, 1719, 1696
Tuaminoheptane sulfate	2439, 2379,[b] 2375, 2345, 2304, 2157, 1766, 1724
Imidazole salt	
Tetrahydrozoline hydrochloride	2643, 2600,[b] 2502, 2464, 2427, 2371, 2332, 2312, 2284, 2267, 2179, 2147, 1683
Phenylethylamine salts	
Amphetamine sulfate	2743, 2469, 2349, 2296, 2258, 2160, 1676
Ephedrine sulfate	2762, 2471, 2439, 2371, 2342, 2286, 2242, 2222, 2184, 2157, 2140, 1676
Mephentermine sulfate	2468, 2404, 2355, 2287, 2251, 2160, 1950, 1676
Metaraminol bitartrate	2651, 2267, 2445, 2296, 2253, 2144, 1663
Methoxyphenamine hydrochloride	2750, 2575,[b] 2474, 2434, 2385, 2338, 2290, 2258, 2162, 1681
Phenmetrazine hydrochloride[a]	2696, 2618, 2570, 2548,[b] 2525, 2484, 2458, 2420, 2369, 2354, 2317, 2302, 2259, 2150, 2137, 1691,[b] 1676
Phenylethylamine hydrochloride	2736, 2540,[b] 2473, 2350, 2280,[b] 2255, 2186, 2162, 1673
Phenylpropanolamine hydrochloride	2692, 2463, 2382, 2302, 2257, 2197, 1676
Phenylpropylmethylamine hydrochloride[a]	2778, 2618, 2464, 2418, 2375, 2323,[b] 2286, 2256, 2182,[b] 2162, 2148,[b] 1686

[a] Solubility was sufficient to obtain spectra in chloroform from 2.32 to 1.70 μ. In addition to the compounds listed in the table, the following sympathomimetic hydrochlorides had sufficient solubility to obtain spectra in chloroform: diethylpropion, methamphetamine, β-phenylpropylamine, and propylhexedrine. [b] Partially resolved peak as a shoulder on a more intense peak.

TABLE III.—MOLAR ABSORPTIVITIES OF AMINE SALTS

Compd.	$\lambda_{max.}$ (ε)
Primary amine salts	
1-Adamantanamine HCl	2298 mμ (2.95); 2223 mμ (1.06)
Isopropylamine HCl	2298 mμ (2.58); 2258 mμ (2.40); 2183 mμ (1.22)
Secondary amine salts	
Diethylamine HCl	2298 mμ (3.02); 2258 mμ (4.24)
Phenmetrazine HCl	2283 mμ (2.52); 2258 mμ (2.69)
Phenylpropylmethylamine HCl	2298 mμ (2.31); 2258 mμ (3.46); 2173 mμ (1.32)

TABLE IV.—SPECTRA OF AMINE HYDROCHLORIDES AND THEIR PARENT AMINES IN CHLOROFORM FROM 2.15 to 1.70 μ

Compd.	Bands, mμ: Amine Hydrochloride	Parent Amine
Primary		
Butylamine	1988, 1902, 1878, 1744	2134, 2112, 2018, 1892, 1814,[a] 1724
tert-Butylamine[b]	1895	2136, 2068, 2026, 1888
Cyclohexylamine	2074,[a] 1986,[a] 1906,[a] 1810, 1786, 1750, 1721	2157, 2126, 2102, 2025, 1892, 1812,[a] 1756, 1728
Isobutylamine	1989,[a] 1898, 1759, 1724, 1707	2107, 2019, 1892, 1826,[a] 1771, 1721
Isopropylamine	1999,[a] 1911,[a] 1875, 1810, 1776, 1724	2187, 2110, 2063, 2023, 1885, 1823, 1767, 1732
Propylamine	1905, 1884, 1796, 1713	2114, 2019, 1887, 1812,[a] 1726
Secondary		
Dibutylamine	2061, 1897, 1765,[a] 1721	2092, 2021,[a] 1893, 1810,[a] 1752, 1725
Diethylamine	2058, 1897, 1810[a]	2097, 2019,[a] 1892, 1820
Dipropylamine	2061, 1976,[a] 1897	2092, 2024, 1892, 1812,[a] 1753, 1723
Diisobutylamine	2063, 1897, 1810,[a] 1761,[a] 1733,[a] 1716	2092, 1892, 1810,[a] 1771,[a] 1740, 1725
Diisopropylamine	2058, 1969, 1896, 1830, 1793, 1752, 1721	2102, 1892, 1815, 1760, 1717
Tertiary		
Tributylamine	2062, 1900, 1756,[a] 1748,[a] 1702	1888, 1800,[a] 1751,[a] 1723
Triethylamine	2058, 2028,[a] 1897, 1878, 1832, 1761, 1711	2045, 2023, 1887, 1713[a]

[a] Partially resolved peak as a shoulder on a more intense peak. [b] A saturated solution of *tert*-butylamine hydrochloride is less concentrated than the normal 1% solutions.

Fig. 2.—Percentage transmittance spectra from 2.15 to 1.70 μ. A, butylamine hydrochloride; B, dibutylamine hydrochloride; C, tributylamine hydrochloride; D, butylamine; E, dibutylamine; F, tributylamine.

interest, chloroform was found to be the most useful. That is, the hydrochloride salts of amines divorced of other polar groups exhibit sufficient solubility to permit the quantitative measurement of these salts in the chloroform transparent portion (2.32 to 2.10) of the region of interest. Indication is given in Tables I and II of the compounds which could be measured in this manner.

Quantitative dilutions of the initial 1 to 3.5% chloroform solutions used to obtain these recorded spectra indicated conformity to Beer's law. Typical compounds were further examined in this regard on a Zeiss single beam instrument and conformity to Beer's law was established. The compounds tested, together with molar absorptivity of major peaks in the 2.32 to 2.10 μ region, are listed in Table III. Thus, 1% concentrations yield absorbance values in the order of 0.1 to 0.3. The use of microcells permits from 0.4 to 0.6 mmole of compound to be measured. For example, 2 mg. of adamantanamine hydrochloride can be determined in a 1-cm. microcell if total volume of solution is limited to the 0.2-ml. volume of the cell.

Spectra of solid state amine salts, especially in the 2.70 to 2.10 μ region, are complex enough and sensitive to small changes in structure to be useful for the "fingerprint" comparison and identification of these compounds. A comparison of the spectra of closely related compounds such as *n*-propylamine hydrochloride to that of isopropylamine hydrochloride (Table I) illustrates this point. The effectiveness of a comparison of this type is also illustrated with the butylamine salt series in Fig. 1. These spectra are a useful adjunct to infrared spectra in this regard.

Although the 2.70 to 2.20 μ region was the most complex and contained the most intensive absorbance bands, it was not readily possible to assign bands due to amine salts. The suspected presence of bands in this region based upon protonated nitrogen is supported by the solid state spectra of ammonium chloride (Table I). However, overtone and combination bands due to CH stretching are also exhibited in this region (1) and tend to obscure the amine salt bands.

Just below 2.20 μ and extending to 2.12 μ, there was observed for every primary amine salt studied, a broad band of medium intensity. A band in this region does not correspond to a calculated overtone for primary amine salt absorption in the infrared (2, 3). However, this band was consistently present in primary amine salts and except for the *N*-methylamines and cyclic amines studied was absent in the secondary and tertiary amine salts. The butylamine salts in Fig. 1 are a typical illustration of this difference in the 2.20 to 2.12 μ region.

The primary amine band consistently exhibits a shift of from 10 to 50 mμ to higher wavelengths when a spectrum in chloroform is compared to the corresponding spectrum of the solid state. The band at 2.25 to 2.27 μ is not shifted in this manner while the band at 2.32–2.27 μ is usually constant but with some compounds exhibits a 15 to 20 mμ hypsochromic shift in chloroform.

To study the weak bands below 2.12 μ, per cent transmission spectra of chloroform solutions recorded on the 90 to 100% expansion scale of the DK 2-A instrument were found to be the most helpful. Chloroform in a 1-cm. cell is transparent below 2.32 μ with the exception of two brief ranges (1.86 to 1.85 μ and 1.70 to 1.67 μ) and, therefore, offered a satisfactory medium for the comparison of amine salts and their parent amines. Concentrations were chosen such that the spectral region from 2.15 to 1.70 μ could be expanded full scale on the 90 to 100% range. For the amine salts, 1% solutions were satisfactory while the parent primary amines were run as 0.05% solutions and the remaining free bases as 0.1% solutions. The results are summarized in Table IV and typical examples are shown in Fig. 2.

All spectra of both amine salts and their parent amines have a band between 1.90 and 1.87 μ. This band exhibits a bathochromic shift of 3 to 10 mμ in the salts as compared to their parent amine and is the major similarity between a given amine salt and its parent amine in this region.

There is no other significant absorption from 2.15 to 1.70 μ for primary amine salts which is in direct contrast to the very strong combination band noted in the present investigation and previously reported for the free base primary amines (1). The absence of bands by this technique in this region for primary amine salts and the presence of a band at 2.05 μ for both secondary and tertiary amine salts offer a second method of distinguishing primary amine salts. Again, the 2.05 μ band does not correspond to an overtone for reported (2, 3) NH stretching bands in the infrared.

SUMMARY

The spectra of both model amine hydrochlorides and pharmaceutical amine salts have been observed in the near-infrared. Solid state spectra and solutions of hydrochlorides in chloroform have been shown to be useful sampling techniques and have

applications for qualitative work. Bands in the 2.15 to 2.32 μ region have been shown to be useful for the quantitative measurement of amine hydrochloride salts. Primary amine salts were distinguished from secondary and tertiary amine salts on the basis of the presence of a 2.18 μ band and the absence of a band at 2.05 μ.

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Applications of the Montmorillonites in Tablet Making

By KEE-NENG WAI, H. GEORGE DEKAY, and GILBERT S. BANKER

The montmorillonites were studied for their use as disintegrants, binders, and lubricants for the manufacture of compressed tablets. It was found that the clays, although traditionally believed to be inert materials, agglomerated with several macromolecules commonly used as tablet binders. When added dry to prepared granulations, magnesium aluminum silicate F was an excellent disintegrant which produced tablets disintegrating twice as fast as those containing an equal amount of cornstarch. Furthermore, the clays contained a lower moisture content and were more compressible than starch. However, the other grades of montmorillonite studied were less effective as disintegrating agents than starch, and wet granulation of the clays with the diluents substantially decreased the effectiveness of these materials as disintegrants.

MONTMORILLONITE is the name given to a clay mineral first found near Montmorillon, France. Essentially, it has the composition $Al_2O_3 \cdot 4SiO_4 \cdot H_2O \cdot xH_2O$. Many minerals of similar properties, but distinctly different chemical compositions, have since been found (1). The clay minerals frequently exhibit properties which are highly desirable for any products used as disintegrants, binders, and fillers, or viscosity imparting agents. They have a high swelling volume in water, form gels at low concentrations, are chemically inert and stable to a wide range of temperatures, and are smooth, white to off-white fine powders. This study was undertaken to investigate the extent of application and limitations of the montmorillonite type of clays in tablet making, based on and in view of selected physical properties of the clays which were previously determined (2).

Bentonite and magnesium aluminum silicate[1] have been studied by several workers as tablet disintegrating agents. Granberg and Benton (3) reported that bentonite was an effective filler and disintegrating agent in thyroid tablets. Gross and Becker (4), on the other hand, found that neither bentonite nor magnesium aluminum silicate, in concentrations up to 17%, produced any disintegrating effect in tablets. However, Firouzabadian and Huyck (5) and Ward and Trachtenberg (6), who conducted comparative studies on the effectiveness of tablet disintegrants, found that magnesium aluminum silicates were among the best disintegrants studied. Nair and Bhatia (7) in another comparative study reported that sulfathiazole tablets containing magnesium aluminum silicate as the disintegrant appeared to have the most rapid distintegration time when most of the clay product was added after granulation and only a small portion before granulation. A suspension of 20% montmorillonite clay has been used as a granulating agent with reported disintegration activity (8).

EXPERIMENTAL AND RESULTS

The three commercial montmorillonites studied in this work will be referred to as clay I, II ,and III,[2] respectively. The composition and properties of these

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[1] Marketed as Veegum by the R. T. Vanderbilt Co., New York, N. Y.

[2] Marketed as Veegum, Veegum F, and Veegum WG by the R. T. Vanderbilt Co., New York, N. Y.

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TABLE I.—COMPATIBILITY OF A 5% MONTMORILLONITE[a] DISPERSION WITH EQUAL VOLUMES OF VARIOUS OTHER TABLET ADJUNCTS

Granulating Agent	pH of Granulating Agent	pH of Granulating Agent with Clay	Appearance of Mixture
Distilled water	5	8	Smooth paste
Starch paste, 5%	6	8	Smooth paste
Starch paste, 2%	5	8	Smooth paste
Dextrose soln., 30%	4	8.5	Smooth paste
Algin,[b] 1%	7	9	Smooth paste
Propylene glycol alginate,[c] H.V., 1%	3	7	Smooth paste
Sodium alginate, 1%	5	8.5	Smooth paste
Simple syrup U.S.P.	5	7	Smooth paste
Acacia, 10%	5	7	Slightly agglomerated
Carboxymethylcellulose, 2.5%	5	8	Slightly agglomerated
Gelatin, 1%	5	8	Agglomerated
Methylcellulose 4000, 1%	5	8	Agglomerated
Polyvinylpyrrolidone, 5%	5	9	Agglomerated and separated

[a] Magnesium aluminum silicate WG. [b] Marketed as Keltose by Kelco Co. [c] Marketed as Kelcoloid by Kelco Co., Clark, N. J.

clays were reported in an earlier paper (2). The clays were oven dried at 50° for 24 hr. prior to use to remove nonbound moisture which may have been sorbed on storage.

Compatibility of Montmorillonite with Various Tablet Adjuncts.—The montmorillonites are frequently used in tablet matrices in combination with other tablet adjuncts with which the montmorillonites might be reactive. Such reactivity could affect tablet disintegration, drug availability, or manufacturing methods.

Two liters of a 5% dispersion of clay III were prepared in hot water. This preparation was permitted to hydrate for 24 hr. and was then divided into 100-ml. portions. To each portion, 100 ml. of another common or previously reported tablet binder was added. The change in pH and appearance of the mixed binders were observed and are summarized in Table I.

In spite of their relative chemical inertness, the montmorillonite clays were incompatible with certain common tablet adjuvants. The montmorillonites apparently interact with many macromolecules. The clays consistently increased the pH of the combined systems from 2 to 4 units. Hydrogen bonding between the clay and the polymer molecules and electrical discharge between two oppositely charged particles (or molecules) may explain the agglomeration phenomenon which frequently occurred between clay and polymer.

Disintegrating Action of Unwetted Montmorillonites.—Sixty-four batches of tablets were prepared, containing various concentrations of the three grades of clay, or starch, blended with a granulation of calcium sulfate. The relationships between the disintegration time and the concentrations of disintegrants, disintegration time and granule size, and between tablet hardness and disintegrant concentration were studied. The calcium sulfate granulation was prepared according to the following formula:

Calcium sulfate	10 Kg.
Ethylcellulose 47 cps. (5% solution in ethanol)	4 L.

The calcium sulfate was granulated in a dough mixer[3] and then passed through an oscillating granulator[3] fitted with a No. 12 screen. The granulation was air-dried. Through a series of hand sizing and screening processes, it was separated into granulations of the following size ranges:

Mesh Size	Quantity, Kg.
12–16	1
16–20	1
20–30	5
30–40	1
40–80	1

Ethyl cellulose was used in granulating the calcium sulfate to produce a granulation which did not readily disintegrate in water or simulated gastric juice. This granulation formula was used because the experiment was designed to study the efficiency of the clays as disintegrants when they were blended with prepared, insoluble, nondisintegrating granulations. Using this granulation, 100-Gm. samples of material were prepared by adding 0.5 Gm. of magnesium stearate and various concentrations (0, 0.5, 1, 2, 4, 8, 15, or 25%) of a disintegrating agent (clays I, II, III, and starch) to the granulation. These granulations were compressed into $^{5}/_{16}$-in. standard cup, 200-mg. tablets with a Colton 120 single punch machine.[4] Tablets of a hardness of about 4.5 to 5.5 Kg. were prepared when possible. Hardness was measured with a Dillon prototype tablet hardness tester[5] and is expressed as the average of 20 measurements. The average disintegration time of six tablets was determined in gastric juice U.S.P. by the official method. The results are summarized in Table II.

Figure 1 represents the relationship found between the disintegration time and the concentration of the four disintegrating agents. The straight lines were drawn with the assumption that the relationship holds when the concentration of the disintegrating agent is between 1 and 20%. The figure also illustrates that tablets containing clay II disintegrate about twice as fast as tablets containing cornstarch as a disintegrating agent in the same percentage. Starch, in turn, is a better disintegrating agent than clay III or clay I. The addition of 2% of clay II reduced the disintegration time of the tablets to below 5 min. The presence of 5% of clay II with

[3] F. J. Stokes Co., Philadelphia, Pa.

[4] Arthur Colton Co., Detroit, Mich.

[5] W. C. Dillon & Co., Van Nuys, Calif.

TABLE II.—THE DISINTEGRATION TIME, HARDNESS, AND WEIGHT OF TABLETS CONTAINING VARIOUS CONCENTRATIONS OF DISINTEGRATING AGENTS (DISINTEGRANTS ADDED DRY TO $CaSO_4$ GRANULATION)

Disintegrating Agent	Mesh Size of Granulation	Tests	Concn. of Disintegrating Agent, %: 0	0.5	1	2	4	8	15	25
Clay III	12–16	Disintegration time, sec.	1373	1343	1238	658	464	457	53	39
		Hardness, Kg.	4.4	4.7	5.1	5.0	5.2	5.1	3.0	3.6
		Wt., mg.	198	198	200	201	204	200	194	204
Clay III	16–20	Disintegration time, sec.	1655	1429	1089	866	219	74	42	34
		Hardness, Kg.	4.8	4.2	4.5	4.6	4.2	3.5	2.7	2.9
		Wt., mg.	199	197	199	200	205	204	210	210
Clay III	20–30	Disintegration time, sec.	1581	1198	1114	752	303	200	49	33
		Hardness, Kg.	4.4	4.3	4.8	4.8	4.6	3.8	4.4	4.0
		Wt., mg.	203	205	209	208	210	206	208	217
Clay III	30–40	Disintegration time, sec.	1221	1173	921	758	277	142	47	40
		Hardness, Kg.	4.7	4.9	5.1	5.0	4.1	5.5	4.9	4.9
		Wt., mg.	195	196	199	200	199	199	195	199
Clay III	40–80	Disintegration time, sec.	1025	947	811	433	228	62	30	27
		Hardness, Kg.	4.4	4.7	5.8	5.0	5.2	5.5	5.5	5.7
		Wt., mg.	202	197	199	202	205	204	200	203
Clay III	20–30 Replicate	Av. disintegration time, sec.	1581	1218	1035	693	298	136	44	35
Clay II	20–30	Disintegration time, sec.	1581	1403	690	282	75	21	10	61
		Hardness, Kg.	4.4	5.1	5.1	4.8	4.6	4.4	4.5	4.3
		Wt., mg.	203	201	200	200	201	200	200	197
Clay I	20–30	Disintegration time, sec.	1581	1434	1068	1150	477	197	66	29
		Hardness, Kg.	4.4	5.1	5.3	4.6	4.9	3.5	3.2	2.0
		Wt., mg.	203	200	201	203	201	197	198	200
Starch	20–30	Disintegration time, sec.	1819	1305	1071	485	133	57	36	12
		Hardness, Kg.	4.8	5.2	4.5	4.4	2.8	1.4	...	...
		Wt., mg.	199	200	197	200	202	196	197	196

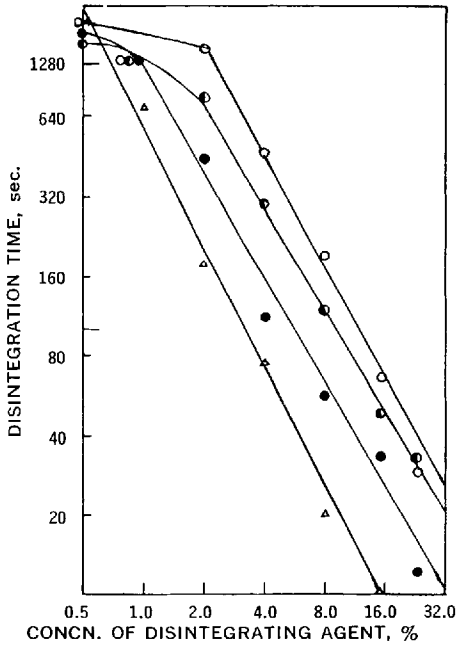

Fig. 1.—Disintegration time of $CaSO_4$–ethylcellulose tablets containing clays I, II, and III and starch as disintegrating agents (prepared from 20/30 mesh granulations). Key: ○, clay I; △, clay II; ◐, clay III; ●, starch.

the granulation reduced the disintegration time to below 1 min.

When the concentration of starch added dry with the lubricant was 8% or higher (Table II) it became very difficult to produce a tablet with a hardness above 5 Kg. Granulations containing more than 15% starch in powder form were practically incompressible, while fairly hard tablets could be produced with the concentration of clay II as high as 25%. Using clay III, in the coarser three granule sizes (Table II) a degree of tablet hardness appeared to be lost at clay concentrations of 8 to 15% or higher.

The particle-size distribution of the clay may be a factor which influences the disintegration rates of the tablets. Clay II had the smallest average particle size (completely passed a number 200 sieve) of the clays studied, and was the best disintegrant; clay I had the coarsest particles (average diameter equal to 373 μ by sieve analysis) and was the poorest disintegrant among the three clays studied (2). The ion-exchange capacity of the clays (0.601, 0.585, and 0.523 mmoles/Gm. for clays I, II, and III, respectively) and the swelling volume of the clays (21.3, 20.0, and 10.8 ml. in gastric juice) (2) showed no apparent relationship to the disintegration action of the clays.

The Disintegrating Action of Montmorillonites When Incorporated Within the Granulation.—The compatibility study (Table I) showed that the clays in this study agglomerated with many other commonly used tablet binders. It was hypothesized that this agglomeration might influence both the binding action of the binder and the disintegrating

action of the clays. Lactose and calcium sulfate were separately used as tablet fillers in this study to represent a soluble and an insoluble tablet matrix. The granulations were prepared as follows.

(*a*) A 10% suspension of clay I was prepared and was used alone, or was mixed with the other granulating agents and the mixture used to granulate the filler. The final granulations contained 1% clay by weight. If the mass was not adequately wet after adding sufficient clay suspension to provide 1% clay in the dry granulation, water was used to complete the massing operation.

(*b*) The fillers alone and with 1% clay added dry were granulated with the various syrup and gum granulating agents. These were the control samples against which the other clay-containing (clay added as granulating agent) tablets were compared. Each of the two diluents, with and without the addition of 1% clay, was also granulated with water alone.

Each batch of granulation contained 200 Gm. of lactose or 300 Gm. of calcium sulfate. The granulation masses were passed through a 12-mesh screen by hand, dried at room temperature for 48 hr., and resized through a 16-mesh screen. Magnesium stearate was added as the lubricant (0.5% for the calcium sulfate granulations and 0.25% for the lactose granulations). The tablets were compressed to a weight of 200 mg. using $^{5}/_{16}$-in. standard concave punches. A compression force was used which would yield tablets having a hardness of 2 to 3.5 Kg. for the calcium sulfate tablets and about 5 to 6 Kg. for the lactose tablets. The formula, granule hardness, disintegration time, hardness, and appearance of each batch of tablets were recorded (Table III). The dried granulations varied in per cent moisture content between 0.2 and 1.8%; most were between 0.4 and 1.2%.

According to Table III the following observations may be made.

(*a*) When the montmorillonite clays alone were used as the granulating agent-binder for calcium sulfate, soft granulations were obtained (formulas

TABLE III.—EVALUATION OF THE MONTMORILLONITES AS GRANULATION BINDERS ALONE AND IN COMBINATION WITH DIFFERENT GRANULATING AGENTS

Formula No.	Tablet Filler Base	Granulating Material	Hardness of Granules[a]	Av. Wt. of Tablets, mg.	Hardness of Tablets, Kg.	Disint. Time, sec.	Description of Tablets
1	$CaSO_4$	Water	Soft	197	1.3	278	Slight capping
2	$CaSO_4$	Clay I[c]	Soft	197	1.8	99	Slight capping
3	$CaSO_4$	Clay II[c]	Soft	205	2.1	80	Slight capping
4	$CaSO_4$	Clay III[c]	Soft	197	2.0	28	Slight capping
5	$CaSO_4$-I[b]	Water	Soft	205	2.3	94	Slight capping
6	$CaSO_4$	Clay I[d]	Medium	200	1.1	100	Slight capping
7	Lactose	Water	Medium	203	6.2	30	Satisfactory
8	Lactose	Clay I	Hard	205	5.9	242	Satisfactory
9	Lactose	Clay II	Hard	206	6.3	185	Satisfactory
10	Lactose	Clay III	Hard	202	6.2	233	Satisfactory
11	Lactose-I[b]	Water	Hard	205	5.0	125	Satisfactory
12	$CaSO_4$	Clay I and syr.[e]	Soft	204	3.5	1340	Satisfactory
13	$CaSO_4$	Syrup	Medium	200	3.4	1100	Satisfactory
14	$CaSO_4$-I	Syrup	Hard	203	3.3	710	Satisfactory
15	Lactose	Clay I and syr.	Hard	205	6.5	664	Satisfactory
16	Lactose	Syrup	Hard	205	4.2	215	Satisfactory
17	Lactose-I	Syrup	Hard	209	3.7	211	Satisfactory
18	$CaSO_4$	Starch[f]	Medium	203	2.4	63	Slight capping
19	$CaSO_4$	Clay I and starch	Medium	195	2.3	51	Satisfactory
20	$CaSO_4$-I	Starch	Medium	202	2.0	68	Satisfactory
21	Lactose	Starch	Hard	199	5.0	40	Satisfactory
22	Lactose	Clay I and starch	Hard	207	5.4	80	Satisfactory
23	Lactose-I	Starch	Hard	204	5.8	97	Satisfactory
24	$CaSO_4$	Acacia[g]	Medium	199	3.1	178	Satisfactory
25	$CaSO_4$	Clay I and acacia	Hard	207	3.5	140	Satisfactory
26	$CaSO_4$-I	Acacia	Hard	206	3.4	203	Satisfactory
27	Lactose	Acacia	Hard	202	4.8	53	Satisfactory
28	Lactose	Clay I and acacia	Hard	204	4.9	145	Satisfactory
29	Lactose-I	Acacia	Hard	202	4.6	119	Satisfactory
30	$CaSO_4$	MeCel[h]	Hard	210	2.1	3600	Slight capping
31	$CaSO_4$	Clay I and MeCel	Hard	206	2.4	2410	Slight capping
32	$CaSO_4$-I	MeCel	Medium	208	2.1	3600	Slight capping
33	Lactose	MeCel	Hard	207	4.3	257	Satisfactory
34	Lactose	Clay I and MeCel	Hard	207	4.9	67	Satisfactory
35	Lactose-I	MeCel	Hard	197	5.2	131	Satisfactory
36	$CaSO_4$	Sodium alginate[i]	Soft	206	1.8	119	Slight capping
37	$CaSO_4$	Clay I and sodium alginate	Soft	204	2.3	115	Slight capping
38	Lactose	Kelgin	Hard	203	5.8	103	Satisfactory
39	Lactose	Clay I and sodium alginate	Hard	206	4.6	125	Satisfactory
40	Lactose-I	Sodium alginate	Hard	203	4.6	134	Satisfactory

[a] Soft granules immediately powdered on handling. Medium granules partially powdered on handling. Hard granules could be handled without appreciable powdering. [b] One per cent of clay I was added dry to $CaSO_4$ or lactose before massing. [c] All clay granulating agents were used as 10% w/v suspensions. [d] Used 10% suspension of clay I as the only granulating agent without supplemental water. [e] Simple syrup U.S.P. [f] Ten per cent starch paste. [g] Ten per cent mucilage of acacia. [h] Ten per cent methylcellulose 4000 solution. [i] Two per cent mucilage of sodium alginate.

2–5, Table III). Capping of these tablets was observed during manufacture and on handling and hard tablets could not be prepared.

(*b*) Simple syrup, starch paste, and acacia were excellent granulating agents, producing hard calcium sulfate granulations. Starch, acacia, or methylcellulose, in equal volume combination with the clay suspension generally did not affect calcium sulfate granule hardness or tablet quality, but the combination granulating materials, although incompatible (Table I), did reduce disintegration time. Sodium alginate[6] reacted with the calcium ions of the calcium sulfate and could not be used successfully as a binder for this compound. All five tablet granulating agents tested appeared to be suitable for granulating lactose.

(*c*) The disintegration times of the tablets produced from formulas 1 to 6 in Table III shows that an addition of 1% of montmorillonite clays *via* the granulating agent, to calcium sulfate reduced the disintegration time of the calcium sulfate tablets from 278 sec. (formula 1) to less than 100 sec. (formulas 2–6).

(*d*) The clays in suspension form as a granulating agent permitted satisfactory granulations and tablets to be prepared of the soluble lactose formula; but so did water (formulas 7–11). One per cent of clay, added as granulating agent, greatly increased the disintegration time of the soluble lactose tablets (formula 7 *versus* 8–11). The clay decreased the time of disintegration of the lactose tablets containing methylcellulose as the binder (formulas 33–35). The slowly hydrating methylcellulose apparently prevents the water from penetrating to the core of the soluble tablets (No. 33 and 35). The methylcellulose was dehydrated and agglomerated in the presence of the clay (No. 34), which resulted in more rapid disintegration while not adversely affecting granule hardness or tablet quality. It is interesting to note that in the insoluble calcium sulfate tablets the combination of methylcellulose and clay, even though incompatible (Table I), also produced tablets superior to those obtained when methylcellulose was used singly.

(*e*) It is obvious from Tables I and III that the combination of compatible granulating agent plus clay suspension as binder (syrup plus clay or starch paste plus clay) offered no apparent advantage. In the syrup–clay systems the combination produced the longest disintegration time with both diluents (formulas 12 and 15), while the clay–starch combination tablets (No. 19 and 22) were either not substantially better or were poorer than tablets prepared from starch paste alone. Acacia and methylcellulose agglomerated with the clay, and the disintegrating action of the combination systems were usually superior to the gum or polymer alone, while the combination systems retained adequate binding properties (formulas 24–29 and 30–35).

The clays were also studied for their use as tablet disintegrating agents when added to the granulation internally with the diluent in concentrations ranging from zero to 25%. Four granulating agents (20% glucose, 5% acacia, half strength simple syrup, 10% clay I suspension) and water and the four disintegrating agents (the three clays plus starch) were studied. Three hundred grams of calcium sulfate and disintegrating agent were mixed and moistened with 80 ml. of one of the selected granulating agents, the mass passed through a 20-mesh screen, oven dried at 100°F. for 24 hr., sized to 20 mesh, and lubricated with 1% magnesium stearate. Tablets were compressed to 200 mg. at a hardness of from 3.5 to 5.0 Kg.

Increasing the montmorillonite clay concentrations from 1–16% with each granulating agent failed to produce an equivalent consistent decrease in disintegration times, which varied inconsistently from about 10 to 30 min. *versus* 5 to 12 min. for starch. This showed that wetting the clay type disintegrating agents during the granulation process reduces the activity of the disintegrating agents. Compared to starch the clays were all relatively poor disintegrating agents when wet granulated together with the filler.

The loss of disintegration activity of the montmorillonite clays after wet granulation may be explained in two ways. First, the clays and the binder may form an adhesive gel on disintegration and thus resist falling through the screen of the disintegration tester. Although the tablets disintegrated, they remained partially suspended inside the basket and, according to U.S.P. specification, disintegration was incomplete. Second, certain granulating agents may form a film surrounding clay particles and reduce the rehydration rate of the clay.

CONCLUSIONS

Montmorillonite suspensions alone were poor granulating agents producing inadequate granule binding.

Although montmorillonite clays are relatively inert chemically, they interact with many organic macromolecules. This interaction reduces but does not destroy the binding properties of certain tablet binders while the interacted systems may produce superior tablets with more rapid disintegration than either binder alone.

The clays are not good disintegrating agents when they are added as a dry powder to the filler and are wet granulated. They are good disintegrating agents and may be superior to starch when added dry to completed granulations, in which case the disintegration time and the concentration of clay added demonstrate an inverse second-order relationship.

[6] Marketed as Kelgin by the Kelco Co., Clark, N. J.

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Mathematical Model of Sustained-Release Preparations and Its Analysis

By EKKEHARD KRUGER-THIEMER* and STUART P. ERIKSEN†

A pharmacokinetic model, involving first-order processes for drug release, absorption, and elimination, may be used for the description of the behavior of sustained-release preparations consisting of slowly and immediately available fractions. The shift of the rate-determining step, as the relative rates of absorption and release are changed, can be shown both mathematically and by analog computer simulation. These theoretical results are used for interpretation of experimental data with a new sulfa drug of low solubility, 2-sulfanilamido-5-methyl-pyrimidine, compressed tablets of which behave as a sustained-release preparation.

THE WIDESPREAD use of sustained-release and depot preparations in drug therapy has made the full understanding of their actions and properties of considerable importance. A number of publications concerned with the actions, the properties, and the importance of such a full understanding have appeared in the last decade [*viz.*, Dost (2), Lazarus and Cooper (3), Levy *et al.* (4–6), Nelson *et al.* (7–9), Parrott (10), Robinson and Swintosky (11), Sjogren and Ostholm (12), Wagner *et al.* (13), Wiegand and Taylor (14), and others].

The most useful method for the examination of the interrelationships between drug preparations and the human body has been found to be through the use of a mathematical model, first fitting it to the actual data, and then examining the model found most useful for its functional behavior. In this way the important implications of the model may be discovered and those found important checked by further laboratory experiments. Because of the complexity of the differential equations involved, direct mathematical study is not always the most useful manner of determining the implications of a mathematical model for absorption, distribution, and excretion, and it has often been found more convenient to make use of an electronic analog computor [see Fish (16), Pace (17), and Garrett *et al.* (18)].

A recent paper by Taylor and Wiegand (15) was concerned with the analog investigation of the following model, which has been found to describe suitably the behavior of sustained-release preparations from which a certain fraction, f_r, of the administered dose becomes available through a first-order process while the remaining fraction, $1-f_r$, is available immediately:

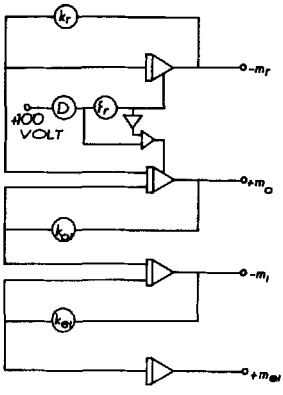

Fig. 1.—Unscaled analog computer program for the system of differential Eqs. for the model used and for initial conditions set forth in Eqs. 1, 2, and 3.

m_r the amount of undissolved drug in the gastrointestinal tract

k_r *the rate constant for the dissolution step*

m_0 the amount of dissolved drug in the gastrointestinal tract

k_{01} *the rate constant for absorption*

m_1 the amount of drug in the body (not including that in the gastrointestinal tract and the urine)

k_{e1} = *the rate constant for elimination* (the sum of the constants for renal excretion and metabolism)

$m_{e1} = m_2 + m_1{}^m$ the amount of the eliminated drug (including metabolized drug)[1]

This model involves three consecutive, irreversible first-order processes with the following initial conditions in the four compartments:

$$m_r{}^0 = Df_r,\ m_0{}^0 = D(1 - f_r),\ m_1{}^0 = 0,\ m_{e1}{}^0 = 0 \qquad \text{(Eqs. 1, 2, 3)}$$

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[1] In this paper the symbols as proposed by Nelson and Kruger-Thiemer (9) are used as far as possible. The use of amounts, m, rather than concentrations, c, throughout this paper should not be construed as reflecting an "amount" driven kinetic situation, but only as a system simplification for these theoretical discussions that does not change the relative results.

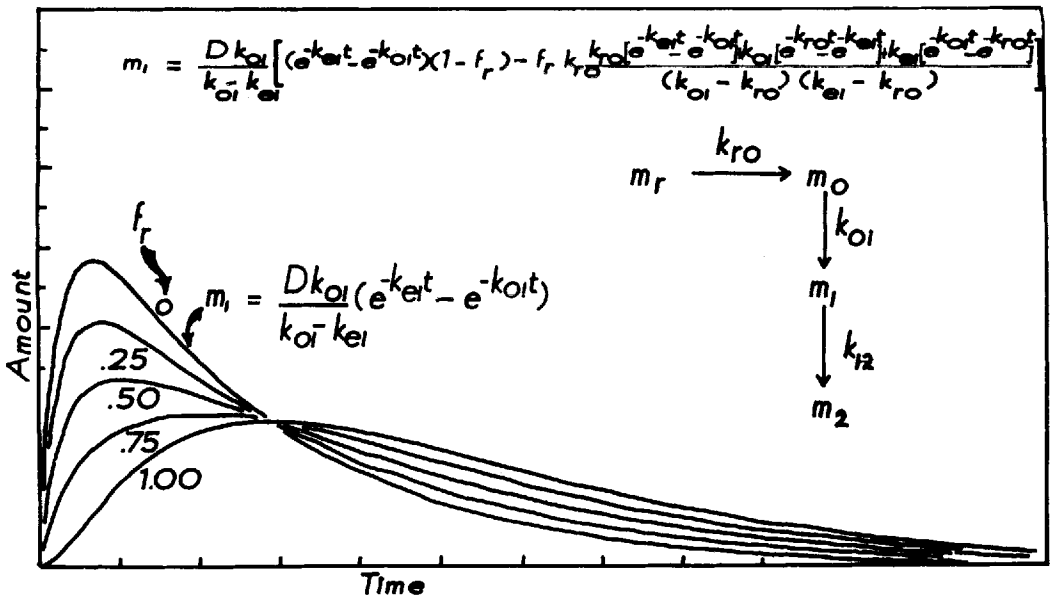

Fig. 2.—Family of computer drawn curves of m (blood amount) showing the effect of regular changes in the fraction of the total dose in sustained form (f_r).

The present paper is concerned with a more complete re-examination of this model.[2]

ADDITIVE BEHAVIOR OF TWO COMPONENTS OF SUSTAINED-RELEASE PREPARATION

Changing the sustained-release fraction, f_r, of the administered dose, D, from 0 to 1, in a stepwise fashion, a family of curves of the type shown in Fig. 2 is obtained. The most interesting feature of this figure is that this family of curves has a point of intersection that might be called the common point, which lies in the maximum point of the lowest curve corresponding to $f_r = 1$. The relationships suggested by this figure may be shown mathematically also.

Substituting $f_r = 0$ into Eq. 2 yields the well-known equation for a model consisting of two consecutive, irreversible first-order reactions, presented previously by Widmark and Tandberg (20), Teorell (19), Dost (2), and Taylor (14):

$$m_1 = \frac{D \cdot k_{01}}{k_{01} - k_{el}} \cdot (e^{-k_{el}t} - e^{-k_{01}t}) \quad \text{(Eq. 4)}$$

The substitution of $f_r = 1$ into Eq. 2 results in Eq. 5 for the model in which the total drug administered is going slowly into solution *via* a first-order process.

$$m_1 = D \cdot k_{01} \cdot k_r \times \frac{(k_{01} - k_{el})\, e^{-k_r t} + (k_{el} - k_r)\, e^{-k_{01}t} + (k_r - k_{01})\, e^{-k_{el}t}}{(k_r - k_{01}) \cdot (k_r - k_{el}) \cdot (k_{01} - k_{el})} \quad \text{(Eq. 5)}$$

[2] The computer curves shown in this work were obtained with the more standard analog programming methods (using the differential forms) rather than the less accurate and more tedious "calculation" method of Taylor and Wiegand (1). The more standard program used by the present authors is shown in Fig. 1 (derived for the Applied Dynamics PB-24 computer, Ann Arbor, Mich.).

The existence of the common point for all curves with the same k_r, k_{01}, k_{el} may be shown by calculating the point(s) of intersection of the two curves for $f_r = 0$ and $f_r = 1$. One equates m_1 from Eqs. 4 and 5 so that the following condition results for the time value, t_i, of the point of intersection:

$$k_r \cdot (k_{01} - k_{el}) \cdot e^{-k_r t_i} + k_{01} \cdot (k_{el} - k_r) \cdot e^{-k_{01} t_i} + k_{el} \cdot (k_r - k_{01}) \cdot e^{-k_{el} t_i} = 0 \quad \text{(Eq. 6)}$$

While Eq. 6 cannot be solved explicitly for t_i, one can see that it contains three solutions for t_i, two trivial ones, $t_i = 0$, $t_i = \infty$, and the intermediate solution in which we are interested. From this equation the time value, t_i, of the point of intersection for actual value of the parameters, k_r, k_{01} and k_{el}, may be calculated using the customary methods for numerical solution of transcendental equations. One obtains the same equation, Eq. 6, for the point of intersection of the curve for $f_r = 0$ (Eq. 4) and *any* other of the curves shown (*e.g.*, any arbitrary value of f_r between 0 and 1). During this calculation f_r cancels out, the mathematical indication of the independency of the point of intersection on the value of f_r; the entire family of curves intersects at a common point.

Proof that the common point is situated in the maximum of the curve for $f_r = 1$ can be verified by setting the first derivative of Eq. 5 with respect to the time equal to zero. This results in exactly the same equation, Eq. 6, as previously obtained, indicating that the time corresponding to this maximum, $t_{max.}$, and the time of the point of intersection of the family of curves, t_i, are identical.

This apparent additivity suggests a rearrangement of the equation obtained by Taylor and Wiegand (1) (our Eq. 2) into an equation representing the sum of Eqs. 4 and 5 and yields Eq. 7:

$$m_1 = \frac{D \cdot (1 - f_r) \cdot k_{01}}{k_{01} - k_{el}} \cdot (e^{-k_{el}t} - e^{-k_{01}t}) + \frac{D \cdot f_r \cdot k_{01} \cdot k_r \cdot (k_{01} - k_{el}) \cdot e^{-k_r t} + (k_{el} - k_r) \cdot e^{-k_{01}t} + (k_r - k_{01}) \cdot e^{-k_{el}t}}{(k_r - k_{01}) \cdot (k_r - k_{el}) \cdot (k_{01} - k_{el})} \quad \text{(Eq. 7)}$$

from which Eqs. 4 and 5 may still be obtained by setting $f_r = 0$ and 1 alternately.

Using the symbols F_4 and F_5 for the right-hand sides of Eqs. 4 and 5, respectively, the structure of Eq. 7 becomes apparent

$$m_1 = F_4 \cdot (1 - f_r) + F_5 \cdot f_r \quad \text{(Eq. 8)}$$

Thus, the amount in the body, m_1, (not including that in gastrointestinal tract and in urine) for any time and for any value of f_r between 0 and 1 is equal to a linear combination of the drug amounts for $f_r = 0$ and $f_r = 1$, the coefficients of which are equal to the two fractions of the dose for instantaneous $(1-f_r)$ and sustained (f_r) release. In terms of the experimental conditions this implies that both parts of the dosage form, that for instantaneous and that for sustained release, behave independently within the body, so that *the final curve of the drug amount within the body is merely the sum of the two curves that would result from separate administration of the two components of the sustained-release dosage form* (*e.g.*, this conclusion might be checked experimentally using a sustained-release preparation one of the two parts of which, either instantaneous or sustained release, is radioactively labeled). The result of this study clearly demonstrates that the functional behavior of the curves described by Eqs. 2 or 7 may be examined best by separate examination of Eqs. 4 and 5.

ASYMPTOTICAL SIMILARITY BETWEEN SLOW SUSTAINED-RELEASE AND SLOW ABSORPTION MODELS

Figure 3 shows two families of curves in which the first (Fig. 3, A) corresponds to the simplified model for $f_r = 0$, *i.e.*, Eq. 4, using values of k_{01} from 1.0 to 0.0078 hr.$^{-1}$, and the second (Fig. 3, B) corresponds to the simplified model for $f_r = 1$, *i.e.*, Eq. 5, using a constant value of $k_{01} = 1.0$ hr.$^{-1}$ and varying values of k_r from infinity to 0.0078 hr.$^{-1}$. In both cases the values of k_{el} have been set equal to 0.1 hr.$^{-1}$.

The curves with the highest maximum in Fig. 3, A and B, are identical, for $k_r = \infty$ means instantaneous release, *i.e.*, in this case Eq. 5 reduces to Eq. 4 by rearrangement of Eq. 5 before the substitution of $k_r = \infty$, or by use of L'Hospital's rule for indeterminate forms of the type ∞ / ∞.

Obviously, the lowest members of the two families of curves (Fig. 3), having identical values for k_{01} and k_r, respectively, are more similar to each other than the intermediate and the higher ones. These curves have suggested an asymptotical similarity of these two different models. The mathematical demonstration of this similarity may be found by substituting very high values of k_{01} (rising to infinity) into Eq. 5. As Eq. 5 has a symmetrical structure with respect to k_{01} and k_r, the mode of calculation is quite the same as above for $k_r = \infty$, so one finally obtains Eq. 9:

$$m_1 = \frac{D \cdot k_r}{k_r - k_{el}} \cdot (e^{-k_{el}t} - e^{-k_r t}) \quad \text{(Eq. 9)}$$

which has obviously the same structure as Eq. 4. The result is not surprising, indicating only the shift in the rate-determining step; it has already been predicted (7). The similarity of Eqs. 4 and 9 is the reason for the asympotic similarity of the lower curves in Fig. 3, A and B, because before going to $k_{01} = \infty$ all the terms in Eq. 5 that are lacking in Eq. 9 have coefficients of the type k_r/k_{01} or k_{el}/k_{01}. The smaller these two ratios, the more similar the curves in Fig. 3, B, will be to the curves in Fig. 3, A.

There is no *real* equality in Fig. 3, but only a similarity between the lower curves. The two before mentioned ratios k_r/k_{01} or k_{el}/k_{01} may become negligible either by decreasing k_r and k_{el} or by increasing k_{01}. As long as k_{01} has a finite value, the initial slopes for the curves in Fig. 3, B, will be zero, while the curves in Fig. 3, A, have initial slopes higher than zero, a fact that may be verified by differentiation of Eqs. 5 and 4 with respect to the time followed by substitution of $t = 0$.

The difference in the initial slopes of the lower curves in the Fig. 3, A and B, are easily visible from these fully drawn theoretical curves. However, it would be nearly impossible to recognize these differences in initial slope from actual experimental data, considering the usual experimental errors.

ESTIMATION OF RATE CONSTANTS OF SUSTAINED-RELEASE OR SLOW ABSORPTION PREPARATIONS

Figure 3 shows another feature of practical importance. In cases of slow absorption and/or slow sustained release a significant absorption may last much longer than anticipated so that the slope of the descending part of the concentration curve in the blood plasma (after the curve maximum) may not reflect the elimination process alone. As pointed out recently by Wagner (21), a graphical estimation or calculation of the rate constant for elimination, k_{el}, from the apparent slope of the descending part of the curve in a semilogarithmical plot may result in too low a value. Since the customary methods for the calculation of the rate constant for absorption [Dost (2), Kruger-Thiemer (22, 23), Nelson (24), Wagner (25), Schlender and Kruger-Thiemer (27)], are based on the knowledge of the rate constant for elimination, the calculated value of k_{01} will be incorrect in such cases, too. This difficulty cannot be overcome by the method of Dost and Medgyesi (26) or even by methods using digital computation (27) unless the error of the points of measurement is much lower than usual. In such cases it seems to be necessary to estimate the rate constant for elimination (and the corresponding biological half-life, $t_{50\%} = \ln 2/k_{el}$) from independent experiments with *intravenous* administration of the same drug, to the same test subject.

Tables I and II and Fig. 4 demonstrate an actual example of that kind. These experiments, the details of which will be published elsewhere (28), were performed with 2-sulfanilamido-5-methylpyrimidine, a sulfa drug on the German market

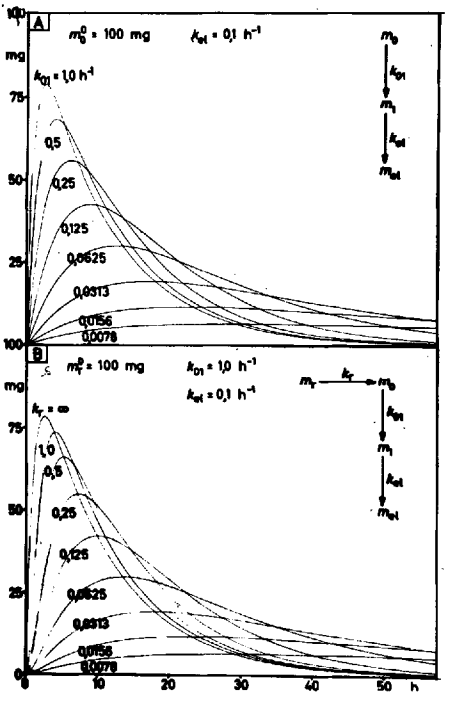

Fig. 3.—Families of computer drawn curves of m (blood amount) for the model used. Key: A, the simplified form of Eq. 4, varying k_{01}; B, the simplified form of Eq. 5, varying k_r.

TABLE I.—BLOOD PLASMA CONCENTRATION OF 2-SULFANILAMIDO-5-METHYL-PYRIMIDINE AFTER ORAL OR INTRAVENOUS ADMINISTRATION OF 2000 mg.

Time After Admin. t, (hr.)	Mode of Administration[a] (Test) Oral[b] (1) c_1' (mg./L.)	Oral[c] (2) c_1' (mg./L.)	Intravenous (3) c_1' (mg./L.)
1.0			579.8
1.33		97.9	
2.0	36.1		
4.0	74.5	149.4	473.4
6.0	88.5	144.8	445.0
8.0	100.0	120.7	412.0
24	68.4	89.6	245.4
32	80.1	68.3	153.2
48	49.9	68.8	82.0

[a] Test subject: male, 45 years, 75.0 Kg. [b] Commercial tablets. [c] Freshly precipitated suspension (neutralized solution of the sodium salt).

since 1961.[3] Table I contains the chemically measured [method of Bratton and Marshall (29)] drug concentrations in the blood plasma, c_1', after oral or intravenous administration of 2000 mg. of 2-sulfanilamido-5-methyl-pyrimidine.

The data in Table II show that the biological half-lives after oral administration are significantly higher than the biological half-life after intravenous administration. The 95% confidence limits of the latter value are much narrower than the confidence limits of the foregoing values. The same situation prevails for the apparent plasma distribution coefficient (apparent relative volume of distribution with respect to the plasma concentration). But it is unlikely that the mode of administration would influence the processes of distribution and elimination (metabolism and renal excretion) so greatly. It seems much more appropriate to assume that the apparent biological half-life and the apparent plasma distribution coefficient after oral administration should have approximately the same values as after intravenous administration. Using the assumption that the differences noted are due to a slow absorption or a slow sustained release, a provisional calculation has been made using the method of Dost and Medgyesi (26), in which the value of the rate constant for elimination after intravenous administration, $k_{el} = 0.0170$ hr.$^{-1}$, has been used (therefore, only two points of measurement, related by $t_2 = 2 \cdot t_1$, are necessary instead of three as in the original method). Using the data from $t = 4$ and 8 hr. of test *1* (Table I) produced a rate constant for absorption (or for sustained release) of k_{01} (or k_r) = 0.1775 hr.$^{-1}$ by substitution into the equation

$$e^{-k_{01}t_1} = \frac{c_1'^2}{c_1'^1} - e^{-k_{el}t_1} \qquad \text{(Eq. 10)}$$

which results from $t_2 = 2 \cdot t_1$ and Eq. 4 or Eq. 9 ($c_1'^2$ denotes the plasma concentration value at the time t_2). The corresponding value of the apparent plasma distribution coefficient, Δ', is 0.166 ml./Gm., which lies within the confidence limits of the corresponding value after intravenous administration (Table II) and supports the assumption underlying this calculation that Eqs. 4 or 9 may be used for the description of this part of the experimental curve. Using these three parameters one predicts 29.9 mg./L. as the expected value of c_1' at 48 hr. after the administration. This value is appreciably lower than the measured value of 49.9 mg./L. and might be explained by the following calculation. Repeating the foregoing calculation with the points of measurement at 24 and 48 hr. we get the rate constant for absorption, $k_{01} = 0.0437$ hr.$^{-1}$, and the apparent plasma distribution coefficient, $\Delta' = 0.149$ ml./Gm., the last of which is again rather close to the confidence limits of the corresponding value after intravenous administration. The apparent

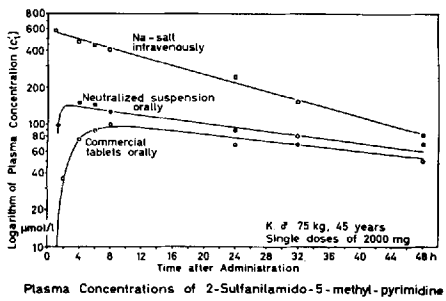

Fig. 4.—Sample experiment showing plasma concentration/time relationship for 2-sulfanilamido-5-methyl-pyrimidine.

[3] Marketed as Pallidin, by E. Merck AG., Darmstadt, Germany. The authors are grateful for the support of these studies by the manufacturer of this drug.

TABLE II.—APPARENT BIOLOGICAL HALF-LIVES, $t'_{50\%}$, APPARENT PLASMA DISTRIBUTION COEFFICIENTS, Δ', ABSORBED FRACTION OF THE DOSE, δ_1, AND RATE CONSTANT FOR ABSORPTION, k_{01}, CALCULATED FROM DATA OF TABLE I

Test	Mode of Admin.	Apparent Biological Half-Life, $t'_{50\%}$, hr.	Apparent Plasma Distribut. Coeff., Δ' (ml./Gm.)	Absorbed Fraction Dose, δ_1	Rate constant for Absorption k_{01} (hr.$^{-1}$)
1	Oral[a]	40.8 (20.8–80.2)[b]	0.910 (0.666–1.243)	0.19 (0.15–0.25)	0.397 (0.222–0.712)
2	Oral[c]	36.3 (22.3–59.0)	0.691 (0.550–0.868	0.25 (0.21–0.31)	3.384 (1.546–7.408)
3	Intra-venous	17.1 (15.7–18.7)	0.174 (0.159–0.189)	1.00[d]	...

[a] Commercial tablet. [b] Figures in parentheses are 95% confidence limits (Student *t* test of the average mean logarithmic values). The calculation of the data in this table was done using a digital computer program for fitting a curve to Eq. 4 (to be published) at the Calculation Center, University of Kiel (Dr. B. Schlender), Germany, with the electronic digital computer XI (Electrologica, Inc., Amsterdam, The Netherlands). [c] Freshly precipitated suspension (neutralized solution of the sodium salt). [d] By definition the entire dose is injected into the body.

rate constant for absorption (or release) for the time interval from 24 to 48 hr. is less than one-third of the value for the time interval from 4 to 8 hr. This relates well to the fact that the usual time of passage through the small intestine is approximately 8 to 12 hr., and that the rate of absorption in the large intestine is slower than in the small intestine [*cf.*, Eriksen *et al.* (30) and Diller and Bunger (31)]. Therefore, it might be necessary for a proper description of the experimental data to use the idea of a variable rate constant for absorption (or release) changing with time. Pharmacokinetic approaches of this type have been given by Wagner and Nelson (21, 32) and Stelmach *et al.* (33). In experiment *1* (Tables I and II) the rate constant for absorption (or release) in the time interval from 24 to 48 hr. is according to the calculation method of Dost and Medgyesi (26) in the same order of magnitude as the rate constant for elimination, $k_{e1} = 0.0404$ hr.$^{-1}$. From Fig. 3 and the foregoing discussion it is clear that this is one of the reasons for the apparently low slope of the curves after oral administration in Fig. 4, resulting in erroneous values for the biological half-life. The other reason is the higher value of the rate constant for absorption within the first 8 hr. Both reasons together allowed the digital computer to find a curve fitting solution according to Eq. 4, as shown in Fig. 4, which contains the erroneously high value of the biological half-life. A more detailed analysis of our experiments (Table I) is not feasible because of the low number of points of measurement.

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Theoretical Formulation of Sustained-Release Dosage Forms

By J. R. ROBINSON and S. P. ERIKSEN*

The present investigation deals with the result of a mathematical and an analog computor analysis of the kinetic relationships governing the rate of release of drugs from sustained-release dosage forms. Two types of release have been considered, those described by zero-order and those that can be described by first-order kinetics. In addition, mathematical equations are derived that permit the calculation of doses and of release constants that will give a blood concentration *versus* time curve most closely approximating an "idealized" curve.

THE APPROACH most often used in the kinetic treatment of biologic data is that involving formulation of a mathematical model, the comparison of this model with *in vivo* findings, and finally the adjustment of the model and its constants to accommodate the *in vivo* results. Once a suitable model has been established, the complete interdependency of the model's parameters can be examined,[1] subject to the suitability of the model chosen. These parameters may be divided into two types, those under the control of the formulator, *i.e.*, the dosage form, release pattern, and rate, etc., and those that are imposed upon the model by the system studied, *i.e.*, the absorption, distribution, and excretion (ADE) pattern for the drug in the body. In the past, the major emphasis has been placed on the ADE parameters with relatively little attention being given to the dosage form release rate and pattern, not only because these studies are more difficult to carry out, but also because initially it was of particular concern to study the suitability of various models as simulations of the body's ADE capabilities. It seemed reasonable at that time to assume that in cases where the drug has been administered by injection or orally in some readily available form, *e.g.*, drug in solution, the effect of the dosage form release pattern might safely be neglected; for nonreadily available forms, particularly sustained-release dosage forms, this assumption cannot be made as the dosage form release pattern and its rate undoubtedly do play *major* roles in the blood concentration *versus* time curve obtained; indeed, it is the exploitation of this effect that makes sustained release possible. In addition to the rate and pattern of release from the dosage form, consideration must also be given to the effect of the relative amounts of the administered initial and maintenance dose on the resultant concentration in the blood. In the face of the recent barrage of studies supporting the suitability of the simpler kinetic models as descriptions of ADE phenomena, attention should now be paid to those aspects of the kinetic path less amenable to analysis, the dosage form release pattern and administered dose fractions. Indeed, a knowledge of the effects of these controllable parameters is essential in order to formulate sustained-release dosage forms having particular blood level characteristics.

Both Wiegand and Taylor (4, 5) and Wagner (6) have shown that the per cent released *versus* time data reported in the literature for many sustained-release preparations follow apparent first-order rate equations. Similarly, others (7, 8) have shown that some sustained-release preparations release drugs by apparent zero-order processes. From an experimental standpoint it would appear that these two mechanisms might adequately describe the rate of release for the majority of existing sustained-release dosage forms, and ADE equations involving both of these release patterns have already been described (5, 6, 9).

In order to obtain a constant blood level for some desired period of time from a sustained-release dosage form, Nelson (10) has stated that a constant (zero-order) rate of release from a dosage form is desired and has developed an approximate equation for calculating the amounts of sustained and initial drug forms required, based upon this assertion.

Utilizing essentially the same model as that of Nelson (10) but assuming first-order release, Wiegand and Taylor (1) have reported computer

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[1] Testing of the effect of various parameters in a model can be carried out very effectively on an analog computer, since variables can be changed and the effect read out immediately with a subjective appraisal being made even if complete mathematical solutions are unavailable. Utilization of the analog computer for this purpose is well documented (1–3), therefore, the required computer technology will not be stressed here. The circuits used in this study are given in the *Appendix*.

drawn curves showing the effect of altering the first-order release constant at a constant rate of absorption and elimination. In addition, they have also shown the effect of variation in the elimination constant on the blood concentration curve at a constant first-order rate of release from the dosage form and a constant rate of absorption. Computer drawn curves showing the effect of the fraction of the initial and maintenance dose at a constant rate of absorption, elimination, and first-order rate of release from the dosage form excretion have also been described (2), again using the same model.

In a recent paper supporting Nelson's assertion, Rowland and Beckett (9) have further claimed that first-order release from a dosage form *cannot* give the "idealized" blood concentration-time curve.

Unfortunately, experience suggests that the majority of sustained-release formulation techniques produce formulations that release drug at roughly a first-order rather than zero-order rate. In order to adequately compare these two availability patterns as to their potential to produce suitable sustained-action forms, a complete investigation of the effects of the various parameters in the models is essential. *Part* of such a study has been done for the first-order release case (1), but to the authors' knowledge a study of the effect of design parameters for a formulation having zero-order release characteristics has not been reported.

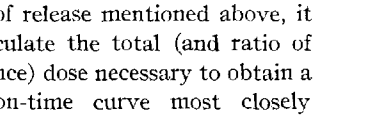
For both types of release mentioned above, it is desirable to calculate the total (and ratio of initial to maintenance) dose necessary to obtain a blood concentration-time curve most closely approximating the "idealized" case. Nelson (10) has given a method for calculating the maintenance dose of a constant rate of release dosage form, based on the biological half-life of the drug and the dose required to give the desired blood level, assuming, however, that the blood level *begins* at the concentration desired. The assumptions of these equations have been criticized recently (9), but completely corrected equations were not given. For first-order release from the dosage form, Wiegand and Taylor (4) have presented equations for calculation of the total dose remaining in a dosage form *in vitro*. These equations, while useful, cannot predict which combination of rate constant, fraction in initial form, and fraction in the maintenance form, will give a particular blood level.

In order to calculate the dosages required, methods must be available to obtain the optimum release rate constant. For zero-order release, one author (10) feels it is the product of the elimination constant and the dose required to give the desired blood level, while another (9) feels it is the product of the elimination constant and the desired blood level itself. For first-order release, there appears to be no suitable method available for obtaining the desired rate of release constant, or the fractions of initial and maintenance dose required.

In an effort to summarize the work in this area, the present investigation was designed to completely characterize the standard model for sustained-release dosage forms. In addition, it is the authors' purpose to report the analog computer, and where possible, mathematical solutions of the equations describing absorption, distribution, elimination, and availability relationships with the over-all goal of devising complete equations suitable for calculation of the doses and rate constants to give a desired blood level based on the type of release pattern employed or available. It will be apparent to the pragmatists among the readers that the ease with which a given type of release can be formulated must always be a consideration and the value of considering only those parameters within the reach of the experimenter will be appreciated.

GENERAL CONSIDERATION OF THE MODEL

In this study, the following model [after Teorell (11)] has been adopted, portions of which have been found to adequately describe actual biological processes.



(Eq. 1)

where

D = concentration of drug remaining in the dosage form,
G = concentration of drug at the sites of absorption,
B = concentration of drug in the fluids of distribution (for purposes of simplicity referred to as blood concentration),
U = concentration of drug in the urine or other permanent drug sink,
E = concentration of drug metabolized,
k_r = rate constant for release of drug from the dosage form, where the superscript 0 and 1 indicate the apparent order of release. The wavy arrow is used with k_r to indicate that the precise *form* of the release is a variable also,
k_a = rate constant for absorption,
k_e = rate constant for elimination of unchanged drug,
k_u = rate constant for elimination *via* all other routes.

For purposes of simplicity k_e and k_u have been combined into one rate constant k_d (where $k_d = k_e + k_u$).

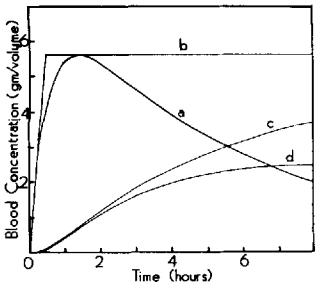


Fig. 1.—Comparable blood level *versus* time curves obtained for (a) an immediately available dose, (b) an "idealized" sustained-action formulation, (c) zero-order release maintenance from, (d) a first-order release maintenance form.

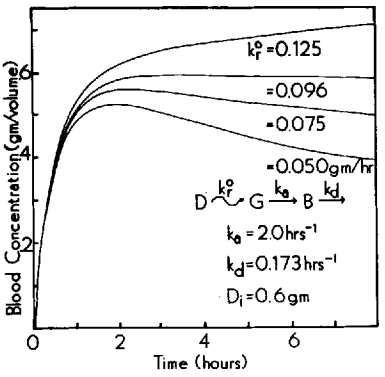


Fig. 2.—Blood level *versus* time curves showing the effect of variation in the zero-order release constant (using the ADE constants from *Reference 9*).

In using a model such as this, a problem arises in the treatment of the various components present from the standpoint of concentrations and compartments. Recent articles (9) have used amounts and concentrations interchangeably, but since the driving force in kinetic equilibria is concentration, amount can be used in its place to describe the kinetic relationships between compartments only when the volumes in each compartment are the same or when the assumption is made that the whole process takes place in the same compartment and volume. Since the one compartment–one volume idea is a useful and common, but tacit assumption, perhaps a brief explanation is necessary. The concept is more easily understood if the relationship of Eq. 1 is viewed as a chemical reaction involving four steps and taking place in a single given volume of solution, *i.e.*, a beaker. The model then becomes volume independent; the concentrations obtained have units of moles per unit volume or grams per unit volume; amounts and concentrations are interchangeable. Since the volume in each compartment of the body is different, conversion from concentrations as described by the equations, to amounts in the body compartments, then requires a knowledge of the relative compartment volumes and assumes complete uniformity within each compartment. Such a

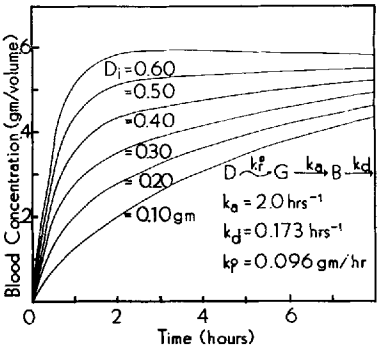


Fig. 3.—Blood level *versus* time curves showing the effect of variation in the initial dose (using ADE constants from *Reference 9*).

change is not of concern if fractions of total dose *only* are to be considered.

In this study the one volume–one compartment idea has been adopted for simplicity and thus the results are subject to the above assumptions. In addition, it is assumed that the equilibria (which must exist) for each component lie far to the right, so that reverse reactions are negligible, the drug is completely absorbed, and that after release it is immediately available.

The concentration of drug at the absorption site at time zero is the initial dose (D_i) and is equal to the fraction in the initial or in the immediately available dose (F_i) times the total dose given (W), *i.e.*, drug being in solution or in some rapidly dissolving drug form. The concentration of drug in the dosage form at time zero is the maintenance dose (D_m) and is that fraction of dose (F_m) required to maintain an optimum and as nearly as possible a constant concentration in the blood for a given length of time times the total dose given (W). It is proposed that release from the maintenance portion of the dosage form can be described by either zero- or first-order kinetics.

RESULTS AND DISCUSSION

Figure 1, curve a, illustrates the blood concentration *versus* time computer curve obtained for an immediately available dose using the model (Eq. 1), based on representative values of k_a = 2.0 hr.$^{-1}$ and k_e = 0.2 hr.$^{-1}$. Curves c and d (Fig. 1) are representative blood concentration curves for maintenance forms releasing drug by zero- and first-order kinetics, respectively.[2] Curve b (Fig. 1) illustrates the desired or "ideal" curve for a sustained-release dosage form, which includes both initial and maintenance dose. The design of a suitable sustained-action dosage form thus depends on finding the combination of a and c or a and d that produces the curve b, if such a combination exists, or as close an approximation as is possible.

The effect on the blood concentration *versus* time curve due to variation of the release rate con-

[2] The computer curves shown in this report were obtained using an Applied Dynamics AD-24-PB computer, a Moseley model 2D-2AM x-y recorder with a type F-1 photo electric curve follower, and an Electro-Instruments model 101-1518 x-y recorder.

stant as well as the effect of varying F_i and F_m are discussed under the appropriate headings for zero- and first-order release from the dosage form, and in addition, the mathematical relationships necessary to calculate both the total dosage and the required rate constant such that as close an approximation to the ideal as possible is obtained are discussed under their appropriate headings.

RELEASE BY ZERO-ORDER KINETICS

General Concepts.—From an immediately available dose, the blood concentration at any time, t, is a function of k_a, k_e, and concentration of drug in the gut (Eqs. 1 and 2),

$$B_t = \frac{D_i k_a}{k_d - k_a} (e^{-k_a t} - e^{-k_d t}) \qquad \text{(Eq. 2)}$$

where B_t is the concentration of drug in the blood at any time, and all other symbols represent quantities previously defined. The peak concentration and time to arrive at the peak are also functions of these parameters. The equation for the peak time (T_p) being:

$$T_p = \frac{2.3}{k_a - k_d} \left(\log \frac{k_a}{k_d}\right) \qquad \text{(Eq. 3)}$$

To obtain a constant blood level, one suspects, and can show mathematically, that a constant rate of availability from the dosage form is desired and once this desired rate is estimated (k_r^0), the required maintenance dose (D_m) may be found as the product of k_r^0, and the time over which sustained action is desired (h),

$$D_m = k_r^0 \times h \qquad \text{(Eq. 4)}$$

The desired rate of availability (k_r^0) can be roughly estimated, from the equations for the model, to be

$$k_r^0 = k_d \times B_d \qquad \text{(Eq. 5)}$$

where B_d is the desired blood level. The rational for this estimate can be shown by considering the differential equation for the blood level obtained from a sustained-action dosage form having both an initial and a zero-order maintenance form. From the general equations of the model in Eq. 1 one can obtain the relationship:

$$dB/d_t = k_r^0 - k_d(B_d) - e^{-k_a t}[k_r^0 - k_a(D_i)] \qquad \text{(Eq. 6)}$$

a constant blood level would require that $dB/d_t = 0$ therefore,

$$k_r^0 = k_d(B_d) - e^{-k_a t}[k_r^0 - k_a(D_i)] \qquad \text{(Eq. 7)}$$

if k_a is very large, *i.e.*, the absorption phase is not at all rate limiting

$$k_r^0 = k_d(B_d) \qquad \text{(Eq. 5)}$$

where B_d is the blood level to which the sustained action is aimed. Note that the assumption made to obtain Eq. 5 is in essence that the blood level *equals* B_d at time zero ($k_a = \infty$). This is of course not the true situation and while the use of Eq. 5 produces a reasonably flat blood level curve, it is *not the desired blood level* used in the calculation, but a somewhat higher one even if k_a is made very large. Variations in k_r^0 indicating this result are shown in Fig. 2 using the absorption and excretion constants of *Reference 9*. The k_r^0 calculated for a $B_d = 0.56$ is $k_r^0 = 0.096$

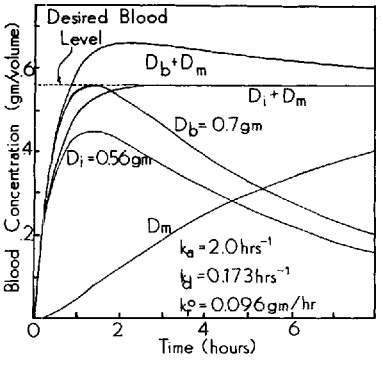


Fig. 4.—Blood level *versus* time curves showing the achievement of the desired blood level by adjustment of the initial dose provided. The blood levels that would be obtained for the initial dose required to obtain the desired level when alone (D_b), when in the presence of the maintenance dose (D_i), and those obtained with a maintenance (D_m) dose alone are also shown.

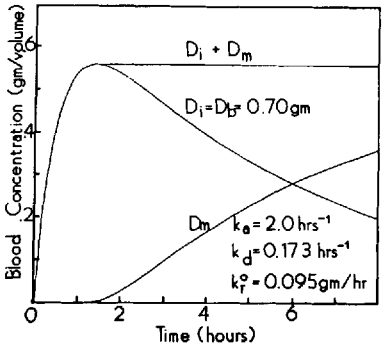


Fig. 5.—Blood level *versus* time curves showing nearly ideal sustained-action obtained by a delayed start of the maintenance dose. The blood levels that would be obtained for the initial dose (D_i) and maintenance dose (D_m) are also shown.

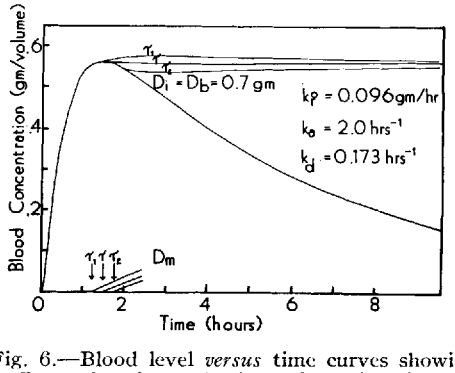


Fig. 6.—Blood level *versus* time curves showing the effects of various selections of starting time for the maintenance dose.

(the actual B_d obtained using these values can be seen to be about 0.59).

If the initial dose (D_i) is varied while a constant k_r^0 is used, a family of curves such as those in Fig. 3 are produced. It would appear that by a proper selection of D_i and k_r^0, a curve corresponding to the "idealized" one should be possible, but it is not. The "idealized" curve with a plateau slope equal to zero over the time period required cannot be obtained with a maintenance dose releasing drug in this fashion, although the slope is sufficiently close to zero to be considered "ideal." This can be seen in Figs. 2 and 3 but can more easily be demonstrated by noting that the derivative of the equation describing the blood level-time relationship (Eq. 6) *has* a real solution at $dB/dt = 0$ (Eq. 7), denoting a true maximum value for this equation (it is of course different from that of the immediately available dose), unless one is able to assume that the time to reach a *maximum* blood level was zero (and $k_a = \infty$).

The initial dose (D_i) of a sustained-release preparation cannot be assumed to be identical to that immediately available dose needed (D_b) to produce a peak equal to the desired blood level. Because the sustained portion of the dose also provides some drug for absorption over this early interval, too much drug becomes available for absorption and consequently a higher blood level is obtained than is desired. A correction on the immediately available dose is needed then such that less drug is initially available for absorption. While this does produce the desired blood level, a slightly longer time is required to reach the desired blood level; both of the above considerations are shown in Fig. 4. The correction needed should concern the time interval from time zero until absorption of the initial dose is complete, but as mathematically, absorption is never complete, for calculation purposes this may be assumed to correspond to the time to achieve the peak height, and simple subtraction of the quantity yielded by the maintenance dose in this interval produces a suitable correction. This correction is equal to $k_r^0 \times T_p$ where T_p is defined in Eq. 3,[3] so that,

$$D_i = D_b - (k_r^0 \times T_p) \qquad \text{(Eq. 8)}$$

This difficulty can be overcome more easily by using a sustained-release dosage form that begins its release of the maintenance drug *not* at time zero, but at the point where absorption of the initial dose is virtually over. This proposal is shown graphically in Fig. 5. For this type of dosage form, the initial dose and the time to reach the desired blood level remain the same, since the maintenance dose is not contributing drug over this time period [note that in the previous sample where both started together, adjustment of D_b to obtain D_i resulted in a slight delay in reaching the desired blood level (Fig. 4)]. If the maintenance form begins release of drug at times before or after the peak height time, the curve will tend to approach the desired blood level concentration at a rate which is dictated by the release constant of the maintenance form as shown in Fig. 6.

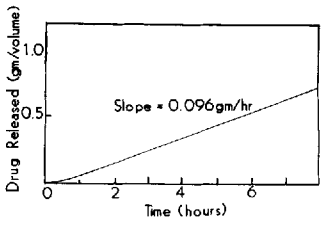


Fig. 7.—Dose release curve necessary to produce an "idealized" blood level *versus* time curve such as that shown in Fig. 5 (labeled $D_i + D_m$).

While a delayed start appears to be a difficult complication, sustained-action medication forms capable of providing it are currently available, *e.g.*, cored tablets, in which the core is commonly the maintenance dose that does not become available until some time after the initial dose has been absorbed. While not actually designed with this in mind, cored sustained-action tablets of superior action may well be assumed to owe their action to this type of behavior.

The general equations for the blood level *versus* time relationship in such a case can be solved to yield:

$$B_t = K_r^0 u(t-\tau)\left\{\frac{1}{k_d}(1 \times e^{-k_d(t-\tau)}) + \frac{1}{k_a - k_d}[e^{-k_a(t-\tau)} - e^{-k_d(t-\tau)}]\right\} + \frac{D_i k_a}{k_a - k_d}(e^{-k_d t} - e^{-k_a t}) \qquad \text{(Eq. 9)}$$

where $u(t-\tau)$ is the so-called "unit step" function whose value is 0 for all values of its argument ≤ 0 and $+1$ for all others. By making this substitution, one can see that before $t = \tau$, B_t describes the expected immediately available dose curve, while after $t = \tau$, the maintenance dose adds its effect onto whatever is left at that time. The value of such a dosage form is more apparent from the computer curves than from the equation.[4]

Calculation of the Desired Zero-Order Rate Constant.—Previous publications (10) have directed that the rate constant necessary for sustained release be set equal to k_d times the dose required to produce B_d. This has been criticized recently (9) and as found in this study the criticism is valid. The correct k_r^0 is the product of the elimination constant and the desired blood level (B_d).[5] The zero-order rate constant necessary can be obtained in another fashion also, utilizing the method of Stelmach, Robinson, and Eriksen (3), where using the desired blood concentration *versus* time curve as a computer

[3] This simple method for making the correction is of course only an approximation. The exact calculation would involve the solution of the complete equation for the blood level obtained from a zero-order sustained-action dosage formulation for the initial dose D_i, at some time after the expected peak. As no *mathematically* flat blood level *versus* time line is ever obtained using this formulation method, the equation is not soluble explicitly and the approximation given becomes the most desirable method for calculation of the correction.

[4] In the interests of complete precision, it must be pointed out that the blood level curves obtained from this type of dosage form are not mathematically straight either as absorption of D_i is mathematically "eternal." They represent major improvements on Eqs. 7 and 8 and also give complete mathematical linearity of B_d only when $k_a = \infty$, as discussed later.

[5] The use of the dose required to produce the desired blood level yields the same result as the blood level itself only if the one compartment–one volume model is used and k_a is assumed very large. In this case $B_d = W$.

input voltage, the dosage release *versus* time curve required is produced as an output. The slope of the dosage release curve can be seen to equal the zero-order rate constant required. This was tested using the plot of Fig. 5 as an input, with results shown graphically in Fig. 7.

The rate constant k_r^0 used for Fig. 5 had been set equal to 0.096 Gm./hr.; the slope of the line in Fig. 7 calculated by the computer as being required to produce the blood level curve (labeled $D_i + D_m$) in Fig. 5, was found to be equal to 0.096 Gm./hr.

The linearity of the blood concentration-time curve with a delayed start can be shown by changing the time variable in Eq. 9 to describe only times *after* the peak time of the immediately available portion, so that

$$= t - \tau$$

and

$$\text{when } \pi = 0,\ B_\pi = B \text{ peak} = B_p$$

under these conditions, Eq. 9 becomes,

$$B_\pi = k_r^0 \left\{ \frac{1}{k_d}(1 - e^{-k_d\pi}) + \frac{1}{k_a - k_d}(e^{-k_a\pi} - e^{-k_d\pi}) \right\} + \frac{D_i k_a}{k_a - k_d}[e^{-k_d(\pi + \tau)} - e^{-k_a(\pi + \tau)}] \quad \text{(Eq. 9}a\text{)}$$

setting the first derivative of B_π with respect to π equal to zero produces an expression for B_π independent of time (and thus flat), only if $e^{-k_a\pi}$ is assumed to be zero ($k_a \to \infty$) and $k_a \gg k_d$. Under those restrictions,

$$\frac{D_i k_a}{k_a - k_d} e^{-k_d\tau} = K_r^0 \left\{ \frac{1}{k_a - k_d} + \frac{1}{k_d} \right\} \quad \text{(Eq. 9}b\text{)}$$

and as the left side of Eq. 9*b* is an approximation for the desired blood level which is produced by the immediately available dose D_i (where $D_i = D_b$), one again finds,

$$k_r^0 \cong B_p k_d \cong B_d k_d \quad \text{(Eq. 5)}$$

Note that the blood concentration after the peak time (B_π) is a constant and identical to the peak blood level if and only if $k_a = \infty$, as one might expect.

Calculation of the Total Dose for Release by Zero-Order Kinetics.—As pointed out previously (10) a dosage form releasing drug at a rate equal to the rate at which drug is eliminated *will* give a very nearly constant blood level, but differentiation must be made between a dosage form releasing drug from time zero and one releasing drug at the peak height time, in the calculation of total dose.

Release from Time Zero.—For a maintenance form releasing drug from time zero, the following equations hold.

$$W = D_i + D_m \quad \text{(Eq. 10)}$$

where $D_i = D_b - (T_p \times k_r^0)$. In this equation $(T_p \times k_r^0)$ is the concentration of drug contributed by the maintenance form that represents the correction on the initial or immediately available dose, and

$$D_m = k_r^0 \times h$$

Therefore

$$W = D_b - (T_p \times k_r^0) + k_r^0 \times h \quad \text{(Eq. 11)}$$

where

- W = total dose,
- D_i = initial dose,
- D_m = maintenance dose,
- D_b = dose required to give the desired blood level, when given in an immediately available form,
- T_p = peak height time,
- k_r^0 = zero-order rate of release constant,
- h = total desired time for sustained action in hours.

Delayed Start Maintenance Dose.—When the maintenance dose begins release of drug at the peak height time, the equation for total required dose (W) becomes

$$W = D_b + k_r^0 \times (h - T_p) \quad \text{(Eq. 12)}$$

and

$$D_i = D_b$$
$$D_m = k_r^0(h - T_p)$$

where the symbols have the same significance as above.

RELEASE BY FIRST-ORDER KINETICS

General Concepts.—The relationship between the initial and maintenance dose of an absorption, distribution, and excretion model with first-order availability is shown in Fig. 8 for various values of k_a (and fractions of dose as maintenance from, F_m) at constant k_r' and k_d. As expected, k_a influences the curve very little but primarily *before* the peak height time; the intersection points remaining essentially in the same place as F_m and k_a change. The common intersection point for various fractions of dose has been recognized and reported previously by Kruger-Thiemer and Eriksen (2). Mathematically, the intersection point lies at the peak time (T_p) for the maintenance dose alone, representing a solution of the equation,

$$k_r'(k_d - k_a)e^{-k_r'T_p} = k_d(k_a - k_r')e^{-k_dT_p} + k_a(k_r' - k_d)e^{-k_aT_p} \quad \text{(Eq. 13)}$$

as has also been noted previously (2).

Figure 9 demonstrates the effect of altering k_r' at a constant k_d and k_a; this effect has also been suggested by previous workers (5). An interesting point may be noted in this family of curves, when k_a is much larger than k_r' and k_d, the intersection point is a function only of k_r' and k_d. The intersection point occurring at later times for smaller values of k_r'. The same observation can be made mathematically by letting k_a become enough larger than k_d and k_r' that its exponential term may be disregarded at an early time and then solving Eq. 13 for the intersection point of T_p

$$T_p = \frac{2.3}{k_r' - k_d} \log \frac{[k_r'(k_a - k_d)]}{[k_d(k_a - k_r')]} \quad \text{(Eq. 14)}$$

or if $k_a \gg k_d$ and k_r',

$$T_p \cong \frac{2.3}{k_r' - k_d} \log \left(\frac{k_r'}{k_d} \right) \quad \text{(Eq. 15)}$$

It becomes rapidly apparent with the computer that no combination of rate constants and/or doses will reduce a flat, constant blood level using a first-order availability model. Mathematically, this can be shown also by considering the solution for the maximum of the equation for the blood level produced by the authors' three-step model with first-order availability:

$$B_t = \frac{D_m k_a k_r'}{(k_d - k_r')(k_a - k_r')}(e^{-k_r' t} - e^{-kdt}) + \frac{D_i k_a - \frac{D_m k_a k_r'}{k_a - k_r'}}{(k_d - k_r')}(e^{-kat} - e^{-kdt}) \quad \text{(Eq. 16)}$$

[after Wiegand and Taylor (5)], and

$$\mathring{B}_t = \frac{D_m k_a k_r'}{(k_d - k_r')(k_a - k_r')}(+k_d e^{-kdt} - k_r' e^{-k_r' t}) + \frac{D_i k_a - \frac{D_m k_a k_r'}{k_a - k_r'}}{(k_d - k_r')}(k_d e^{-kdt} - k_a e^{-kat}) \quad \text{(Eq. 17)}$$

Although the actual solution for t at $\mathring{B}_t = 0$ can only be found by successive approximation, this equation obviously has three solutions, two trivial ($t = 0$ and ∞) and one real; a plot that has a maximum cannot be flat.

A sustained-release product having a *satisfactorily* flat blood level curve using a first-order release pattern can be designed, however, and that design depends upon the proper selection of both the dose fraction in each form and the maintenance dose release constant. The closest approach to the "idealized" blood level can be found by computer experimentation to require a combination of initial and maintenance does such that the intersection (T_p, Eqs. 14 and 15) occurs at a time equal to or greater than the desired sustained-action interval (h) (Fig. 10). In addition, the further past the desired time for sustained release this point lies, the more combinations of F_i and F_m are available that will give the desired type of blood concentration curve. From the intersection (T_p) equation (Eq. 14) it is observed that the smaller the value of k_d the larger the value of k_r' may be and still produce a satisfactory dosage form. For cases where k_d is large, however, the value of k_r' necessary to give an intersection point at or beyond the desired time can be very small, and, thus, the necessary dose present in the maintenance form may become quite high.

The rather remarkable improvement afforded in the zero-order release case, by delaying the start of the maintenance dose, suggests its use here also with the results shown in Fig. 11. As in the zero-order case, the blood level is obtained at a rate determined by the initial dose, and the subsequent levels by the maintenance dose. Quite opposite to the observation with first-order release started at time zero, however, the most "uniform"[6] blood levels are obtained, when the elimination rate (k_d) is high, by making k_r' high, too. The generally "sustained" shape of this curve suggests that the delayed start principle height be extremely useful for sustained release here too, especially for drugs with large elimination (k_d) constants.

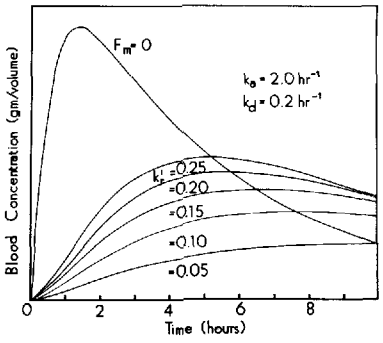


Fig. 9.—Blood level *versus* time curves showing the change in intersection point for various first-order release rate constants.

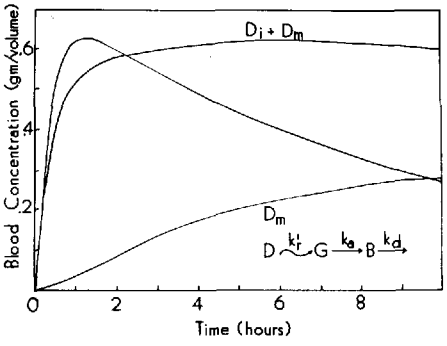


Fig. 10.—Blood level *versus* time curve showing the degree of sustained action obtainable with first-order release. The blood levels *versus* time curves obtained from the initial (D_i) and maintenance (D_m) doses are also shown. Key: $K_a = 3.0$ hr.$^{-1}$; $k_d = 0.10$ hr.$^{-1}$; $K_r' = 0.065$ hr.$^{-1}$.

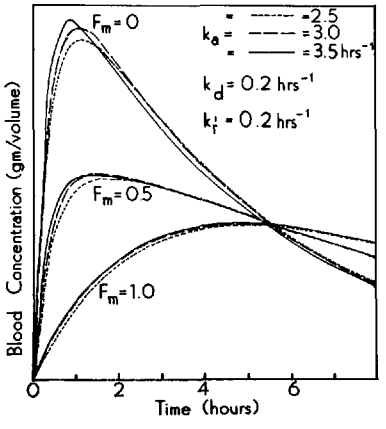


Fig. 8.—Blood level *versus* time curves showing the same intersection point despite variation in absorption rate constant (k_a) for several maintenance dose/initial dose ratios.

[6] A "uniform" or "sustained" blood level in this case represents a nonhorizontal blood level oscillating about some average value.

The general equation for the blood level *versus* time relationship when a delayed start of release and first-order kinetics is used is:

$$B_t = \frac{D_m k_r'}{(k_a - k_r)} u(t - \tau) \left\{ \frac{k_a}{(k_d - k_r')} [e^{-k_r'(t-\tau)} - e^{-k_d(t-\tau)}] + \frac{k_a}{(k_a - k_d)} [e^{-k_a(t-\tau)} - e^{-k_d(t-\tau)}] \right\} + \frac{D_i k_a}{(k_d - k_a)} \left\{ e^{-kat} - e^{-kdt} \right\} \quad \text{(Eq. 18)}$$

using the same "unit step" concept discussed before and τ the time of the delayed start. The similarity in form to Eqs. 9 and 16 are apparent.

Calculation of the First-Order Rate of Release Constant.—It was stated earlier that the intersection points of the blood level curves obtained for immediate and sustained-release forms should be equal to or longer than the desired time for sustained release (h), in order to achieve the closest approximation to the "idealized" blood concentration-time curve when a maintenance dosage beginning immediately is used. As this intersection point depends essentially on k_d and k_r', (if k_a is assumed large) and k_d is a parameter over which the formulator has no control, k_r' must be altered to move this intersection point to the desired time. With k_a and k_d given, Eq. 14 (or Eq. 15) may be used to solve for the rate constant required, to make the intersection point equal to, or greater than, the desired sustained-action time (h). Alternatively, as has been suggested for zero-order release, calculation of the necessary first-order availability constant can be carried out using the desired blood level curve and the computer method of Stelmach, Robinson, and Eriksen (3).

When a delayed start is used, two approaches to the desired k_r' are possible; the maintenance dosage form can provide drug in an approximately zero-order fashion (but *actually* first order) with an over-all rate of $k_d B_d$, or the peak of the blood level produced by a maintenance dose alone may be positioned at a point between the initial dose peak and the desired sustained-action time.

In the first case, the sustained portion should begin releasing at the T_p of the initial portion. The drug released from the dosage form in t hours may be set equal to the drug lost over the same interval assuming constant blood level,

$$D_m(1 - e^{-k_r' t}) = k_d B_d t \quad \text{(Eq. 19)}$$

and the approximation relating the release rate and the administered dose,[7]

$$k_r' D_m \cong k_d B_d = k_r^0 \quad \text{(Eq. 20)}$$

results. Using the maximum dose that the patient will swallow or that can be accommodated in the dosage form, k_r' can be estimated; for any satisfactory degree of sustained action a first-order release form will require roughly 10× the dose required for a zero-order form.

In the second case, a more rapid release rate may be used and a later time of onset tolerated producing, in general, a more practical dosage form. Experimentation on the computer suggests that for the ADE constants normally found, the point where 99% of the initial dose has been absorbed is the most suitable onset point for the maintenance dose,

$$\tau = \frac{4.6}{k_a} \quad \text{(Eq. 21)}$$

The k_r' required to obtain a maintenance dose generated peak at roughly the midpoint between the

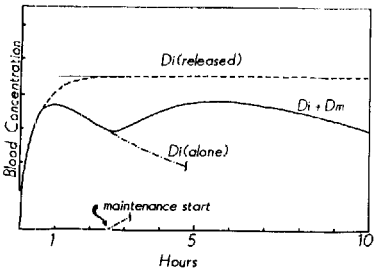


Fig. 11.—Blood level *versus* time curves showing the degree of sustained action obtained by a delayed start of a first-order release maintenance dose. Portions of the blood level *versus* time curves obtained from the initial (D_i) and maintenance (D_m) doses are also shown.

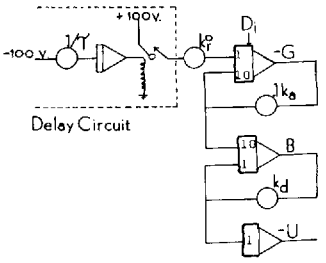


Fig. 12.—Scaled analog computer program for the system shown in Eq. 1, using a zero-order delayed start maintenance dose.

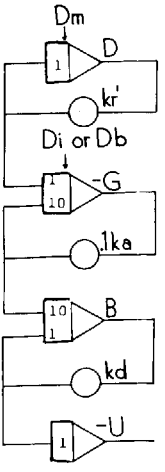


Fig. 13.—Scaled analog computer program for the system shown in Eq. 1, using a first-order immediate start maintenance dose.

[7] This is the "rate in equals rate out" equation of Nelson (10) and again implies $k_a = \infty$ and its applicability one can also verify by solving the maintenance only portion of Eq. 16 for B in the case where k_a and $k_d >> k_r'$.

initial dose blood level peak and the desired sustained-action time (h) may be estimated solving Eqs. 14 or 15 for k_r' at

$$T_p = \frac{h - \tau}{2} \qquad \text{(Eq. 21}a\text{)}$$

Calculation of the Total Dose for Release by First-Order Kinetics.—The total dose required for adequate sustained release (W) will again be described by Eq. 10 and may be approximately solved for both methods of first-order release.

Release from Time Zero.—

$$D_i = D_h - D_{correction} \qquad \text{(Eq. 22)}$$

as before, a correction ($D_{correction}$) for D_h is required which may be thought of as equal to the amount of drug contributed to the blood by the maintenance dose, during the phase controlled by the initial dose, and which can be estimated using reasoning similar to that used in deriving Eqs. 19 and 20.

$$D_{correction} = D_m(k_r'T_p) \qquad \text{(Eq. 23)}$$

Thus, Eq. 22 is found to be approximately[8]

$$D_i = D_h - D_m \times k_r' \times T_p \qquad \text{(Eq. 24)}$$

The maintenance dose required to keep the blood level at approximately the desired value can be calculated by equating the desired "rate in" (k_dB_d) with the actual "rate in"[8]

$$k'_rD_me^{-k_r't} = k_dB_d$$

$$D_m = \frac{k_dB_d}{k_r' - (k_r')^2t} \cong \frac{k_dB_d}{k_r'} \qquad \text{(Eq. 25)}$$

The approximation is sufficiently accurate for most purposes, although the first is better, the time when the desired blood level is reached being used for t.

The total dose (W) then is

$$W = D_h - D_mk_r'T_p + \frac{k_dB_d}{k_r'} \qquad \text{(Eq. 26)}$$

Delayed Start Maintenance.—The immediately available dose (D_i) completely dictates the attained blood level as described for the care of release by zero-order process; but the maintenance dose required for a satisfactorily "uniform" blood level depends on the delay time, τ, and the placing of the maintenance peak.

A precise delay time value is not critical; it may be calculated easily from Eq. 21, and k_r' calculated from Eqs. 14 or 15 as mentioned before (to place the "maintenance peak" at roughly the midpoint of the desired sustained-action time, h, Eq. 21a).

The maintenance dose required to produce a secondary blood level peak equal to the first may be calculated as the maintenance dose (alone) required to produce a peak sufficient to increase the blood level remaining from the initial dose to the desired value. At the desired time for the secondary peak T_p^*, the residual blood level is

$$B_r = \frac{D_ik_a}{k_a - k_d}\,(e^{-k_dT_p^*} - e^{-k_aT_p^*}) \qquad \text{(Eq. 27)}$$

and the peak blood level from the maintenance dose is that calculated from Eq. 16 at $t = (T_p^* - \tau)$ and $D_i = 0$. If it is assumed as before that at the time involved $e^{-k_aT_p}$ becomes negligible, both these equations can be simplified and D_m easily calculated. Under these circumstances,

$$B_r \cong \frac{D_ik_a}{k_a - k_d}\,(e^{-k_dT_p^*}) \qquad \text{(Eq. 28)}$$

Then

$$D_m = \frac{k_d}{k_r'}\,(B_d - B_r)e^{+k_r'(T_p - \tau)} \qquad \text{(Eq. 29)}$$

Figure 10 shows the computer generated blood level produced for the ADE constants used before, calculated for such a delayed first-order start. In addition, the drug delivered from the dosage form is also shown to indicate the separation of starting times.

SUMMARY AND CONCLUSIONS

The general phenomena involved in the ADE kinetics of many drugs are found to be described by rather simple "overall" expressions and these phenomena according to the descriptive equations that happen to fit, *i.e.*, absorption is described as "first order," etc.; while there is little real doubt that such a naive approach is incorrect, the fact that such simple equations *can* adequately describe the concentrations of biologic interest, should be of real use (if not importance) in the formulation of effective sustained-action dosages. As discussed in this report, however, even these "simple" equations cannot be mathematically solved to produce explicit solutions for the dosage fractions and rate constants required, but instead suitable approximations must (and can) be made that permit *useful* solutions for the single sustained-release dose case. These approximations and the assumptions upon which they have been made have not always been explicitly described when (and if) they have been published before and the present authors felt sufficient benefit would accrue from collecting them both in one place that this has been done for the two theoretical cases described before: (a) simultaneous start of initial and "zero-order" sustained dosages and (b) simultaneous start of initial and "first-order" sustained dosages. The methods for calculating the dose fractions of both the initial and the sustained portions as well as the rate constant most suitable for producing a sustained blood level are described fully.

Secondarily, but of interest in order to complete the picture, the effect of delaying the start of the sustained portion of the dosage form has been shown to produce in one case (c) the best theoretical sustained-action blood level picture available and in the other (d) a novel and perhaps not unusable blood level situation: (c) initial release followed by "zero order" sustained dosage after some delay and (d) initial release followed by "first order" sustained dosage after some delay.

The aim and the result of the analysis in this report has been to show the theoretically available blood level situations resulting from a *single* complete (sustained *and* initial) dosage form designed to produce the most constant blood level over the desired sustained-action time and but for the irksome (though real) vagaries of the human gastrointestinal tract would describe the blood level–time pictures actually observed.

[8] The approximations made here are similar to those made for the zero-order case (see *Footnote 3*). The exact solution is similarly not possible and experimentation convinces us that for practical situations, this approximation is suitable.

APPENDIX

Figures 12 and 13 show the computer circuits used in simulating the solutions shown in this work. Of prime concern and utility here is the "nonlinear delay input" by programmed relay shown in Fig. 12. The remaining programs required (immediate start, zero-order, and delayed start, first-order) have been published and discussed previously (3).

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Kinetics of Deterioration of Trimethylene Bis-(4-formylpyridinium Bromide) Dioxime in Dilute Aqueous Solutions

By R. I. ELLIN, D. E. EASTERDAY, P. ZVIRBLIS, and A. A. KONDRITZER

The degradation of trimethylene bis-(4-formylpyridinium bromide) dioxime, TMB-4, occurs by two mechanisms. The first is a hydrolytic reaction of the acid form of the oxime catalyzed by hydrogen ion, and the second, a dehydration reaction. The latter may proceed by either of two pathways—a hydroxyl ion catalyzed dehydration of the acid form of the oxime, or a spontaneous dehydration of the oximate species. Velocity and various thermodynamic constants were determined for each of the mechanisms postulated. General equations were derived that relate the half-life of TMB-4 solutions to pH and temperature.

FOLLOWING THE introduction of pyridinium oximes for the management of intoxication by organophosphorus anticholinesterase compounds, an active search has taken place for more effective compounds. As a result, 1,1′-trimethylene bis-(4-formylpyridinium bromide) dioxime, referred to as TMB-4, was synthesized and shown to be a potent reactivator of phosphorylated cholinesterases (1, 2). Grob has reported (3) that TMB-4 in one-tenth to one-fifteenth the dose of 2-PAM iodide was more effective in humans against the weakening of the response of a muscle to electrical stimulation of its motor nerve. A better therapeutic index for TMB-4 has been demonstrated in laboratory animals (4), and the use of a mixture of 2-PAM and TMB-4 has been reported to be superior to any single oxime tested (5). In view of these reports the establishment of the conditions of maximal stability of TMB-4 in aqueous solution becomes important and worthwhile.

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Mechanisms for the degradation of pyridinium aldoximes in aqueous solution have been postulated in previous reports (6, 7). Recent studies by Kosower (8) support the conclusion that the rate-limiting step for the degradation of pyridinium oximes in basic solution is the formation of cyanopyridinium ion. The mechanisms postulated for the breakdown of TMB-4 are presented in Scheme I. The reaction of TMB-4 in basic solution may be explained by an ElcB unimolecular elimination mechanism (9). Hydroxyl ion attack at each methine hydrogen atom results in the removal of a proton and the formation of a carbanion as the rate-controlling step. Subsequent loss of hydroxide ion from the oximino nitrogen leads to the formation of a triple bond; for TMB-4 the corresponding dinitrile would be the final product of this process. Hydroxide ion attack on the cyano group or addition to the pyridine ring forms dicarbamido and dihydroxy dipyridinium ions, respectively. The latter, on further reaction with hydroxide ion, readily lose a proton to form the corresponding dipyridone. Reaction of TMB-4 with hydrogen ion leads to various states of equilibrium involving the splitting out of hydroxylamine and the formation of

APPENDIX

Figures 12 and 13 show the computer circuits used in simulating the solutions shown in this work. Of prime concern and utility here is the "nonlinear delay input" by programmed relay shown in Fig. 12. The remaining programs required (immediate start, zero-order, and delayed start, first-order) have been published and discussed previously (3).

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Kinetics of Deterioration of Trimethylene Bis-(4-formylpyridinium Bromide) Dioxime in Dilute Aqueous Solutions

By R. I. ELLIN, D. E. EASTERDAY, P. ZVIRBLIS, and A. A. KONDRITZER

The degradation of trimethylene bis-(4-formylpyridinium bromide) dioxime, TMB-4, occurs by two mechanisms. The first is a hydrolytic reaction of the acid form of the oxime catalyzed by hydrogen ion, and the second, a dehydration reaction. The latter may proceed by either of two pathways—a hydroxyl ion catalyzed dehydration of the acid form of the oxime, or a spontaneous dehydration of the oximate species. Velocity and various thermodynamic constants were determined for each of the mechanisms postulated. General equations were derived that relate the half-life of TMB-4 solutions to pH and temperature.

FOLLOWING THE introduction of pyridinium oximes for the management of intoxication by organophosphorus anticholinesterase compounds, an active search has taken place for more effective compounds. As a result, 1,1′-trimethylene bis-(4-formylpyridinium bromide) dioxime, referred to as TMB-4, was synthesized and shown to be a potent reactivator of phosphorylated cholinesterases (1, 2). Grob has reported (3) that TMB-4 in one-tenth to one-fifteenth the dose of 2-PAM iodide was more effective in humans against the weakening of the response of a muscle to electrical stimulation of its motor nerve. A better therapeutic index for TMB-4 has been demonstrated in laboratory animals (4), and the use of a mixture of 2-PAM and TMB-4 has been reported to be superior to any single oxime tested (5). In view of these reports the establishment of the conditions of maximal stability of TMB-4 in aqueous solution becomes important and worthwhile.

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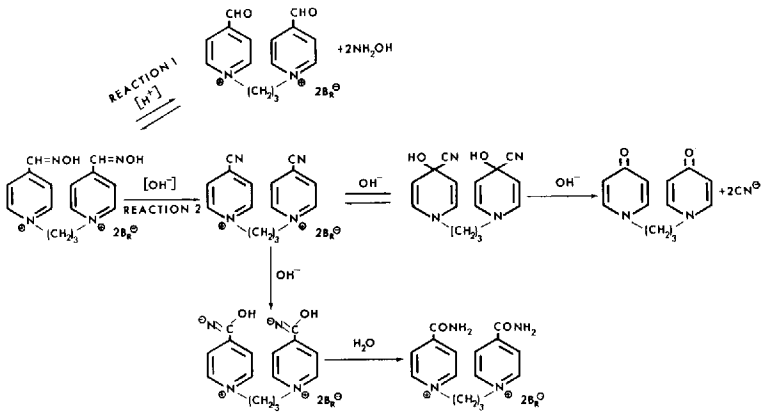
Mechanisms for the degradation of pyridinium aldoximes in aqueous solution have been postulated in previous reports (6, 7). Recent studies by Kosower (8) support the conclusion that the rate-limiting step for the degradation of pyridinium oximes in basic solution is the formation of cyanopyridinium ion. The mechanisms postulated for the breakdown of TMB-4 are presented in Scheme I. The reaction of TMB-4 in basic solution may be explained by an ElcB unimolecular elimination mechanism (9). Hydroxyl ion attack at each methine hydrogen atom results in the removal of a proton and the formation of a carbanion as the rate-controlling step. Subsequent loss of hydroxide ion from the oximino nitrogen leads to the formation of a triple bond; for TMB-4 the corresponding dinitrile would be the final product of this process. Hydroxide ion attack on the cyano group or addition to the pyridine ring forms dicarbamido and dihydroxy dipyridinium ions, respectively. The latter, on further reaction with hydroxide ion, readily lose a proton to form the corresponding dipyridone. Reaction of TMB-4 with hydrogen ion leads to various states of equilibrium involving the splitting out of hydroxylamine and the formation of



Scheme I

the dialdehyde. Compounds isolated, or identified, or both, from the degradation of TMB-4 were the picrate derivative of the corresponding dipyridone, hydroxylamine, and inorganic cyanide.

The purpose of the present investigation was to determine the stability of TMB-4 in aqueous solution. Kinetic studies of the degradation of TMB-4 were made at various pH values and temperatures in order to obtain equations that would permit the prediction of the stability of the oxime at any pH and temperature.

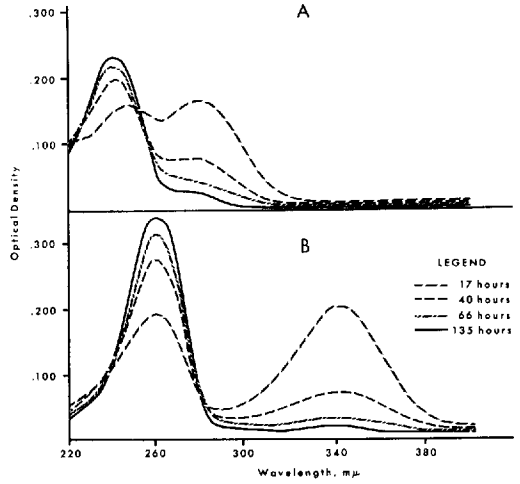


Fig. 1.—Ultraviolet absorption spectra of TMB-4 solutions undergoing degradation. Key: A, in 0.1 *N* perchloric acid, 71°; B, 0.1 *N* sodium hydroxide, 71°.

EXPERIMENTAL

The degradation of TMB-4 was carried out at five different temperatures in buffer solutions of various pH values, as well as in dilute solutions of perchloric acid and sodium hydroxide. Essentially the same buffer systems used previously (7) were employed in this study. The pH of each system was determined at the temperature at which the experiments were performed. The TMB-4 used in these experiments was recrystallized from ethyl alcohol to a melting point of 224–226° dec. (Thomas-Hoover, U.S.P. substances of class I).

Molar absorptivities of this oxime were found to be 4.82×10^4 at 345 mμ in 0.1 *N* alkaline and 3.39×10^4 at 281 mμ in 0.1 *N* acid solutions. Figure 1, B, shows that the intensity of the TMB-4 peak at 345 mμ in basic solutions diminishes with time and that simultaneously a single peak appears at 259 mμ and increases in intensity. Figure 1, A, shows a similar occurrence with TMB-4 in acid solution: the gradual diminution of the peak at 281 mμ and the corresponding appearance of a peak at 239 mμ. A solution of the breakdown products of TMB-4 gives practically no absorbance at 345 mμ in alkaline solution. Therefore, the residual TMB-4 in the

Table I.—Observed Rate Constants k in hr.$^{-1}$ for Spectrophotometrically Determined Degradation of TMB-4

71°		81°		90°		59.7°		50°	
pH	k, hr.$^{-1}$	pH	k, hr.$^{-1}$	pH	k, hr.$^{-1}$	pH	k, hr.$^{-1}$	pH	k, hr.$^{-1}$
0.1 *N* NaOH	4.68×10^{-2}	0.1 *N* NaOH	1.49×10^{-1}	0.1 *N* NaOH	2.95×10^{-1}	0.42	8.28×10^{-1}	0.42	5.11×10^{-1}
11.07	4.12×10^{-2}	10.90	1.34×10^{-1}	10.59	2.73×10^{-1}	0.18	1.52	0.18	8.96×10^{-1}
8.43	3.58×10^{-2}	8.50	9.43×10^{-2}	8.49	2.44×10^{-1}	0.11	2.75	0.11	1.14
7.03	9.92×10^{-3}	7.01	4.01×10^{-2}	6.97	8.25×10^{-2}	...	...	...	...
5.99	1.17×10^{-3}	6.09	5.06×10^{-3}	6.03	1.40×10^{-2}	...	...	...	...
5.01	2.50×10^{-4}	5.08	8.17×10^{-4}	4.98	1.35×10^{-3}	...	...	...	...
1.02	4.95×10^{-1}	...	...	...	...	...	...	...	...
0.72	8.76×10^{-1}	...	...	...	...	...	...	...	...
0.42	2.45	...	...	...	...	...	...	...	...
0.18	4.00	...	...	...	...	...	...	...	...

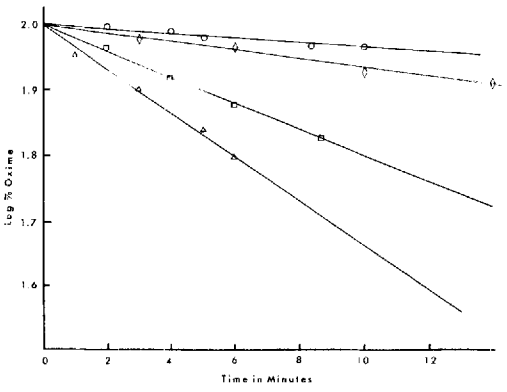


Fig. 2.—Degradation of TMB-4 in perchloric acid solutions at 71°. Key: ○, 0.096 *N*; ◇, 0.19 *N*; □, 0.38 *N*; △, 0.70 *N*.

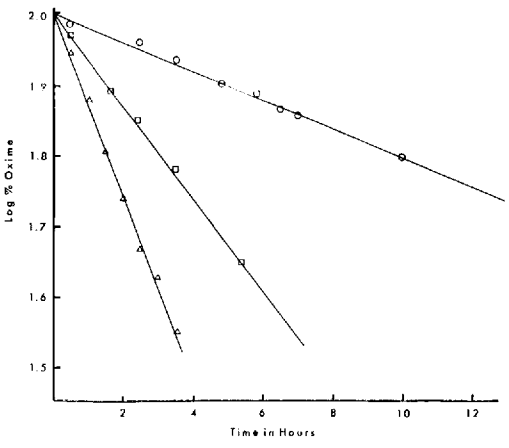


Fig. 3.—Degradation of TMB-4 in 0.1 *N* sodium hydroxide. Key: ○, 71°; □, 81°; △, 90°.

experimental solutions may be determined quantitatively by measuring the absorbance at 345 mμ in 0.1 *N* alkaline solution.

Buffered solutions were placed in a thermostatically controlled bath and were allowed to reach the temperature of the bath. A relatively small volume of a TMB-4 solution was added to obtain a final concentration of 1 mg./ml. Samples of 0.5 ml. were removed and diluted to 100 ml. with 0.1 *N* sodium hydroxide and the concentrations of the oxime were determined spectrophotometrically at 345 mμ.

RESULTS

Influence of Oxime Concentration.—The rate of disappearance of TMB-4 in both acid and alkaline solutions was found to be directly proportional to the concentration of the oxime, indicating that the degradation is of the first order with respect to the concentration of the oxime. The calculated reaction rate constants obtained at the various temperatures and hydrogen-ion concentrations are given in Table I. Typical plots of the results are shown in Figs. 2 and 3.

Temperature Dependency.—The velocity constants of the degradation of TMB-4 at 50, 59.7, and 71° in perchloric acid solutions ranging from 0.1 to 1.0 *N* (reaction 1 of Scheme I) were used to calculate the respective values for the catalytic constant, k_{H^+}, at normal hydrogen-ion concentration for each of the temperatures used. In Fig. 4 the rate constants are plotted against the reciprocal of the absolute temperature. The straight line relationships obtained indicate that the mechanism of the hydrolysis reaction is not altered by changes in temperature.

The temperature dependency of reaction 2 in Scheme I is graphically illustrated in Fig. 5 and indicates that the postulated dehydration mechanism is not affected by changes in temperature.

The effect of pH on the rate of degradation of TMB-4 at various temperatures is shown in Fig. 6. Between approximately pH 4.5 and 8, the rate is linear with hydroxyl-ion concentration; above approximately pH 8, the reaction becomes nonlinear. This phenomenon may be interpreted as representing

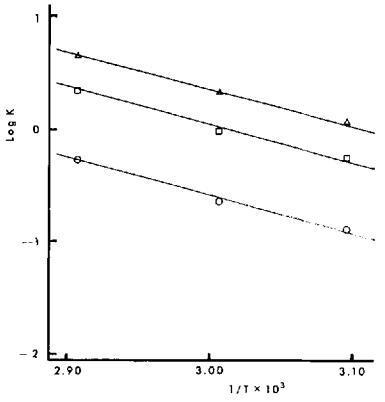


Fig. 4.—Arrhenius plot of hydrogen-ion catalysis of decomposition of TMB-4. Key: △, pH 0.11; □, pH 0.42; ○, pH 1.0.

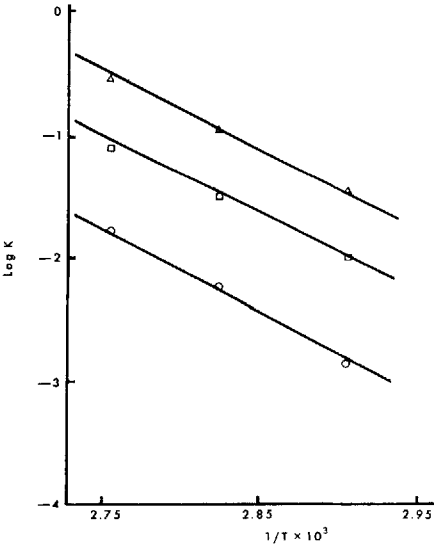


Fig. 5.—Arrhenius plot of hydroxyl-ion catalysis of decomposition of TMB-4. Key: ○, pH 6; □, pH 7; △, pH 10.

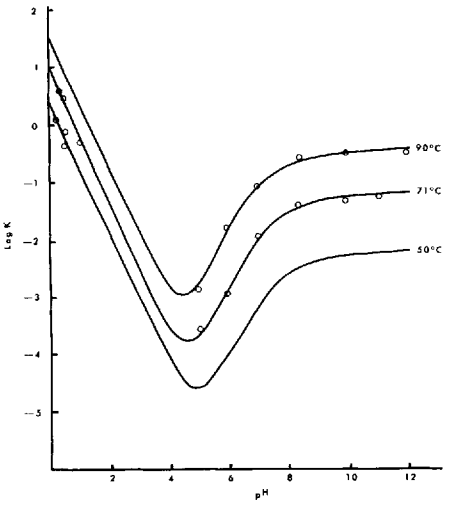


Fig. 6.—The pH profile for the breakdown of TMB-4 at 50, 71, and 90°. The curves represent values calculated from the equation derived from experimental data. The illustrated circles represent experimental values.

either spontaneous degradation of the oximate species or dehydration of the oxime catalyzed by hydroxyl ion. Either possibility would support the conclusion that above approximately pH 4.5 the mechanism of degradation can be due predominately to reaction 2 (Scheme I). On the assumption that the rate of the water reaction, *i.e.*, k_{H_2O} is negligible, and by use of the initial rate constants obtained for the reaction in acid solution and the rate constants for the reaction in alkaline solution, one obtains a general expression for the observed velocity constant of the degradation of TMB-4 (Eqs. 1 and 2).

$$\text{rate} = k_{H^+}(H^+)\frac{(H^+)}{K_a + (H^+)} + k_{OH^-}\frac{K_w}{K_a + (H^+)} \quad \text{(Eq. 1)}$$

or

$$\text{rate} = k_{II^+}(H^+)\frac{(H^+)}{K_a + (H^+)} + k_{Ox}\frac{K_a}{K_a + (H^+)} \quad \text{(Eq. 2)}$$

where k_{H^+} = catalytic constant for the hydrogen-ion catalysis, k_{OH^-} = catalytic constant for the hydroxyl ion catalysis, k_{Ox} = rate constant for degradation of oximate, and K_a = acid dissociation constant,

Table II.—Thermodynamic Constants for Degradation Reactions at 25° C.

Reaction	E_a Kcal./mole	ΔH^* Kcal./mole	ΔF^* Kcal./mole	ΔS^* cal./mole-deg.	A, hr.$^{-1}$
$TMB(H)^+ + H_3O^+$	14.6	14.0	23.2	−30.8	1.01×10^{10}
$TMB(H)^+ + OH^-$	22.8	22.2	20.2	6.7	1.70×10^{18}
$TMB(O)^-$ spontaneous	24.2	23.6	27.3	−12.5	1.13×10^{14}

Table III.—Predicted Stability of TMB-4 at pH of Minimum Decomposition

t, °C.	$pH_{Min.}$	$t_{1/2}$, yr.
0	6.13	2400
10	5.90	570
20	5.69	140
25	5.60	80
30	5.49	40
40	5.31	12
50	5.13	4
60	4.96	1.3
70	4.81	0.5
80	4.67	0.2
90	4.52	0.08
100	4.39	12 days

oxime ⇄ oximate, calculated from the equation, $-\log K_a = 3.41 + 1422/T$, which was derived from titration with alkali between 25 and 90°.

If the reaction is determined in solutions wherein the hydrogen ion is the only effective catalyst, Eqs. 1 and 2 reduce to rate = $k_{H^+}(H^+)$; at relatively high pH values, Eq. 1 reduces to rate = $K_w k_{OH^-}/K_a$ and Eq. 2 to rate = K_{Ox}.

The activation energies, E_a, were determined from the slopes of the Arrhenius-type plots in Figs. 4 and 5. The relationship between the rate constant and the equilibrium constant, ΔK^*, was used to determine the free energy of activation, ΔF^*, the frequency factor, A, and the entropy of activation, ΔS^*. The values for the latter two characteristics were calculated from Eqs. 3 and 4.

Table IV.—Predicted Stability of TMB-4 in Aqueous Solutions, $t_{1/2}$ (Half-Life)

Temp., °C.	pH 3	4	5	6	7	8	13
10	1.4 yr.	14.4 yr.	140 yr.	560 yr.	93 yr.	13 yr.	3.3 yr.
20	0.6 yr.	5.9 yr.	57 yr.	115 yr.	15 yr.	2.2 yr.	0.8 yr.
30	95 days	2.6 yr.	24 yr.	23 yr.	2.7 yr.	146 days	73 days
40	44 days	1.2 yr.	9.6 yr.	4.7 yr.	197 days	37 days	19 days
50	21 days	212 days	3.7 yr.	1.0 yr.	44 days	10 days	6 days
60	11 days	106 days	1.3 yr.	91 days	11 days	3 days	2 days
70	5.5 days	56 days	170 days	25 days	3 days	21 hr.	16 hr.
80	2.9 days	29 days	54 days	7 days	18 hr.	7 hr.	5 hr.
90	1.7 days	16 days	17.5 days	2 days	7 hr.	2.6 hr.	2.2 hr.

$$k = \frac{RT}{Nh} e^{\Delta S^*/R} \cdot e^{-\Delta H^*/RT} \quad \text{(Eq. 3)}$$

and

$$A = \frac{RT}{Nh} e^{\Delta S^*/R} \quad \text{(Eq. 4)}$$

The calculated values for the thermodynamic constants are listed in Table II. By substituting the appropriate constants into Eqs. 1 and 2, the observed reaction rate can be expressed, in terms of hr.$^{-1}$, as either Eqs. 5 or 6.

$$k = 1.01 \times 10^{10} e^{-14,600/RT}(H^+) + 1.70 \times 10^{18} e^{-22,800/RT}\left[\frac{K_w}{K_a + (H^+)}\right] \quad \text{(Eq. 5)}$$

or

$$k = 1.01 \times 10^{10} e^{-14,600/RT}(H^+) + 1.13 \times 10^{14} e^{-24,200/RT}\left[\frac{K_a}{K_a + (H^+)}\right] \quad \text{(Eq. 6)}$$

Agreement of the values calculated by means of the equations with those obtained experimentally is shown in Fig. 6, where the lines are calculated and the open circles represent experimental data.

pH of Minimum Hydrolysis.—The equation for the hydrogen-ion concentration at which the rate of degradation is minimal can be derived by differentiating Eqs. 1 and 2 with respect to (H^+) and equating to zero, as described previously for 2-PAM iodide (7). It may be expressed as:

$$pH_{Min.} = {}^1/_2\left(pKa - 4.05 + \frac{2102}{T}\right)$$

Calculated values for $pH_{Min.}$ at various temperatures are given in Table III and vary inversely with temperature, ranging from 6.13 to 0° to 4.39 at 100°.

The over-all reaction rate of the decomposition of TMB-4 can be expressed as the half-life, $t_{1/2}$, of the reaction; half-lives can be calculated for any pH and temperature. Predicted stability data for pH from 3 to 13 and for temperatures from 40 to 90° are shown in Table IV.

Isolation and Identification of Degradation Product.—Three grams of TMB-4 and 300 ml. of 0.1 *N* sodium hydroxide were heated for 24 hr. in a water bath at 90°. About 3% of the original oxime remained when determined by ultraviolet analysis. To a 25-ml. aliquot, enough picric acid solution in ethanol was added to produce a definite turbidity. The mixture was heated for 15 min. on a steam bath and then was cooled. The resulting yellow precipitate was collected by filtration, dried, and recrystallized from alcohol–water and then from water. The product melted at 234–238°. The elemental analysis for the compound was: C, 43.6%; H, 3.1%; O, 37.2%. The analytical results agree with the calculated elemental content of the dipicrate of 1,1′-trimethylene bis-(4-pyridone): C, 43.6%; H, 2.9%; O, 37.2%.

DISCUSSION

The decomposition of TMB-4 proceeds both by a proton catalyzed hydrolysis mechanism and a dehydration reaction in which the cyanopyridinium intermediate is formed. The experimental data indicate that the dehydration may be explained equally well either by a hydroxyl-ion catalyzed reaction of the oxime, represented by k_{OH^-}, or by spontaneous dehydration of the oximate ion, represented by k_{Ox}.

A comparison of the thermodynamic constants for the dehydration reaction of TMB-4 with those found for the monoquaternary pyridinium aldoxime, 2-PAM (7), may lead to the conclusion that the predominant mechanisms involved differ for the two compounds. In the dehydration reaction for 2-PAM, activation energies assigned for k_{Ox} and k_{OH^-} are 28 and 17 Kcal., respectively. The activation energy for k_{Ox} is determined directly from the observed reaction rates in 0.1 *N* alkali; whereas, that for k_{OH^-} is determined from extrapolated values of observed rates at lower pH. The activation energy for the dehydration reaction of TMB-4 is about 24 Kcal. for both k_{OH^-} and k_{Ox}. The entropy of activation, ΔS^*, for the dehydration reaction of 2-PAM is more positive for k_{Ox} than for k_{OH^-}. In the case of TMB-4 the opposite is true; ΔS^* for k_{OH^-} has the more positive value. If it is assumed that the least sterically hindered mechanism would occur more readily, a base-catalyzed mechanism could be assigned to the degradation of TMB-4 and a spontaneous degradation mechanism to 2-PAM.

The data in this study, with that given in the investigation of 2-PAM (7), provide a means for optimizing conditions for long-term storage of a mixture of the two compounds in solution. The pH-rate profiles of the two aldoximes are similar but not congruent. TMB-4 is the more stable of the two with respect to the dehydration reaction; whereas, 2-PAM is the more stable with respect to the proton catalyzed hydrolysis mechanism. The pH of maximum stability for the two compounds differs by about 1.2 pH units. A pH of 4.5 to 5.0 would be practical for the prolonged storage of mixtures of the two oximes in solution, provided storage temperatures were below 30°. At temperatures appreciably higher than 30°, the stabilities of both oximes in solution decrease to such an extent that long-term storage becomes impractical. If storage temperatures above 30° are anticipated, then the mixture should be packaged as a dry powder; provisions and directions for preparing solutions extemporaneously should be supplied.

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Effects of Aging and Surfactant Concentration on the Rheology and Droplet Size Distribution of a Nonaqueous Emulsion

By RICHARD D. HAMILL* and ROBERT V. PETERSEN

Six olive oil-in-glycerin emulsions were prepared wherein ammonia and 2-amino-2-methyl-1,3-propanediol (AMP), in various concentrations, served as saponifying agents. Six-tenths, 2, and 6 mg. ammonia/100 ml. and 2, 6, and 20 mg. AMP/100 ml. were used in making the emulsions. The emulsions were stored at room temperature (25°), and droplet size, rheology, and emulsion stability studied on days 1, 2, 4, 7, 14, 30, and 60. Droplet size, as measured from photographs taken by phase contrast photomicrography, were inversely related to amine concentration. Growth in droplet size over the period of study was more striking in AMP emulsions than in ammonia emulsions. Rheology studies with the Brookfield LVT viscometer revealed the existence of non-Newtonian pseudoviscous flow. The emulsions were very stable, as indicated by logarithmic graphs of viscosity and time. It is suggested that the data may be extrapolated to predict viscosity over longer time periods.

WHEN AN EMULSION is aged there is usually a distinct growth in droplet size before the dispersed phase separates in bulk (1). This growth can be attributed to coalescence between neighboring droplets and has been characterized as following first-order kinetics (2, 3).

Some of the factors which have an effect on droplet size in an emulsion are the method of preparation, phase–volume ratio, temperature during mixing, viscosity (4–8), and surfactant type and concentration (9). Conversely, the effects that droplet size and droplet size distribution have on emulsion viscosity have been explored. For example, the effects of homodispersity (10–14), flocculation (15–21), surfactant properties and concentration (22), and phase–volume relative to surfactant concentration (23, 24) on viscosity in oil–water emulsions have been studied and reported.

Previous reports (25, 26) have described the emulsifying effects of several ionic and nonionic surfactants on the nonaqueous, binary, immiscible system, glycerin and olive oil. More recently (27) this system was studied with regard to the effect of surfactant concentration on the effective size of two anionic amine surfactants formed *in situ* by the combination of either ammonia or 2-amino-2-methyl-1,3-propanediol (AMP) with the free fatty acids of olive oil. The present study was designed to learn more about the stability of this emulsion system by examining the effects of aging and amine concentration on both rheology and droplet size distribution.

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EXPERIMENTAL

Six olive oil–glycerin emulsions were prepared with varying concentrations of amine. Samples 1, 2, and 3 contained 0.6, 2, and 6 mg. of ammonia/100 ml. of emulsion, respectively; whereas samples 4, 5, and 6 contained 2, 6, and 20 mg. of AMP/100 ml. of emulsion, respectively. These concentrations were chosen because 0.6 mg. of ammonia/100 ml. and 2 mg. AMP/100 ml. correspond to the minimum amine concentrations needed for stable emulsifications at phase volumes of 0.40 as previously reported (27). Concentrations of 6 mg. of ammonia/100 ml. and 20 mg. AMP/100 ml. represent a tenfold increase above these minima. The two remaining samples contained concentrations intermediate between the minimum concentrations and the tenfold increase. The olive oil and glycerin used in making the emulsions and the order of mixing were the same as those previously described (25–27). All emulsions were made with phase volumes of 0.40. Emulsion type was confirmed by the phase dilution method.

The emulsions were stored at room temperature (25 ± 2°) for 2 months. A testing schedule was established wherein the six samples were tested at days 1, 2, 4, 7, 14, 30, and 60. Day 1 corresponds with the day the emulsions were made.

Photographs were taken by means of phase contrast photomicrography on the days chosen in the testing schedule. A Wild phase contrast microscope providing 480× magnification and equipped with a Polaroid camera was used. The wavelength of the light source was 475 mμ.

A glass microscope slide was prepared by placing a drop of emulsion on the slide after which a cover glass was gently rested on the emulsion. Care was taken not to apply any pressure on the sample other than that exerted by the weight of the cover

glass. Pressure on the cover glass could cause distortions or force some droplets to coalesce in the emulsion, thus altering the droplet diameter results. The prepared slide was set aside for 1 hr. This time period was required to allow movement in the sample virtually to stop so that a clear photomicrograph could be obtained. The low intensity of the light source used in this type of microscopy required a several-second exposure time. Photographic prints were obtained using Polaroid Polapan 200 film. The photograph taken was immediately available for inspection to insure a picture of best possible clarity.

Two or three 1-in. squares were arbitrarily selected from each photomicrograph and the diameters of 200 to 300 droplets were classified within these areas. The average droplet diameter (D_m) was calculated using Eq. 1 (22).

$$D_m = \sqrt[3]{\frac{\Sigma n D^3}{\Sigma n}} \qquad \text{(Eq. 1)}$$

where n is the number of droplets with a diameter of D.

The rheology of each emulsion was studied on the test days using a Brookfield LVT viscometer. Day 120 also was included in the rheology testing schedule. Each sample was poured into the test container which was then placed into a constant-temperature water bath, maintained at 25 ± 0.2°, and allowed to equilibrate for 45 min. Spindle No. 2 provided with the Brookfield viscometer was chosen for testing the emulsions because it allowed the widest range of scale readings over a large number of r.p.m. (shear rate). Dial readings (shear stress) at spindle speeds of 0.6, 1.5, 3, and 6 r.p.m. were recorded for each sample. A test was conducted to determine the presence of thixotropy by running the viscometer at 60 r.p.m. for 10 min. after having measured speeds from 0.6 up to 6 r.p.m. After this period of time the dial readings were again recorded, but this time from 6 down to 0.6 r.p.m. In samples where thixotropy was present, the amount of standing time required before the "up" readings could be duplicated was noted.

To utilize effectively the Brookfield viscometer on non-Newtonian materials, various methods of calculation have been proposed (28, 29). The method described by Runikis *et al.* (29) takes into account the entire flow data obtainable with the Brookfield instrument. Their "n value" method utilizes Eq. 2 which they derive and explain.

$$\text{viscosity} = \frac{(K_B)\,(n)\,(\text{scale reading})}{(\text{r.p.m.})} \qquad \text{(Eq. 2)}$$

Since $K_B = 300$ when spindle No. 2 is used with the Brookfield LVT viscometer, Eq. 2 becomes

$$\text{viscosity} = \frac{300\,(n)\,(\text{scale reading})}{(\text{r.p.m.})} \qquad \text{(Eq. 3)}$$

A logarithmic plot of r.p.m. *versus* scale reading is a convenient method of calculating n since the reciprocal of the slope of the line formed in such a plot equals n.

All viscosity data reported in this study have been calculated from Brookfield data using Eq. 3 and recorded in centipoise (cps.).

A brief definition of some rheology terms which will appear in subsequent parts of this paper is necessary to avoid confusion later. Non-Newtonian flow can be classified according to the presence or absence of a yield value. A yield value represents that amount of force necessary before a liquid begins to flow. If a material does not exhibit a yield value, it is referred to as viscous flow and if it does, it is called plastic flow (30, 31). This division of flow type is rarely referred to in pharmaceutical literature, but it represents more precisely the fundamental types of flow with less ambiguity. According to this definition, a material which thins out or becomes less viscous as the rate of shear is increased is referred to as pseudoviscous rather than pseudoplastic, a term more commonly employed. The term pseudoplastic is reserved for that type of flow which becomes less viscous upon an increase in shear rate, but in addition, also exhibits a yield value. Dilatant materials, which become more viscous upon an increase in shear rate, must be identified further as dilatant-viscous or dilatant-plastic.

RESULTS

The effects of shear rate and age on the viscosity of the ammonia samples are shown in Table I. In sample 1 (0.6 mg. ammonia/100 ml.) the viscosity on day 1 at 0.6 r.p.m. was 3700 cps.; however, as the rate increased to 6 r.p.m. the viscosity dropped to 3630 cps. The influence of shear rate on viscosity became more marked in samples 2 (2 mg. ammonia/100 ml.) and 3 (6 mg. ammonia/100 ml.). Sample 2 decreased 220 cps. going from 3860 cps. at 0.6 r.p.m. to 3640 cps. and 6 r.p.m. Sample 3 exhibited a decline of 530 cps. as the rate was increased over the same interval. There were individual variations in each sample over the period of study, but no meaningful pattern could be defined.

Table I also shows that thixotropy appears to have developed by day 14 in sample 1. Although it could not be demonstrated on day 30, it appeared again on days 60 and 120. Samples 2 and 3 did not appear to display thixotropy until day 60, but after it developed it appeared to remain throughout the duration of the study.

The effect of shear rate on viscosity is further illustrated in Table II. The decrease in viscosity when the shear rate was increased from 0.6 to 6 r.p.m. is listed for each sample according to the day on which it was determined. The last column represents the mean decrease in viscosity for each sample. As shown in the table, the mean decrease in viscosity of sample 1 was 210 cps., whereas that for sample 3 was 590 cps. As was expected, the mean decrease in viscosity of sample 2 was intermediate to these values (340 cps.). Thus, there appears to be an inverse relation between amine concentration and susceptibility of the emulsions to shear rate.

Table III shows the effects of shear rate and age on the viscosities of the AMP emulsions. Pseudoviscous flow was also displayed by these emulsions. The influence of shear rate on the viscosities of samples 4, 5, and 6 (corresponding to 2, 6, and 20 mg. AMP/100 ml., respectively) is demonstrated in the same manner as for samples 1, 2, and 3. Sample 4 decreased in viscosity 130 cps. (from 3720 to 3590 cps.) as the shear rate was increased from 0.6 to 6 r.p.m. The effect of shear rate on viscosity

TABLE I.—EFFECT OF SHEAR RATE AND AGE ON THE VISCOSITY OF AMMONIA[a] EMULSIONS

Sample	Shear Rate (Spindle Speed, r.p.m.)	Viscosity, cps.[b] Day 1	Day 2	Day 4	Day 7	Day 14	Day 30	Day 60	Day 120
1	0.6	3700	4070	4060	4000	4000[c] / 3560	3960	3950 / 3320	4090 / 3790
	1.5	3680	3920	3920	3910	3850 / 3450	3810 / 3790	3810 / 3270	4030 / 3850
	3	3670	3860	3880	3840	3780 / 3400	3750 / 3730	3770 / 3200	3930 / 3800
	6	3630	3820	3840	3810	3730 / 3430	3720 / 3690	3740 / 3230	3890 / 3790
2	0.6	3860	4150	4270	4250	4060	4010	4010	4010 / 3620
	1.5	3750	3850	4060	4040	3850	3770	3770 / 3710	3830 / 3440
	3	3660	3750	3990	3980	3750	3710	3700 / 3640	3790 / 3360
	6	3640	3660	3920	3920	3710	3650	3650 / 3590	3720 / 3300
3	0.6	4250	4320	4530	4470	4320	4320	4230 / 4020	4470 / 4340
	1.5	3940	4000	4200	4160	3990	3950	3880 / 3650	4190 / 4000
	3	3810	3850	4020	4020	3830	3810	3670 / 3460	4020 / 3840
	6	3720	3750	3920	3910	3720	3700	3530 / 3350	3930 / 3730

[a] Samples 1, 2, and 3 contain 0.6, 2, and 6 mg./100 ml., respectively. [b] Viscosity measured with the Brookfield viscometer is reproducible to ±1%. [c] Thixotropy is represented by two figures for the same day and spindle speed. The upper figure represents the "up" viscosity determined when the spindle speed was increased from 0.6 to 6 r.p.m. The lower figure represents the "down" viscosity determined when the spindle speed was decreased from 6 to 0.6 r.p.m.

TABLE II.—VISCOSITY DECREASE IN AMMONIA[a] EMULSIONS RESULTING FROM SHEAR RATE INCREASE FROM 0.6 TO 6 r.p.m.

Sample	Day Examined 1	2	4	7	14	30	60	120	Mean Decrease
1	70	250	240	190	270	240	210	200	210
2	220	490	350	330	350	360	360	290	340
3	530	570	610	560	600	620	700	540	590

[a] Samples 1, 2, and 3 contain 0.6, 2, and 6 mg./100 ml., respectively.

was not as apparent in samples 4 and 5 as it was in samples 1 and 2; however, sample 6 showed a marked effect when compared with sample 4. Over the same shear rate interval sample 6 decreased 470 cps. The effect of shear rate on viscosity also varied with age. These variations did not follow a meaningful pattern, however.

Thixotropy appeared on day 14 in all AMP emulsions and continued through day 120 in sample 4 but could not be demonstrated in sample 5 on day 30 nor in sample 6 on day 120. In each case where thixotropy was present, the emulsion showed complete structural recovery within 2 hr. after the test.

Table IV, constructed like Table II, illustrates the net effect of viscosity decrease with shear rate for each AMP sample according to age. Sample 4 showed a mean decrease of 240 cps. There was no change in the mean viscosity decrease between samples 4 and 5. Sample 6 does show greater mean decrease in viscosity than shown for samples 4 and 5. These data are consistent with those of the ammonia emulsions.

The effect of age on the droplet size in each emulsion studied is shown in Table V. The figures listed show the droplet size as per cent within a size class on the day examined for each sample studied. Upon inspection of the data for sample 1 it is easily seen that the per cent of droplets less than 0.5 μ decreased from 2.5 on day 1 to 0.6 on day 60, whereas the per cent of droplets 1.0 μ increased from 94.3 on day 1 to 96.0 on day 60. There was no significant change in the per cent of droplets with a diameter of 2.0 μ. Sample 2 shows the same pattern but to a lesser extent. Since the magnification in the photomicrographs was 480×,

TABLE III.—EFFECT OF SHEAR RATE AND AGE ON THE VISCOSITY OF AMP[a] EMULSIONS

Sample	Shear Rate (Spindle Speed, r.p.m.)	Viscosity, cps.[b] Day 1	Day 2	Day 4	Day 7	Day 14	Day 30	Day 60	Day 120
4	0.6	3720	3720	4140	4090	4000[c] / 3820	4140 / 3960	4110 / 3950	3980 / 3840
	1.5	3630	3670	4010	4010	3910 / 3760	4030 / 3730	3930 / 3750	3710 / 3620
	3	3600	3620	3960	3960	3840 / 3690	3950 / 3610	3890 / 3680	3510
	6	3590	3600	3900	3910	3780 / 3650	3900 / 3680	3820 / 3630	3470
5	0.6	3730	3500	3920	3870	3830 / 3520	3640	3770 / 3140	3920
	1.5	3690	3390	3780	3780	3650 / 3330	3490	3590 / 3060	3760 / 3720
	3	3620	3380	3700	3710	3510 / 3270	3440	3550 / 2980	3700 / 3680
	6	3590	3340	3660	3690	3410 / 3250	3410	3510 / 2960	3670 / 3640
6	0.6	4070	3720	4340	4290	4390 / 4160	4390 / 4310	4260 / 3960	4390
	1.5	3830	3610	4080	4060	4040 / 3870	4080 / 4020	3980 / 3760	4040
	3	3670	3630	3970	3940	3920 / 3750	3980 / 3900	3890 / 3630	3950
	6	3600	3520	3890	3890	3830 / 3670	3880 / 3820	3830 / 3580	3880

[a] Samples 4, 5, and 6 contain 2, 6, and 20 mg./100 ml., respectively. [b] Viscosity measured with the Brookfield viscometer is reproducible to ±1%. [c] Thixotropy is represented by two figures for the same day and spindle speed. The upper figure represents the "up" viscosity determined when the spindle speed was increased from 0.6 to 6 r.p.m. The lower figure represents the "down" viscosity determined when the spindle speed was decreased from 6 to 0.6 r.p.m.

TABLE IV.—VISCOSITY DECREASE IN AMP[a] EMULSIONS RESULTING FROM SHEAR RATE INCREASE FROM 0.6 TO 6 r.p.m.

Sample	Day Examined 1	2	4	7	14	30	60	120	Mean Decrease
4	130	120	240	180	220	240	290	510	240
5	140	160	260	180	420	230	260	250	240
6	470	200	450	400	560	510	430	510	440

[a] Samples 4, 5, and 6 contain 2, 6, and 20 mg./100 ml., respectively.

1 mm. was equivalent to 1.92 μ. The droplets in sample 3 were so small (in the range of 0.25 mm.) that they were impossible to measure; consequently, droplet size could not be obtained. It can be stated, however, that the droplets in this sample were less than 0.5 μ.

The emulsion having the lowest concentration of AMP is sample 4. As aging occurs, droplet size distribution appears as a decrease in per cent of droplets in smaller sizes and as an increase of larger sizes. The droplet size range narrows in sample 5; in sample 6 the droplet size became so small (as it did in sample 3) that a count could not be made. The droplet size range in sample 6 fell entirely below 0.5 μ.

The mean droplet size is reported in Table VI for each sample on the day it was determined. This table shows that even though a significant rise in droplet size with age cannot be demonstrated, the increase in mean droplet size with increased amine concentration is readily apparent.

DISCUSSION

It has been said that, with very few exceptions, all emulsion systems display some type of non-Newtonian flow characteristics especially when the phase volume exceeds 0.4 to 0.5 (23). The emulsions examined in this study proved no exception to this statement. A pronounced pseudoviscous type flow with the appearance of thixotropy was seen during the study. Thixotropy has been attributed to the formation of extended flocculated networks capable of giving the emulsion necessary structural properties before thixotropy can be seen (15, 16). Since flocculation is an important factor (15-21), its effects are seen to some extent in most

emulsions. Thus, it was not unexpected to see thixotropy displayed in all of the emulsions used in this study.

The increased influence of shear rate on viscosity as the rate was increased, as shown in both the ammonia and AMP emulsions, can undoubtedly also be explained on the basis of increased interaction between droplets or between the droplets and the dispersion medium.

The over-all increase in viscosity as the concentration of amine was increased agrees with previously cited work by Sherman (22). He concluded that the emulsifier can influence emulsion viscosity in several ways depending on its solubility in the two liquid phases, its chemical composition, the concentration used, and the physical properties of the film adsorbed around the droplets. The latter property has been studied on nonaqueous systems and will be described in a separate publication.

The effects of droplet size are also important here. A decreased mean droplet size corresponds to an increased total interfacial area. Both a decreased mean droplet size and an increased homodispersity was seen in the ammonia emulsions and also in the AMP emulsions. The effect of this on viscosity has been explained on the basis of a more uniform droplet size offering more resistance to flow, thus increasing viscosity (10), and by an increased interaction among droplets due to an increased interfacial area (12) with the effect being more pronounced when droplet dispersity becomes more uniform (13, 14).

Levius and Drummond (32) found that droplet size frequency analysis yielded more complete and reliable information about the internal state of the emulsion than was obtained from mean droplet size data only. This is in agreement with the results presented in Tables V and VI. The mean droplet size of each sample listed in Table VI gives the impression that little growth occurs between days 1 and 60. Table V reveals that droplet growth did continue through the entire period of study.

TABLE V.—EFFECT OF AGE ON DROPLET SIZE[a]

Diam., μ	Day Examined: 1	2	4	7	14	30	60
Sample 1, 0.6 mg. NH_3/100 ml.							
<0.5	2.5	2.6	4.3	3.5	3.2	0.8	0.6
1.0	94.3	94.4	92.6	92.3	93.9	95.9	96.0
2.0	3.2	3.0	3.1	4.2	2.9	3.3	3.4
Sample 2, 2 mg. NH_3/100 ml.							
<0.5	95.5	95.5	95.5	94.3	94.3	94.3	94.4
1.0	4.5	4.5	4.6	5.7	5.7	5.7	5.6
Sample 3, 6 mg. NH_3/100 ml.							
<0.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Sample 4, 2 mg. AMP/100 ml.							
1.0	45.1	40.4	34.5	31.5	32.2	30.2	34.0
2.0	52.1	54.9	59.3	60.1	60.2	61.8	57.3
3.0	2.8	4.3	5.7	8.4	7.4	8.1	8.7
4.0	...	0.4	0.5	...	0.4	...	...
Sample 5, 6 mg. AMP/100 ml.							
<0.5	42.6	41.6	34.7	31.6	27.6	21.5	22.3
1.0	57.4	58.4	64.9	67.0	71.9	77.9	77.3
2.0	...	...	0.4	1.0	0.5	0.5	0.4
Sample 6, 20 mg. AMP/100 ml.							
<0.5	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a] Distribution is represented as per cents of total droplets measured in each size class.

TABLE VI.—EFFECT OF CONCENTRATION ON MEAN DROPLET SIZE[a]

Sample[b]	Day Examined: 1	2	4	7	14	30	60
1	2.04	2.04	2.04	2.05	2.04	2.05	2.05
2	0.80	0.80	0.81	0.84	0.84	0.84	0.84
3				Less than 0.5			
4	3.36	3.53	3.65	3.74	3.73	3.74	3.73
5	1.61	1.62	1.70	1.77	1.77	1.80	1.79
6				Less than 0.5			

[a] Numbers represent droplet diameters calculated in microns using Eq. 1. [b] Samples 1, 2, and 3 contain 0.6, 2, and 6 mg. NH_3/100 ml., respectively. Samples 4, 5, and 6 contain 2, 6, and 20 mg. AMP/100 ml., respectively.

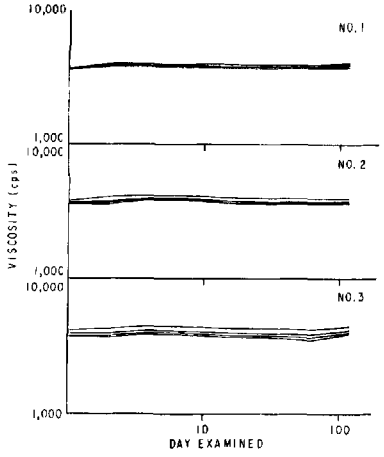

Fig. 1.—The effects of age on the viscosities of ammonia emulsions (logarithmic scale). Samples 1, 2, and 3 represent ammonia concentrations of 0.6, 2, and 6 mg./100 ml., respectively.

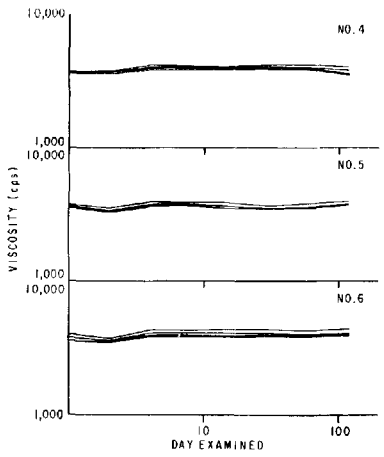

Fig. 2.—The effects of age on the viscosities of AMP emulsions (logarithmic scale). Samples 4, 5, and 6 represent AMP concentrations of 2, 6, and 20 mg./100 ml., respectively.

The fact that samples 2 and 5 both showed a smaller mean droplet size and droplet distribution than was seen with samples 1 and 4, respectively, infers a greater degree of stability between the emulsions as the amine concentration was increased. Even though a droplet size could not be calculated in either sample 3 or 6, the fact that both samples had sizes completely below 0.5 μ can have significant implications as to their stability.

Several published reports have shown the importance and usefulness of time plots on a logarithmic scale (33–36) in stability studies on dispersed systems. These studies demonstrated that linear relations existed when the logarithm of some property to be followed in the system was graphed with the logarithm of time. For example, Wood and Catacalos (36) applied this concept to studies on viscosity and concluded that changes in viscosity expected to occur after 1 year can be predicted from data on only 100 days.

In order to perform a similar evaluation of the data obtained in this study, logarithm time plots of viscosity and age for the various ammonia emulsions and AMP emulsions were prepared and are represented in Figs. 1 and 2, respectively. Figure 1 shows a fairly linear relation for each sample. Sample 3 takes a slight rise between day 60 and day 120. However, this increase, if continued, would not result in an appreciable rise after 1 year. Figure 2 also shows fairly good linearity. Therefore, no appreciable viscosity change should be seen after 1 year.

SUMMARY

Six olive oil-in-glycerin emulsions were prepared wherein ammonia and 2-amino-2-methyl-1,3-propanediol (AMP), in various concentrations, served as saponifying agents. Six-tenths, 2, and 6 mg. ammonia/100 ml. and 2, 6, and 20 mg. AMP/100 ml. were used in making the emulsions. The emulsions were stored at room temperature (25°) and droplet size, rheology, and emulsion stability studied on days 1, 2, 4, 7, 14, 30, and 60. Day 1 corresponded to the day the emulsions were prepared.

Droplet size, as measured from photographs taken by phase contrast photomicrography, was inversely related to amine concentration. This work also demonstrated that a droplet size frequency analysis was much more meaningful to a stability study than was mean droplet size data alone.

Rheology was studied on the respective test days with a Brookfield LVT viscometer. Viscosity was calculated from Brookfield data using the "*n* value" method described by Runikis and coworkers. These studies revealed the existence of non-Newtonian pseudoviscous flow with occasional thixotropy.

Logarithmic graphs of viscosity and time illustrate stability in the emulsion systems. These graphs also suggest that no significant changes in viscosity would be expected over longer time periods.

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Effect of Surfactant Concentration on the Interfacial Viscosity of a Nonaqueous System

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The interfacial viscosities of the glycerin–olive oil interface after either ammonia or 2-amino-2-methyl-1,3-propanediol (AMP) had been added were studied by means of a viscous traction interfacial viscometer. The amine concentrations used were 0.6, 2, and 6 mg. ammonia/100 ml., and 2, 6, and 20 mg. AMP/100 ml. The results indicate that more rigid films were produced at the interface as amine concentrations were increased. The original viscosities and the increased viscosities resulting from increased amine concentrations were as would be predicted from fatty acid films. However, viscosities were not so high as thought to be necessary for stability against coalescence in emulsions.

PREVIOUS STUDIES from this laboratory have demonstrated that nonaqueous emulsions of glycerin and olive oil can be prepared by means of anionic, cationic, or nonionic surfactants (1, 2). It has further been pointed out that a high degree of emulsion stability can be achieved with extremely low concentrations of saponifying amines such as ammonia or 2-amino-2-methyl-1,3-propanediol (AMP). Indeed, concentrations barely sufficient to produce monomolecular films of the amine soap at the interface produced stable emulsions (3).

The effect on emulsion stability of a rigid interfacial film as a mechanical barrier to coalescence has been studied by several investigators. In 1941, King (5) proposed that the strength and compactness of the interfacial film in an emulsion were the most important factors favoring stability. A year earlier, Schulman and Cockbain (6, 7) implied the necessity of high interfacial viscosity for emulsion stability by stating several conditions essential for optimum stability including the need for a "condensed" interfacial film. Becher (8) has also suggested that the formation of a rigid interfacial film is a mechanism in stabilizing emulsions. Sumner (10) supports this concept by suggesting that the mechanical strength of the film of emulsifying agent around the droplets is important. Blakey and Lawrence (11) found a partial correlation between emulsion stability and surface viscosity at the solution–air surface. More recently, the mechanical resistance of the film and emulsion stability have been related (12, 13).

Because of the remarkable stabilizing effect of ammonia and AMP and because it has been repeatedly suggested that a relation exists between emulsion stability and the interfacial viscosity and mechanical strength of the film in oil–water systems (4–13), it seemed important to study the interfacial viscosities of the glycerin–olive oil system containing varying concentrations of amine to determine if a similar relation exists which could help to explain the unusual stability of emulsions of these components.

EXPERIMENTAL

The term interfacial viscosity, as used throughout this study, is defined as the shear viscosity of a monolayer spread or adsorbed at the interface

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Effect of Surfactant Concentration on the Interfacial Viscosity of a Nonaqueous System

By RICHARD D. HAMILL* and ROBERT V. PETERSEN

The interfacial viscosities of the glycerin–olive oil interface after either ammonia or 2-amino-2-methyl-1,3-propanediol (AMP) had been added were studied by means of a viscous traction interfacial viscometer. The amine concentrations used were 0.6, 2, and 6 mg. ammonia/100 ml., and 2, 6, and 20 mg. AMP/100 ml. The results indicate that more rigid films were produced at the interface as amine concentrations were increased. The original viscosities and the increased viscosities resulting from increased amine concentrations were as would be predicted from fatty acid films. However, viscosities were not so high as thought to be necessary for stability against coalescence in emulsions.

PREVIOUS STUDIES from this laboratory have demonstrated that nonaqueous emulsions of glycerin and olive oil can be prepared by means of anionic, cationic, or nonionic surfactants (1, 2). It has further been pointed out that a high degree of emulsion stability can be achieved with extremely low concentrations of saponifying amines such as ammonia or 2-amino-2-methyl-1,3-propanediol (AMP). Indeed, concentrations barely sufficient to produce monomolecular films of the amine soap at the interface produced stable emulsions (3).

The effect on emulsion stability of a rigid interfacial film as a mechanical barrier to coalescence has been studied by several investigators. In 1941, King (5) proposed that the strength and compactness of the interfacial film in an emulsion were the most important factors favoring stability. A year earlier, Schulman and Cockbain (6, 7) implied the necessity of high interfacial viscosity for emulsion stability by stating several conditions essential for optimum stability including the need for a "condensed" interfacial film. Becher (8) has also suggested that the formation of a rigid interfacial film is a mechanism in stabilizing emulsions. Sumner (10) supports this concept by suggesting that the mechanical strength of the film of emulsifying agent around the droplets is important. Blakey and Lawrence (11) found a partial correlation between emulsion stability and surface viscosity at the solution–air surface. More recently, the mechanical resistance of the film and emulsion stability have been related (12, 13).

Because of the remarkable stabilizing effect of ammonia and AMP and because it has been repeatedly suggested that a relation exists between emulsion stability and the interfacial viscosity and mechanical strength of the film in oil–water systems (4–13), it seemed important to study the interfacial viscosities of the glycerin–olive oil system containing varying concentrations of amine to determine if a similar relation exists which could help to explain the unusual stability of emulsions of these components.

EXPERIMENTAL

The term interfacial viscosity, as used throughout this study, is defined as the shear viscosity of a monolayer spread or adsorbed at the interface

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between two liquids. The term surface viscosity, on the other hand, is the shear viscosity of a monolayer spread or adsorbed at the surface of a liquid (14). Both interfacial and surface viscosities have the dimensions MT^{-1}, instead of $ML^{-1}T^{-1}$ as for bulk viscosity (where M, L, and T represent mass, in Gm., distance, in cm., and time, in sec., respectively), and are measured in units of Gm. sec.$^{-1}$ called "surface poise" (s.p.).

Interfacial viscosity was measured by means of the instrument shown in Fig. 1. Except for the diameter of the wire (0.078 cm. instead of 0.064 cm.) used to form the concentric rings and the driving mechanism (Bodine speed reduction motor and appropriate pulley arrangement), this instrument was constructed according to the specifications of the modified viscous traction interfacial viscometer of Davies and Mayers (15). The specifications of the instrument will be mentioned briefly. The two concentric stainless steel wire rings were held in place by the larger support rods

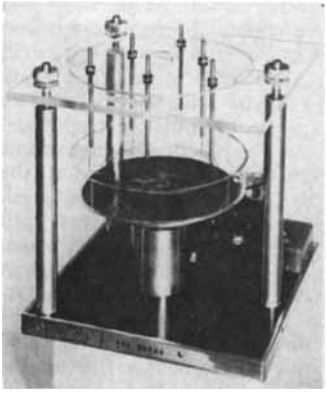

Fig. 1.—Interfacial viscometer.

attached to the upper plastic platform. Three wires of the same diameter as used for the rings were soldered to each ring and made to lie in the plane of the rings for a short distance (about 1.5 cm.) before they were bent upward and soldered to the support rods. The diameters of the outer and inner rings were 12.5 and 11.6 cm., respectively. A Pyrex dish, 18.5 cm. in diameter and 4 cm. high, was centered on the turntable and held the liquids which formed the interface to be studied. The turntable rotated at 1 rev. in 38 sec. The wire rings were held stationary by both the support rods and the upper platform, but the platform could be adjusted, thereby raising and lowering the rings to the desired position.

The methods employed in the use of the instrument were also patterned after those described by Davies and Mayers (15). The results obtained are dependent upon the amount of retardation of the film flowing in the canal when the interface is clean and when an additive is present. To measure retardation, talc particles were placed at the interface and the time for 1 rev. was measured. The rings were raised slightly above the interface producing a meniscus, which acted as a trough to insure that the talc particles remained in the canal.

In order to determine if the difference in wire diameter modified the calibration curve used by Davies and Mayers when applied to this instrument, three experiments were performed. First, the two interfaces (benzene/water and ethyl acetate/water) utilized by the original investigators when calibrating their instrument at the oil–water interface were formed and duplicate results were obtained. Second, Davies and Mayers reported interfacial viscosities of monolayers adsorbed from 4×10^{-3} M sodium lauryl sulfate solutions at the benzene–water interface as 1.5×10^{-4} s.p. at 20° and as 1×10^{-4} s.p. at 22°. The viscometer constructed for this study gave an interfacial viscosity of 1.2×10^{-4} s.p. at 21°. Third, Davies (4) reported the surface viscosity of stearic acid on 0.01 N HCl as 6.25×10^{-4} s.p. at 20.1 Å.^{2}. A similar film was used to verify this value with the present instrument. A benzene solution of stearic acid was applied to the surface of 0.01 N HCl using a micrometer syringe. Surface area calculations were corrected for the small area occupied by the wire rings. A surface viscosity of 6.5×10^{-4} s.p. was obtained when measuring this film. These data suggest that the results obtained with this instrument are remarkably similar to those obtained by Davies and Mayers and that their calibration curve can be used within the range of duplicate results.

Six solutions of amine in glycerin were prepared to correspond with the concentrations of ammonia (0.6, 2, and 6 mg./100 ml.) and 2-amino-2-methyl-1,3-propanediol (AMP) (2, 6, and 20 mg./100 ml.) used in emulsions studied and reported in a separate publication (16). These concentrations were based on the total amount of olive oil and glycerin contained in the dish. The ratio of glycerin to olive oil was 60:40 analogous to a phase volume of 0.40 used in the olive oil-in-glycerin emulsions previously studied (16).

The mean time of 1 rev. at the clean glycerin–olive oil interface was determined. Following this, mean revolution times were measured at the glycerin–olive oil interface after varying amounts of ammonia or AMP had been added. All determinations were made at 20°.

Each interface was measured within a few minutes after it was formed and at intervals over the next 2 hr. Several revolutions were timed and averaged to obtain the mean time of 1 rev. The mean time of the clean glycerin–olive oil interface was subtracted from the mean time of each sample to determine the amount of retardation. The calibration curve (Fig. 2) was then used to obtain the interfacial viscosity in s.p.

RESULTS

Table I lists the results of the interfacial viscosity studies at the glycerin–olive oil interface.

When the interface was clean (no amine added) the mean time of 1 rev. was 171 sec., corresponding to no retardation and therefore, no interfacial viscosity. Upon the addition of 0.6 mg. ammonia/100 ml. to the glycerin phase, the mean time of 1 rev. at the interface was 185 sec. representing a 14-sec. retardation. This was equivalent to an interfacial viscosity of 1.2×10^{-4} s.p. The mean revolution time rose to 204 sec. with the addition of 2 mg. ammonia/100 ml. to the glycerin phase. This represented a retardation of 33 sec. and a

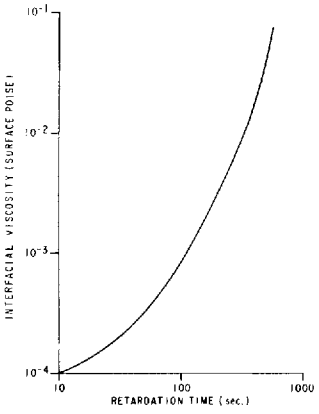

Fig. 2.—Calibration curve for the interfacial viscometer. [After Davies and Mayers (15).]

viscosity of 2.2×10^{-4} s.p. A 52-sec. retardation time was shown when 6 mg. ammonia/100 ml. was added to glycerin. This corresponded to a mean revolution time of 223 sec. and an interfacial viscosity of 3.4×10^{-4} s.p.

Upon the addition of 2, 6, and 20 mg. AMP/100 ml. to the glycerin phase, mean revolution times of 206, 217, and 225 sec., respectively, were obtained; these represent retardation times of 35, 46, and 54 sec., and interfacial viscosities of 2.3×10^{-4}, 3.0×10^{-4}, and 3.6×10^{-4}, respectively.

All interfaces formed with either ammonia or AMP in the glycerin phase showed constant mean revolution times within 30 min.

DISCUSSION

This study was undertaken to measure the effects of various concentrations of ammonia or AMP upon the interfacial viscosities of the glycerin–olive oil interface.

The existence of surface viscosity was first observed by Plateau in 1869 (14). Plateau observed that a fluid surface exhibited resistance to deformation or flow from the difference in damping rates of a compass needle oscillating on the surface and in the interior of a liquid. Since that time several methods for the measurement of surface and interfacial viscosities have been developed (4, 15, 17–22). However, the modified "flow through a canal" method of Davies and Mayers was selected for this study because of several inherent advantages. Ewers and Sack (21) have discussed the validity of the "flow through a canal" technique with soluble layers. They pointed out that where surface pressure is applied to soluble layers, part of the adsorbed film merely dissolves in the interior of the liquid instead of passing through the canal. The "viscous traction" modification of Davies (4) and Davies and Mayers (15) overcomes this disadvantage by allowing the liquid substrate to cause the film to move. Another advantage in the use of this modification is that the width of the canal can be varied and made as narrow as required to increase the sensitivity when working with monolayers of very low viscosity. Joly (14) states that this method is considered best for measuring films of low Newtonian viscosity. The existence of both Newtonian viscosity and viscosity of a low magnitude (10^{-4} s.p.) can easily be established for the glycerin–olive oil system. The very presence of olive oil with its free fatty acid content makes it reasonable to hypothesize that the surfactant found at the interface is a fatty acid type. Furthermore, Joly (23) states that practically all monolayers of fatty acids and triglycerides are Newtonian and are of a low order of magnitude.

The disadvantage in the use of this instrument involves the mathematical interpretation of the hydrodynamics of the process. Since this is extremely complicated, Davies and Mayers calibrated their instruments using films of known surface viscosities. After calibrating the instrument at the air–water surface, they applied it to the oil–water interface. In the first instance, a benzene water interface was measured and flow in the canal was found to take only 10 sec. longer than at the clean air–water surface. When ethyl acetate was used in place of benzene, 1 rev. took only 5 sec. longer. Because of this approximate equality, they concluded that the viscous drag, which the rotating oil exerts on the talc in the canal, balances the extra drag on the interface by the stationary canal walls *via* the adjacent oil. Therefore, the same calibration curve which was determined at the air–water surface was used at the oil–water interface. Davies

Table I.—Interfacial Viscosity of Glycerin–Olive Oil Interface After the Addition of Either Ammonia or AMP

Additive	Mean Time of 1 rev., sec.	Retardation, sec.	Interfacial Viscosity, $\times 10^{-4}$ s.p.
No additive	171 (170–175)[a]	0	0
0.6 mg. NH_3/100 ml.	185 (182–187)	14	1.2
2 mg. NH_3/100 ml.	204 (203–205)	33	2.2
6 mg. NH_3/100 ml.	223 (221–223)	52	3.4
2 mg. AMP/100 ml.	206 (202–209)	35	2.3
6 mg. AMP/100 ml.	217 (215–219)	46	3.0
20 mg. AMP/100 ml.	225 (223–229)	54	3.6

[a] Numbers in parentheses indicate the range of observed revolution times from which mean time was calculated.

and Mayers claim that their instrument can be used to study interfacial viscosities from 10^{-4} to 10^{-1} s.p. The only portion of the curve utilized in this study was between 10^{-4} and 10^{-3} s.p., which was essentially within the range where duplicate results were obtained with known films.

The results obtained demonstrate that there is a measurable increase in viscosity when the concentration of ammonia is raised from 0.6 mg./100 ml. to 6 mg./100 ml. At 0.6 mg./100 ml. the interfacial viscosity was 1.2×10^{-4} s.p. This increased to 2.2×10^{-4} s.p. at 2 mg./100 ml. and rose again at 6 mg./100 ml. to 3.4×10^{-4} s.p.

The addition of AMP to the glycerin phase showed a similar increase in interfacial viscosity. At 2 mg./100 ml., the interfacial viscosity was 2.3×10^{-4} s.p. The interfacial viscosity rose to 3.0×10^{-4} at 6 mg./100 ml. and to 3.6×10^{-4} s.p. at 20 mg./100 ml.

Davies and Rideal (24) state that monolayers of fatty acid esters which give "gaseous" films of great areas are characterized by Newtonian flow and low viscosities (10^{-5} to 10^{-4} s.p.). Monolayers of higher fatty acid esters and of oleic acid also show Newtonian flow and viscosities of this order, but show an increase in viscosity as the concentration is increased and the available surface area decreased producing a more "condensed" film.

Davies (25) reports that an interfacial viscosity of at least 10^{-2} s.p. *seems* to be required if the interfacial film is not to allow coalescence during the close approach of two droplets. The results of this study indicate that a more rigid film is produced at the interface as the amine concentration is increased. The results also indicate that the orders of magnitude of the viscosities and their increases over the concentrations studied were similar to viscosities predictable from Newtonian fatty acid films, but that they were not so large as Davies suggests is needed for coalescence inhibition.

The knowledge that a more viscous interfacial film is present in the glycerin–olive oil system is not meaningful in itself, but when correlated with published work on the stability of glycerin–olive oil emulsions (16), a significant relation can be hypothesized. When either ammonia or AMP was employed in the emulsion system, stability as seen by droplet size distribution data, increased as the added amine was increased. This study then has demonstrated that stability can be achieved in these emulsions with interfacial viscosities which are on the order of 100 times lower than that considered necessary.

SUMMARY

A viscous traction interfacial viscometer, patterned after the one described by Davies and Mayers, was used to measure interfacial viscosity at the glycerin–olive oil interface after either ammonia or 2-amino-2-methyl-1,3-propanediol (AMP) had been added. The concentrations of amine employed corresponded with those used in an olive oil-in-glycerin emulsion stability study.

The results indicate that a more rigid film was produced at the interface as the amine concentration was increased. The interfacial viscosity increased from 1.2×10^{-4} s.p. at 0.6 mg. ammonia/100 ml. to 3.4×10^{-4} s.p. at 6 mg./100 ml. The interfacial viscosity of the AMP film increased similarly. At 2 mg. AMP/100 ml., the interfacial viscosity was 2.3×10^{-4} s.p. This rose to 3.6×10^{-4} s.p. at 20 mg./100 ml.

The viscosities measured and the increases seen are as would be predicted from known fatty acid films. However, the viscosities were on the order of 100 times smaller than that thought necessary for stability against coalescence in emulsions.

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Determination of 7-Bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one (Ro 5-3350) in Blood by Gas–Liquid Chromatography

By J. ARTHUR F. DE SILVA and J. KAPLAN

A method for the determination of Ro 5-335 0 [7-bromo-1,3-dihydro-5-(2-pyridyl)-2H-1,4-benzodiazepin-2-one] in whole blood of humans is described involving selective extraction into ether, acid hydrolysis, and analysis by gas–liquid chromatography. The method has good sensitivity (0.07 to 0.10 mcg./ml., blood), is specific for Ro 5-3350 after it is hydrolyzed to 2-amino-5-bromo-benzoylpyridine, and has an acceptable recovery of the order of 61.0 ± 3.0 per cent. The method has been successfully applied to the determination of Ro 5-3350 in samples obtained from a blood level fall-off study.

Ro 5-3350 [7-BROMO-1,3-DIHYDRO-5-(2-PYRIDYL)-2H-1,4-BENZODIAZEPIN-2-ONE] (1) is a new psychotherapeutic drug of the 1,4-benzodiazepine class of compounds (2, 3). It is undergoing clinical evaluation as a psychotropic drug in the control of ambulatory schizophrenics.

The compound is chemically related to chlordiazepoxide[1] and diazepam.[2] Ro 5-3350 undergoes strong acid hydrolysis to an aromatic primary amine which can be measured by a Bratton-Marshall procedure as is chlordiazepoxide (4) (Scheme I).

Studies of Schwartz and Baukema (5) with ^{14}C-labeled Ro 5-3350 (labeled in the benzodiazepine ring at $\diagdown C{=}O$, Scheme I) conducted in dogs revealed that the maximum concentration of the compound in blood after a 3.5-mg./Kg. oral dose totaling 36 mg. was 0.90 mcg./ml. 4 hr. after administration of the drug. This indicated the need for an assay capable of detection in the submicrogram range and suggested the use of gas–liquid chromatography (GLC).

Preliminary work showed that intact Ro 5-3350 could not be determined by GLC because of thermal decomposition at the high flash temperatures required for volatilization. However, strong acid hydrolysis of the compound to 2-amino-5-bromo-benzoylpyridine (ABBP), Scheme I, reaction 3, gave a good response to electron-capture detection when chromatographed on a 2-ft. column of 2% Carbowax 20M-TPA (6). The method finally adopted utilized the electron-capture detector response to ABBP for the determination of the parent compound. Ro 5-3350 in blood or plasma was extracted at pH 9.0 into diethyl ether and then re-extracted from ether into 6 N H_2SO_4. The Ro 5-3350 in this acid medium was hydrolyzed to 2-amino-5-bromo-benzoylpyridine (ABBP) and glycine, after which the acid phase was neutralized, and the ABBP was extracted into diethyl ether. The residue after the evaporation of ether was taken up in a known volume of n-hexane, an aliquot of which was analyzed by GLC. The area of the ABBP peak was used to determine the concentration of Ro 5-3350 in the sample.

EXPERIMENTAL

Operational Parameters.—Instrument: Jarrell-Ash chromatograph, Universal model 28-700 with a 100 mc. titanium tritide electron-capture detector (No. 28-750). Column: a 2-ft. column of 2% Carbowax 20M–TPA on silanized Gas Chrom P 100–120 mesh contained in stainless steel 1/4-in. tubing. Carrier gas: nitrogen passed through a molecular sieve before entering the column and adjusted to a flow rate of 250–300 ml./min. Column head pressure: 25–27 psig on a second stage of gas regulator. Conditions of column head pressure and flow rate may be varied so as to obtain a retention time for ABBP between 6 and 7 min. for effective separation from adjacent peaks. Temperature of oven: 220 ± 2° (isothermal). Temperature of injection port: 250 ± 2°. Temperature of detector: 210 ± 2°. Amplifier range: 1.0×10^{-9} amp. full-scale deflection. Optimum detector voltage for ABBP: 20–30 v. d.c. Output on recorder: 1.0 mv. (Brown-Honeywell.) Chart speed: 1.25 cm./min. = 30 in./hr. Time constant: 1 sec. Minimum detectable amount of ABBP = 5.0×10^{-9} Gm. (5.0 ng.). Retention time (R_t) of ABBP = 6–7 min.

Preparation of Column Substrate.—The inert support Gas Chrom P 100/120 mesh (Applied Science Labs, State College, Pa.) was silanized, and coated with Carbowax 20M-TPA (Wilkens Instrument and Research, Inc.) to give a 2% loading on a weight–weight basis (7).

Packing and Conditioning the Chromatographic Column.—A 2-ft. piece of stainless steel tubing was

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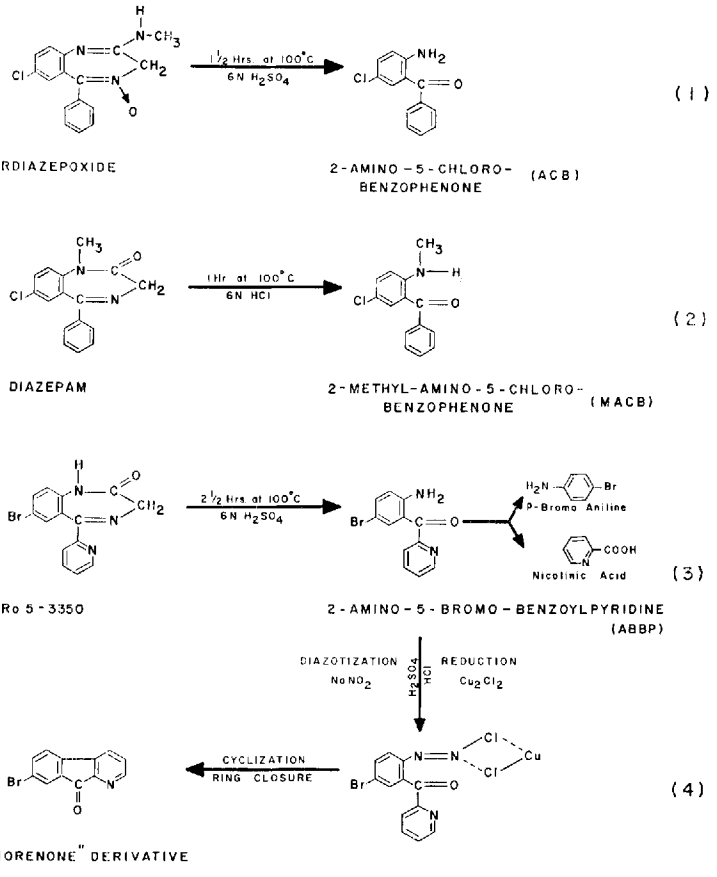
[1] Marketed as Librium by Hoffmann-LaRoche, Inc., Nutley, N. J.

[2] Marketed as Valium by Hoffmann-LaRoche, Inc., Nutley, N. J.



Scheme I
Chemical Reactions of Chlordiazepoxide, Diazepam, and Ro 5-3350.

packed with the prepared substrate and conditioned for 24–48 hr. at 240° (7). A properly conditioned and sensitized column had a useful life-span of about 6 months of continuous use.

Determination of Standard Curve of ABBP

2-Amino-5-bromo-benzoylpyridine synthesized by Fryer *et al.* (1) of >98% purity was dissolved in hexane to yield a stock standard solution of 1 mg./ml. From this solution suitable dilutions were made with *n*-hexane to yield final standard solutions covering the range of 5 ng./10 μl. to 30 ng./10 μl. Three 10-μl. aliquots of each of the final solutions were injected and from their average peak areas a standard curve of peak area (cm.²) *versus* nanograms of ABBP was drawn as shown in Fig. 1. A standard curve should be determined for each day of analysis because column performance and detector response to ABBP changes with time.

Procedure

Reagents.—All reagents must be of reagent grade purity (>98%) and all inorganic reagents were made up in triple-distilled water. 6 *N* H_2SO_4 in triple-distilled water. 6 *N* NaOH in triple-distilled water.

1 M H_3BO_3–Na_2CO_3–KCl Buffer.—Dissolve 62.8 Gm. of boric acid (H_3BO_3) and 74.6 Gm. of KCl/L. of triple-distilled water. Dissolve 106.0 Gm. Na_2CO_3/L. of triple-distilled water. To 630 ml. of the boric acid–KCl solution add 370 ml. of the Na_2CO_3 solution to make a liter of buffer solution. Shake well and check pH, buffer it up to pH 9.0 if necessary with the Na_2CO_3 solution. This solution is 1 *M* with respect to H_3BO_3–Na_2CO_3–KCl. The solution should be stored at about 35–37° to prevent crystallization of the salts out of the solution.

Diethyl Ether.—Analytical reagent grade ether (absolute) containing not more than 0.0005% residue after evaporation and peroxide content not more than 0.00005% (Mallinckrodt) must be used from a freshly opened can on the day of use or up to 3 days after opening.

n-Hexane.—Reagent grade "spectranalyzed" hexane (Fisher) containing not more than 0.0003% residue after evaporation must be used.

Into a 40-ml. glass-stoppered centrifuge tube, add 1 ml. of sample of blood containing Ro 5-3350, 2 ml. of triple-distilled H_2O, and 5 ml. of pH 9.0 1 *M* borate buffer, and mix well by tapping.

Add 15 ml. of ether, seal stopper with triple-distilled water, and shake on a reciprocating shaker for 10 min. Centrifuge at 0–5° (refrigerated centrifuge) for 5 min. at 2100 r.p.m. to separate the layers and transfer ether phase into another 40-ml. glass-stoppered tube.

Re-extract the blood with another 10 ml. of ether and combine the ether extracts.

Add 5 ml. of 6 *N* H_2SO_4 to the combined ether extract, shake for 10 min., and centrifuge for 5 min. to effect a quantitative separation of the ether and acid phases. Remove the ether layer by aspiration with a capillary pipet.

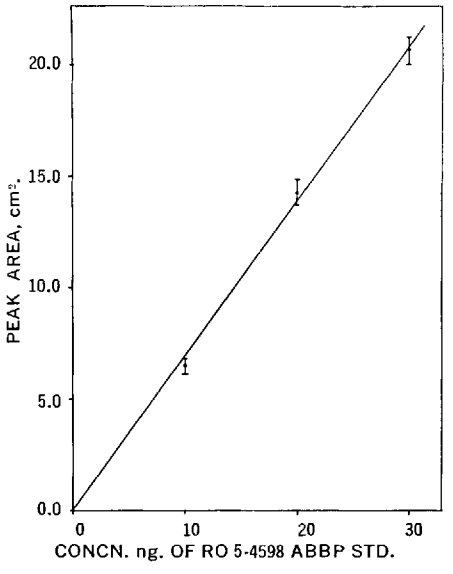


Fig. 1.—Linear response of ABBP standards on a 2-ft. column of 2% Carbowax 20M-TPA on silanized Gas Chrom P.

Extract the acid phase once by shaking with 10 ml. of ether for 10 min., followed by a second extraction with 10 ml. of ether for 5 min. After each extraction centrifuge for 5 min. to separate the phases and remove the respective ether layers by aspiration with a capillary pipet.

Place the tube containing the ether-washed H_2SO_4 extract (unstoppered) in a hot water bath (80–90°) for 10 min. to drive off all the remaining ether. Then stopper the tube (seal with triple-distilled water) and place it in a boiling water bath for 2.5 hr. to hydrolyze the Ro 5-3350 to ABBP.

After hydrolysis, cool the sample in ice, and add 1 drop of bromthymol blue indicator (0.1% in 50% alcohol). Neutralize the cooled H_2SO_4 extract in ice with 6 *N* NaOH to a blue color.

Add 10 ml. of ether to the neutralized sample, seal the stopper with triple-distilled water, and shake for 10 min. on a reciprocating shaker. Centrifuge for 5 min. to separate the phases and transfer the ether layer into a 15-ml. glass-stoppered conical centrifuge tube, and evaporate the ether down to a volume of about 1 ml.

Re-extract the sample with another 10 ml. of ether and combine the ether extracts in the 15-ml. centrifuge tube.

Centrifuge the combined ether extracts for 5 min. and carefully remove any aqueous layer present using a hypodermic syringe fitted with a cannula (BD-20, 15 cm. in length).

Evaporate the combined ether extracts to dryness in a hot water bath and dry the residue in a vacuum desiccator for 30 min. The samples must not be maintained under vacuum for more than 1 hr. or loss of ABBP is incurred.

Dissolve the residue in 100 μl. of *n*-hexane and ensure uniform distribution and solution of the material by tapping the tube for 60 sec.

A suitable-sized aliquot, 1–10 μl., of the final 100 μl. of *n*-hexane extract of the sample residue is chromatographed and the ABBP peak is identified by its retention time.

The area of this peak is determined and the nanogram amount of ABBP present is obtained from a standard curve. The nanogram amount of Ro 5-3350/ml. of blood or plasma is determined from the total nanograms of ABBP × 1.142 [conversion factor, based on the molecular weights of Ro 5-3350 (316.16) and ABBP (276.9), respectively]. The amount of compound present in the samples should be corrected for the per cent recovery of internal standards run concurrently.

RESULTS AND DISCUSSION

Preparation of the Sample for GLC.—Two problems were encountered in this phase of the study. The first one was to effect a quantitative extraction of Ro 5-3350 from blood and back into 6 *N* H_2SO_4, the second was the complete hydrolysis to ABBP and the extraction of this compound into ether. Because of the basic nature of the compound it was found that Ro 5-3350 was quantitatively extracted (97–99%) into diethyl ether from blood buffered to pH 9.0 and back into 2 *N* HCl as determined by its U.V. absorbance values in 2 *N* HCl, $A_{240}^{\text{max.}} = 0.057$/mcg. The recovery from blood into ether was also determined to be 98–99% of added Ro 5-3350 using ^{14}C-labeled material determined by radioisotope scintillometry (Table I). When the ether phase was extracted with 6 *N* H_2SO_4, all the radioactivity (dpm) was quantitatively removed into the acid.

The next step, conversion of Ro 5-3350 to ABBP by acid hydrolysis in 6 *N* H_2SO_4 and the extraction of ABBP into ether was studied, and was followed by thin-layer chromatography (TLC) on Silica

Table I.—Recovery of ^{14}C-Ro 5-3350 Added to 1 ml. of Blood

Concn., mcg.	Determined by Radioisotope Scintillometry: Activity Added, dpm	Activity Recovered, dpm	Recovery, %
50	18,200	18,042	99.1
		18,307	100.6
		18,371	101.0
100	36,000	36,204	100.5
		35,211	97.8
		34,218	95.1
		35,679	99.1
		Over-all av.	99.0 ± 2.1

Table II.—Recovery of Ro 5-3350 After Hydrolysis to ABBP in 6 *N* H_2SO_4

Ro 5-3350 Added, ng.	Determined by Gas Liquid Chromatography: Total Ro 5-3350[a] Recovered, ng.	% Hydrolysis
300	224	75.0
	226	75.0
500	391	78.0
	389	77.0
	393	79.0
	407	82.0
	365	73.0
	407	82.0
	Over-all av.	77.6 ± 3.3

[a] Total ng. ABBP × 1.142 = Ro 5-3350 equivalent.

Gel G (Stahl) with a fluorescent indicator in two solvent systems: *n*-heptane–chloroform–ethanol (10:10:1) and in ethyl acetate. By running separate standards of Ro 5-3350 and ABBP alongside the hydrolysate extract and by viewing the developed plate under shortwave U.V. light, the position of each compound was made visible so that the segments of Silica Gel G containing them could be scraped off the plate and either counted by radioisotope scintillometry or analyzed by GLC. Using ^{14}C-labeled Ro 5-3350 extracted from blood into ether and back in 6 *N* H_2SO_4, and also added directly to 6 *N* H_2SO_4 and hydrolyzed for 2.5 hr. at 100°, no significant radioactivity was measurable in the Ro 5-3350 area on TLC. This indicated that the compound was quantitatively converted. The area on the TLC plate having the same R_f as the ABBP standard showed an intense yellow band which was scraped off, extracted into ether, and analyzed by GLC. This compound had the same retention time as the ABBP standards and was quantized from a standard curve of ABBP.

Studies on the kinetics of the hydrolysis of Ro 5-3350 to ABBP showed that 6 *N* H_2SO_4 produced the more reproducible and optimal yield of the compound when compared with 6 *N* HCl. The average recovery of Ro 5-3350 after hydrolysis for 2.5 hr. at 100° (boiling water bath) was determined by GLC and was found to be of the order of 77% ± 3% of initially added compound (Table II). This yield could not be improved, and it is suspected that a loss of ABBP under the hydrolysis conditions to *p*-bromoaniline and nicotinic acids (Scheme I, reaction 3) occurs with the resultant loss in yields of ABBP. The conversion of Ro 5-3350 to ABBP is quantitative as there was no measurable amount of residual Ro 5-3350-^{14}C left after hydrolysis, as determined by TLC and radioisotope scintillometry. The amount of ABBP recovered after hydrolysis was determined by GLC. The possible interference of *p*-bromoaniline with the GLC determination of ABBP was investigated. The compound does not interfere with the quantization of ABBP. The use of 6 *N* H_2SO_4 over 6 *N* HCl also gave cleaner chromatograms for control blood after a 2.5-hr. hydrolysis (Fig. 2, curve A).

During the investigation of suitable clean-up methods for the purpose of eliminating interfering peaks, it was found that centrifuging at each extraction gave clean separation of ether and aqueous phases resulting in a minimal contamination of the ether extracts with water-soluble material. Washing the H_2SO_4 with ether, centrifuging the final ether extract, and careful removal of any droplets of water and alkali carried over in the transfer operation very effectively cleaned up the extract and gave a very clean chromatogram in the area of the ABBP peak.

Parameters for Gas Chromatography

The development of GLC parameters for the determination of ABBP involved the investigation of several phases of which Carbowax 20M (CBW 20M) and ethylene glycol adipate (EGA) proved to be the most satisfactory. Using 2-ft. columns of 2% CBW 20M or 2% EGA on silanized Gas Chrom P 100/120 mesh, the minimum detectable amounts of ABBP were in the order of 50 to 100 ng. by electron capture. This level was not sensitive enough since the blood levels from the dog experiments (5) indicated the need for an assay in the 10–20 ng. order of sensitivity for ABBP.

The compound ABBP has an aromatic amine group and the pyridyl-*N* moiety, both of which are electrophilic groups, and thus would tend to reduce the electronegativity of the Br and carbonyl groups. This would tend to reduce the response of ABBP to electron-capture detection (ECD) by GLC. The formation of a derivative (8) which would reduce or eliminate such effects was attempted (Scheme I, reaction 4) but was unsuccessful in the submicrogram range. A few grams of the "fluorenone" derivative was synthesized (9) for use as an analytical standard. It showed excellent response to ECD with a minimum detectable range of 5–10 ng.

The use of lightly loaded columns of 2-ft. 0.20–0.25% Carbowax 20M on micro glass beads 100–120 mesh gave better results on GLC and enabled the determination of 10–20 ng. of ABBP as minimum detectable amounts. The major disadvantage with this preparation was a marked tailing of the ABBP peak and short column life of 4–6 weeks after which the entire column had to be discarded. Finally the use of 2% Carbowax 20M-TPA as the

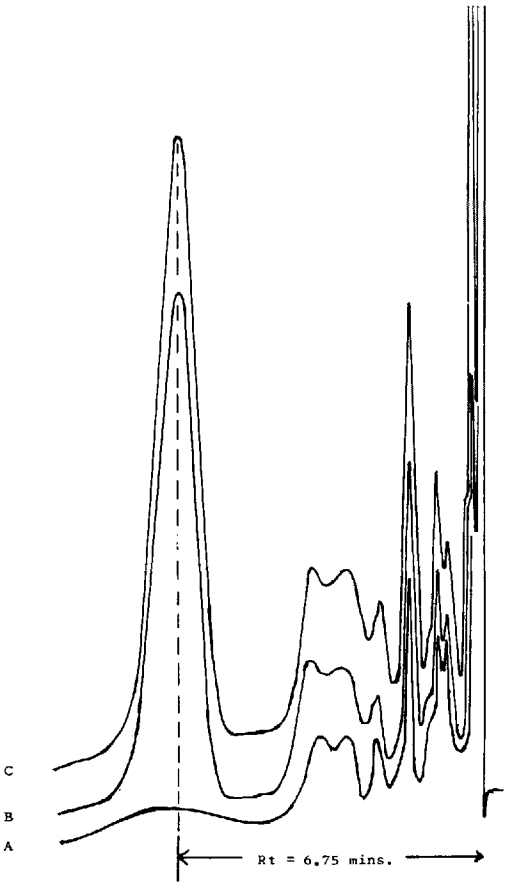


Fig. 2.—Chromatogram of control blood (A) and control blood containing 400 ng. (B) and 500 ng. (C) of added Ro 5-3350 recovered as the ABBP compound (10/100 μl.).

TABLE III.—RECOVERY OF RO 5-3350 ADDED TO 1 ML. OF BLOOD DETERMINED BY GLC

Ro 5-3350 Added, ng.	Total Ro 5-3350[a] Recovered, ng.	% Recovered
200	116	58.0
	116	58.0
300	183	61.0
	185	62.0
	179	59.5
	174	58.0
	201	67.0
	199	66.0
400	249	62.0
	219	55.0
	242	57.0
500	307	61.0
	299	60.0
	297	59.0
	313	63.0
	290	58.0
	295	59.0
	301	60.0
	320	64.0
	322	64.0
	315	63.0
	Over-all av.	61.0 ± 3.1

[a] Total ng. ABBP × 1.142 = Ro 5-3350 equivalent.

liquid phase gave the best results, with well-resolved symmetrical peaks, and a minimum detectable limit of 5–10 ng. of ABBP. It is believed that the terminal TPA moiety of this polyester phase inactivates adsorbent sites still remaining exposed after silanizing and produces a very thin uniform coating which enhances resolution and sensitivity by minimizing losses on the column due to adsorption.

Gas Liquid Chromatography of Sample Extracts.—The response of the electron capture detector to ABBP standards of concentrations ranging from 10.0–30.0 ng. was found to be linear when chromatographed on a properly conditioned column of 2% Carbowax 20M-TPA, as is demonstrated by the standard curve (Fig. 1). Using a constant-injection volume of 10 μl. it was possible to reproduce repeated injections of the same standard within very close limits. Since the response of the detector changes with time possibly due to contamination of the foil with a resultant change in standing current, it was necessary to plot a standard curve for each day of use. A properly conditioned and sensitized column has an average useful life span of about 6 months of continuous use, after which it begins to show signs of deterioration. When the peak response of the column to any given ABBP standard has dropped by 10% or more of its previously determined peak value it is advisable to discharge the column and repack it with fresh substrate and repeat the conditioning operation.

Recovery Experiments

The over-all recovery of known amounts of Ro 5-3350 added to blood and taken through the entire procedure was determined by gas chromatography as the ABBP compound, Fig. 2, curves B and C. The average over-all recovery of 200–500 ng. of Ro 5-3350 added to 1 ml. of blood was in the order of 61% ± 3.0% (Table III). This represents the product of a 98–99% recovery of Ro 5-3350 from blood into 6 *N* H_2SO_4, and an average hydrolysis and recovery of the compound as ABBP of 77%. This represents a theoretical over-all recovery of 75–76%, but in actuality only a 61% recovery is obtained. The discrepancy of 15% recovery has not been satisfactorily accounted for as yet. However, the practical recovery obtained is very reproducible. The minimum detectable amount of ABBP is 5–10 ng./10 μl., and the sensitivity limit of the method is of the order of 0.07–0.10 mcg./ml. of blood or plasma. Blood specimens from a patient who had received a single 15-mg. oral dose of the compound were pooled and extracted into ether using 1 *M* pH 9.0 buffer. The extract was analyzed by thin-layer chromatography (TLC) in two solvent systems: ethyl acetate–NH_4OH–(97:3, v/v) and chloroform–heptane–ethanol (10:10:5, v/v) and showed the presence of Ro 5-3350 which yielded ABBP on strong acid hydrolysis and GLC analysis. This demonstrates the specificity of the extraction procedure and GLC assay for Ro 5-3350 (Fig. 3).

Application of the GLC Method to Biological Samples

Blood Level Fall-Off Curves of Ro 5-3350 Following the Administration of a Single 15-mg. Oral Dose in Man.—Three patients were each given a

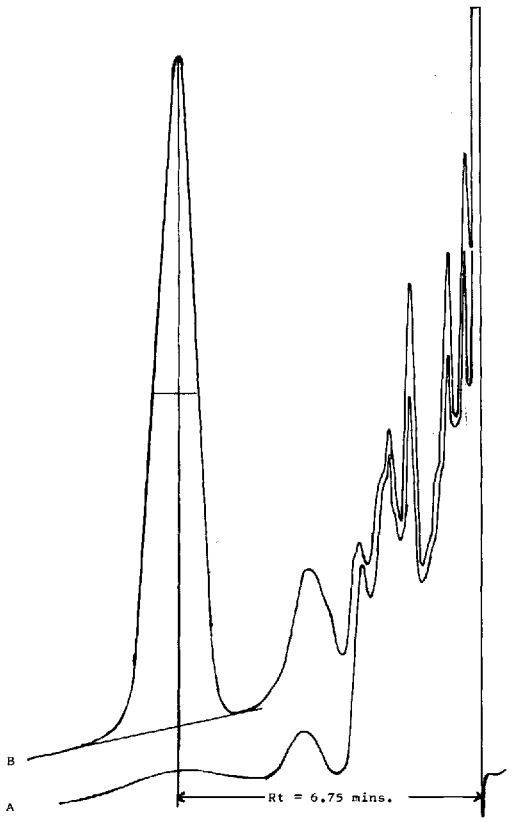


Fig. 3.—Chromatogram of blood extracts from a patient (E.M.) prior to medication, control blood (A), and 4 hr. after a single 15-mg. oral dose of Ro 5-3350; B, recovered as the ABBP compound (10/100 μl.).

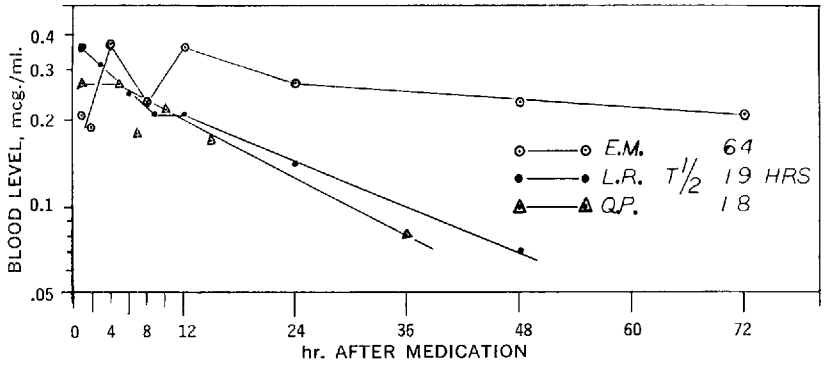


Fig. 4. — Blood level fall-off curves in three humans following the administration of a single 15-mg. oral dose of Ro 5-3350.

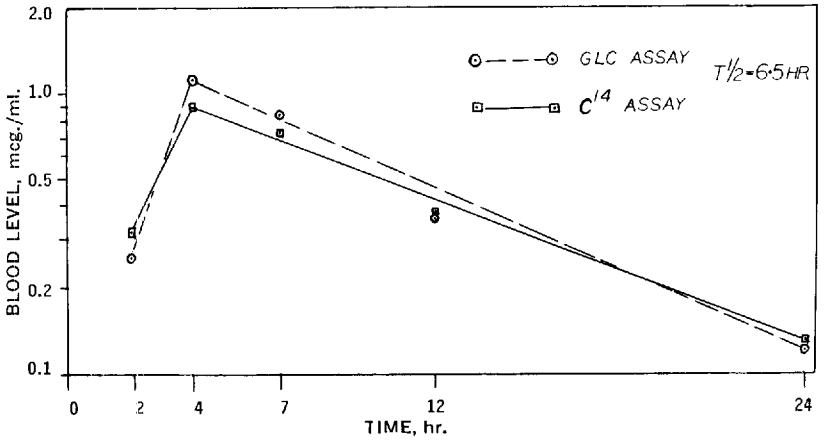


Fig. 5. — Blood level fall-off curve in a dog given a single oral dose (3.5 mg./Kg.) 36 mg. of Ro 5-3350-^{14}C determined by liquid scintillation counting and by GLC.

single oral 15-mg. dose of Ro 5-3350 and specimens of whole blood collected prior to medication (control), and thereafter at suitable intervals of time covering a period of 72 hr. (3 days) from which blood level fall-off curves could be constructed (Fig. 4). In two out of three patients blood level maxima were obtained 1 hr. after dosage of 0.27 (Q. P.) and 0.36 mcg./ml. (L.R.) after which a progressive decline with time was seen with half-lives of 18 hr. and 19 hr., respectively. One patient (E.M.), showed a level of 0.21 mcg./ml. at 1 hr. which rose to 0.37 mcg./ml. at 4 hr., which declined to 0.23 mcg./ml. at 8 hr., rose to 0.36 mcg./ml. at 12 hr., after which a very gradual decline was seen with a half-life of about 64 hr. This erratic behavior may be due to a metabolic factor inherent in this patient.

Blood Level Fall-Off of Ro 5-3350 in the Dog.— The blood level fall-off pattern of Ro 5-3350 was determined in a dog given a single oral dose of 3.5 mg./Kg. totaling 36 mg. of Ro 5-3350-^{14}C. Specimens of whole blood were taken prior to medication and thereafter at 1, 2, 4, 7, 12, and 24 hr. after medication (5) and analyzed for intact Ro 5-3350 by (*a*) liquid scintillation counting and (*b*) GLC. The fall-off curves are shown in Fig. 5 and demonstrate the close reproducibility of the two methods. A blood level maximum of 1.11 mcg./ml. (GLC) and 0.90 mcg./ml. (scintillometry) was obtained 4 hr. after dosage, which declined with a half-life of 6 hr. (GLC) and 7 hr. (scintillometry), indicating an average half-life of about 6.5 hr. in blood.

SUMMARY

The GLC method for the determination of Ro 5-3350 in blood was developed and involves the selective extraction of the compound from blood with pH 9.0 1 *M* borate–Na_2CO_3–KCl buffer using ether as the solvent, followed by extraction of the ether with 6 *N* H_2SO_4. The compound was hydrolyzed in 6 *N* H_2SO_4 for 2.5 hr. at 100° (boiling water bath) to convert it into 2-amino-5-bromobenzoylpyridine (ABBP) which was quantized by GLC.

The average over-all recovery of 200 to 500 ng. of Ro 5-3350 added per milliliter of blood was in the order of 61 ± 3.0%. The minimal detectable amount of ABBP by electron capture was 5–10 ng.

The method was applied to the determination of blood levels in a dog and in man following single oral doses of Ro 5-3350.

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Changes in Ergoline Alkaloids in Seeds During Ontogeny of *Ipomoea violacea*

By K. GENEST

Ipomoea violacea L. (*Convolvulaceae*) (heavenly blue) plants were grown under greenhouse conditions. Seed samples were taken following the tenth day of fertilization until maturity was reached. Their content of total alkaloids, lysergic acid amide, isolysergic acid amide, and clavine alkaloids was determined. The alkaloid values were highest (about 0.1 per cent of dry seed) during the early stages of seed development. Chanoclavine was the most abundant alkaloid in the immature seed. With increasing maturity the lysergic acid amide/chanoclavine ratio increased. Experimental findings are discussed to illustrate biogenetic interrelationships between ergoline alkaloids in *I. violacea* in the light of earlier studies on ergot alkaloids.

THE FORMATION of alkaloids during various stages of plant growth has been investigated in several species of medicinal importance, notably in *Solanaceae* (1, 2). Our interest in the ontogeny of alkaloids in seeds of *Ipomoea violacea* stems from earlier work which dealt with the examination of various commercial samples of morning glory seeds for alkaloids, the toxicity of crude seed extracts, and the composition of the lipid fraction of these seeds (3–6). The active principles of *I. violacea* can be classified as ergoline derivatives, most of which occur also in ergot. Extensive biogenetic work elucidated the origin of the ergoline ring in ergot alkaloids (7, 8). Biogenetic interrelationships between these alkaloids have also been investigated (9–11). Gröger *et al.* (12) reported on the biogenesis of ergoline derivatives in *I. rubro-caerulea* Hook, which is considered to be synonymous with *I. violacea*, and found that in young excised plants L-tryptophan and mevalonic acid can be considered precursors of the ergolines, a pathway proposed earlier for the biogenesis of ergot alkaloids. Taber and Heacock (13) reported that in the seeds of *Rivea corymbosa*, another species of the *Convolvulaceae*, ergoline alkaloids are concentrated in the embryo. The alkaloids were also found in vegetative tissues of mature plants of *R. corymbosa* (14) and *I. violacea* (15) but in lower concentrations than in the seeds. The morphology of the seed development of *I. rubro-caerulea* and *I. violacea* has been described (16, 17), but no chemical studies during ontogeny can be found in the literature. Investigation of the seeds is of importance because frequently their misuse, stimulated by accounts of the alleged psychotomimetic action of the active principles, has been reported (18–20). A recent study by Isbell and Gorodetzky (21) with former morphine addicts indicates that the ergolines in *I. violacea* have predominantly sedative properties. It is the purpose of this paper to report on changes of the alkaloid content in the seeds of *I. violacea* which occur during ontogeny of the plant.

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EXPERIMENTAL

Procedure.—Plants employed in this study were grown under greenhouse conditions. Seeds from a commercial source of *I. violacea* L. (heavenly blue) were germinated in culture flats (March 10, 1965). After 10 days the plants were transplanted into clay pots containing sandy loam and then fed with 20-20-20 fertilizer at regular intervals. Three plants (*A*, *B*, and *C*) were selected for this study. Seed collections were made for each plant separately. Greenhouse temperatures were as follows: sunny days, 70° F.; cloudy days, 65° F.; nights, 60° F. No artificial light was employed. The first flowers appeared on June 10, 1965. From then on pollination was carried out daily by hand during the time of maximal opening of the corolla (10 a.m. to 12 p.m., depending on daylight conditions). The pollinated flowers were marked. Plants yielded from 1 to 22 flowers daily. Collection of fruit capsules was begun 10 days after fertilization and continued at 10-day intervals for 57–62 days when maturity was reached. Seeds were freed from locules of the fruiting body, and placed in a deep freeze refrigerator (−25°) under nitrogen within 1 hr. after collection. One to seven days after harvesting, the seeds were freeze-dried to constant weight and stored under nitrogen at −25° until used for analysis. There were 36 to 305 seeds per plant harvested at each stage of collection.

Alkaloid Analysis.—The dry seeds were ground in a Wiley laboratory mill to pass a 0.037-in. screen and defatted with petroleum ether (b.p. 30–60°). Analyses for total alkaloids, lysergic acid amide (LAA), isolysergic acid amide (isoLAA), and clavines, and examinations of alkaloidal patterns obtained by thin-layer chromatography (TLC) were carried out by methods described previously (3, 6). Samples (0.25–1 Gm.) of dried seeds were analyzed in duplicate for total alkaloids. Values for LAA, isoLAA, and clavines were obtained by averaging

results from densitometric scannings of 2–4 thin-layer plates per sample, each containing nine spots of test material. TLC systems used were: sytem *1* (alumina G and $CHCl_3$–EtOH, 96:4), system *2* (Silica Gel G and acetone–piperidine, 9:1) for densitometric method; system *3* (Silica Gel G and acetone–ethylpiperidine, 9:1); system *4* (alumina G and acetone–ethylpiperidine, 9:1); system *5* (Silica Gel G and acetone–ethyl acetate–dimethylformamide, 5:5:1).

RESULTS

Flower and Seed Production.—The pattern of flower and seed production was very similar in all three plants. While the first flowers appeared during the 13th week after sowing, the first peak of flower production was reached within 3 weeks after flowering had begun. At that stage approximately 100 flowers per plant were produced each week. Until the end of July when flower production had subsided the plants had produced 284, 278, and 291 flowers, respectively. During this period they produced 230, 238, and 228 fruit capsules. Seed samples derived from flowers fertilized from June 6 to July 31 are referred to as first crop. After the first a second, smaller, flowering peak developed. Seed samples derived from flowers fertilized from August 11 to September 23 are referred to as second crop. Since the second crop was only 12.5% of the first, the mature seeds in this group only were collected. Fresh and dry weights of the maturing seeds are plotted in Fig. 1. The fresh weight increased rapidly after fertilization, reached a plateau after 20 days, and fell rapidly during the latter stage of maturity, while the dry substance rose gradually until the 50th day after fertilization, leveling off during the last 10 days of the maturation process.

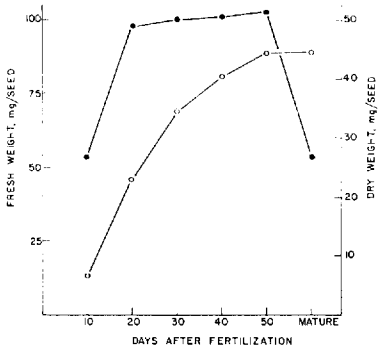

Fig. 1.—Fresh and dry weight of average seed of *I. violacea* L. (heavenly blue) during increasing maturity. Average from three plants. Key: ●, fresh weight, mg.; O, dry weight, mg.

Total Alkaloids.—The total ergoline alkaloids in seeds with increasing maturity are represented in Fig. 2. Their concentrations are highest during the early stage of seed development. From the 30th day after fertilization until full maturity was reached the alkaloid content in the dry seed substance decreased slowly. If calculated on a per seed basis (Fig. 3), the total ergoline alkaloids increased rapidly from the 10- to the 40-day samples and then dropped slowly again as maturity increased. Values for the second crop were always higher than those for the first crop.

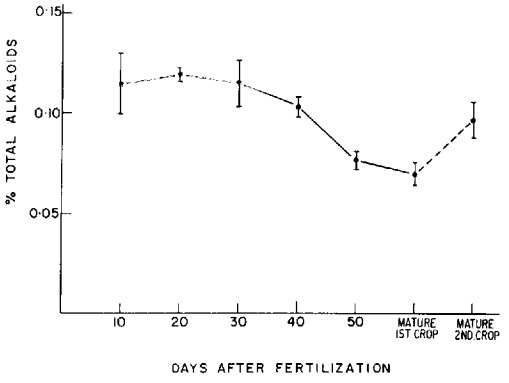

Fig. 2.—Total ergoline alkaloids as per cent of dry seeds during increasing maturity. Average value from three plants and standard deviation are indicated schematically.

Individual Alkaloids.—Results of the determination of LAA, clavines, and isoLAA in seeds collected during increasing maturity are given in Table I and Fig. 4.

As shown in Table I all three alkaloidal components were present from the first sampling date until maturity of the seed was reached. Their relative composition, however, changed. In the early stages after fertilization the clavines were most abundant. Between the 20th and 30th day after fertilization LAA became the predominant constituent which it remained until full seed maturity was reached. The steady decrease of clavine alkaloids after the 20-day sampling can been seen in Fig. 4. LAA reached its maximum concentration at about the 40th day after fertilization, and

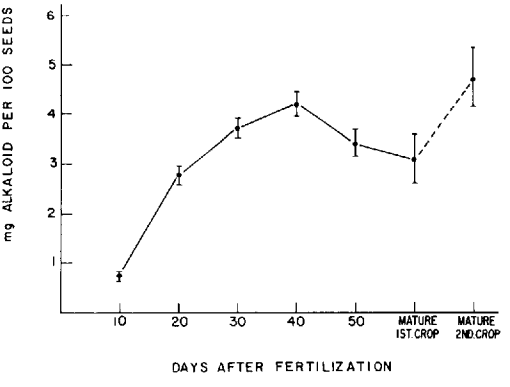

Fig. 3.—Total ergoline alkaloids (mg.) in 100 dry seeds during increasing maturity. Average value from three plants and standard deviation are indicated schematically.

TABLE I.—LYSERGIC ACID AMIDE, CLAVINE ALKALOIDS, AND ISOLYSERGIC ACID AMIDE (mg.) PER 100 DRY SEEDS OF THREE PLANTS OF *I. violacea* L. (HEAVENLY BLUE)

Days After Fertilization	Compd.	Plant *A*	Plant *B*	Plant *C*
10	LAA	0.23	0.29	0.24
	Clavines	0.35	0.37	0.31
	isoLAA	0.08	0.10	0.09
20	LAA	0.86	1.13	1.19
	Clavines	1.23	1.17	1.28
	isoLAA	0.31	0.47	0.55
30	LAA	1.77	1.52	2.31
	Clavines	1.01	1.43	1.51
	isoLAA	0.47	0.88	0.86
40	LAA	2.25	2.15	2.66
	Clavines	0.93	1.21	1.07
	isoLAA	0.72	1.03	0.87
50	LAA	1.72	2.09	2.08
	Clavines	0.87	0.96	0.67
	isoLAA	0.43	0.63	0.53
Mature 1st crop	LAA	1.86	2.08	1.44
	Clavines	0.85	0.81	0.57
	isoLAA	0.41	0.43	0.57
Mature 2nd crop	LAA	3.22	3.42	2.31
	Clavines	1.11	1.00	1.16
	isoLAA	0.50	0.57	0.65

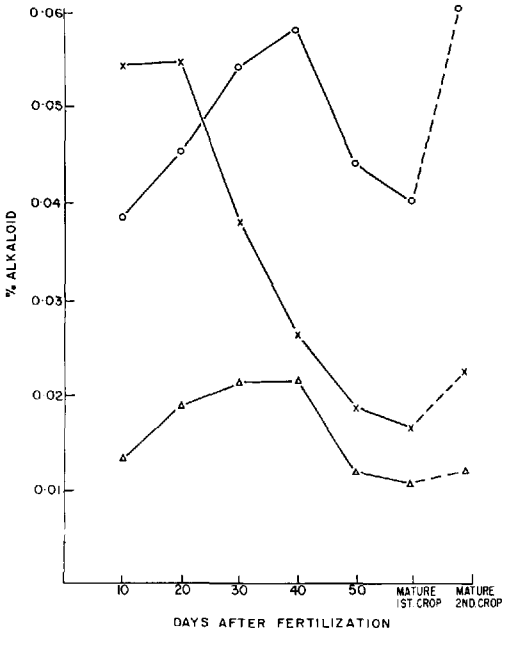

Fig. 4.—Content of lysergic acid amide (LAA), clavines and isolysergic acid amide (isoLAA) in maturing seeds expressed in per cent alkaloid in dry seeds. Averages from three plants are plotted. Key: O, LAA; ×, clavines; △, isoLAA.

its concentration decreased in the later stages of maturity. The change in concentration of isoLAA was similar to that of LAA during increasing maturity but on a lower level. IsoLAA was always the smallest component of the three groups investigated. It too reached its maximum concentration about halfway through maturity. Ergometrine included in the values for clavines, was also present during all stages of seed development. In some cases it was measured separately from the clavine alkaloids. Thus, in seed samples of plant *C* obtained 30 days after fertilization and in the mature seed of plant *B*, 33.0 and 39.4%, respectively, of the clavine fraction consisted of ergometrine. For plant *A* a complete set of ergometrine analyses was carried out. Comparison of results shown in Table II with those recorded in Fig. 2 demonstrates that the decrease of the percentage of ergometrine in the dry seeds was accompanied by a decrease of total alkaloids during seed development. On a per seed basis the ergometrine content is rather constant following the 20th day of fertilization. The ergometrine concentration in the clavine fraction is, however, higher in the more mature samples as compared to the samples closer to the fertilization stage.

The increase in alkaloids in the second crop samples is due to higher values in all three alkaloidal groups as can be seen in Table I and Fig. 4.

Qualitative TLC Patterns.—More information on the clavine alkaloids, which in the densitometric method were estimated as one group, could be

TABLE II.—ERGOMETRINE CONTENT IN SEEDS OF PLANT *A* DURING INCREASING MATURITY

Age of Seeds, Days	10	20	30	40	50	Mature
Ergometrine in dry seeds, %	0.0108	0.0132	0.0107	0.0078	0.0064	0.0061
Ergometrine in 100 dry seeds, mg.	0.06	0.29	0.36	0.31	0.28	0.27
Ergometrine in clavine fraction, %	17.4	23.8	35.7	33.0	32.2	32.0

obtained by chromatography in other TLC systems. Chromatography of the extracts of seeds in various stages of maturity indicated that the changes observed in the clavine group were mainly due to a decrease of the chanoclavine content (Fig. 5) with increasing seed age. This was also observed in systems *1* and *4*. The sum of lysergol, elymoclavine, and penniclavine, which compounds were present in trace amounts only, was lower than the content of ergometrine in all stages of seed development. Lysergic acid methyl carbinolamide, if present, would also be included with the clavine group in the TLC system used for densitometry. R_f values obtained in five systems for this carbinol are shown in Table III. The carbinol can be separated from the other known alkaloidal constituents of *I. violacea* using systems *3* or *5* after rechromatography to separate it completely from penniclavine. As indicated in Fig. 5, no appreciable amounts of the carbinol could be detected at any stage of seed maturity. In addition to the known ergoline alkaloids, at least three spots were observed having higher R_f values than any other alkaloid (R_f system *5*: 0.63, 0.75, 0.79). All these spots showed blue fluorescence under U.V. light and the characteristic blue color given by ergot alkaloids following treatment with dimethylaminobenzaldehyde. Although none of them were identical with agroclavine, there were very faint spots in the agroclavine area when using systems *1*, *3*, *4*, or *5* for the analysis of some seed extracts. In the area where penniclavine is expected, only weak greyish-bluish spots developed 30 min. after spraying.

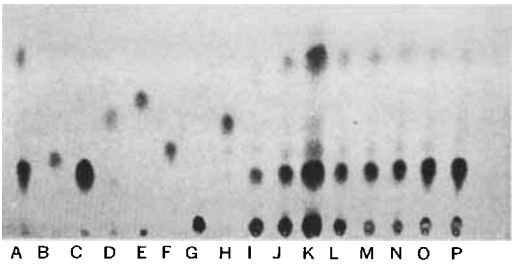

Fig. 5.—Thin-layer chromatogram of ergoline alkaloids and seed extracts of *I. violacea* in system *5*. Key: the spots from left to right are: A, LAA + isoLAA; B, lysergol; C, elymoclavine; D, penniclavine; E, agroclavine; F, ergometrine; G, chanoclavine; H, lysergic acid methylcarbinolamide (with some impurities of ergometrine and LAA). Seed extracts: I, 10; J, 20; K, 20 (3× concentration); L, 30; M, 40; and N, 50 days after fertilization; O, mature seeds (1st crop); P, mature seeds (2nd crop).

TABLE III.—R_f VALUES OF LYSERGIC ACID METHYL CARBINOLAMIDE IN FIVE TLC SYSTEMS

System	*1*	*2*	*3*	*4*	*5*
R_f value	0.30	0.36	0.55	0.23	0.38

DISCUSSION

Many investigations have been carried out on the biogenesis of ergoline alkaloids in parasitic and saprophytic cultures of various ergots or strains of *Claviceps*. The origin of the ergoline nucleus has been attributed to L-tryptophan and mevalonic acid. Several possible biogenetic interrelationships have been discussed. While Abe (22) suggested that all ergot alkaloids derive from a common precursor, a hypothetical lysergic acid aldehyde, which then would be oxidized to lysergic acid or reduced to elymoclavine, agroclavine, and chanoclavine, Rochelmeyer postulated (23, 24) that penniclavine and chanoclavine were possible precursors. The partial pathway, agroclavine → elymoclavine → penniclavine, was established by Agurell and Ramstad (10) in tracer experiments with an ergot strain isolated from *Pennisetum typhoideum*. This observation eliminated the possibility of clavine formation *via* reduction. According to Voigt (11) chanoclavine plays a key role in the development of biogenetic alkaloidal patterns as shown by experiments with rye ergot in various stages of maturation. Further contributions to the biogenetic interrelationships between various clavine alkaloids were made by Abou-Chaar *et al.* (25), Baxter *et al.* (26–28), and Agurell and Ramstad (29). The conversion of clavine alkaloids into lysergic acid alkaloids was proven in experiments with parasitic and saprophytic cultures of *Claviceps* (30, 31). The various findings in this field could partially be explained by the use of different nutrients for saprophytic cultures of fungi (32), but differences in strains producing different alkaloidal patterns have to be considered. The occurrence of alkaloids in varying concentrations during different stages of seed development in *I. violacea* could also be due to transformations of alkaloids into one another. Problems on possible transformation paths are complicated because all alkaloids occur simultaneously in all phases of seed development, and little information has yet been gained on the movement of the alkaloids in the plant. Although only limited conclusions in relation to the biogenesis of ergoline alkaloids are possible from our experimental data, findings in the much more thoroughly investigated field of ergot alkaloids could have some bearing on the elucidation of a biogenetic pathway for lysergic acid derivatives in *I. violacea*. The relatively high concentration of alkaloids and their pattern during the early stages of seed development could indicate that preformed alkaloids are predominantly deposited in the seeds, and that the seed is not necessarily the original site of alkaloid synthesis. Gröger *et al.* (12) reported in tracer experiments that while L-tryptophan and mevalonic acid were incorporated into excised *I. rubro-caerulea* plants to form ergoline alkaloids, their specific rate of incorporation into alkaloids isolated from seeds was much smaller than that into alkaloids isolated from stems. Also radioactive elymoclavine could be converted into penniclavine by detached stems of the same plant (33). Comprehensive tracer studies proving to what degree, if at all, seeds in *I. violacea* can synthesize ergoline alkaloids remain to be carried out and are not only important for finding the site of alkaloid synthesis but also for the elucidation of biogenetic interrelationships between alkaloids in this plant. Indications that chanoclavine may be the precursor of LAA and its epimer are seen in our results on individual alkaloids (Figs. 4 and 5). Ergo-

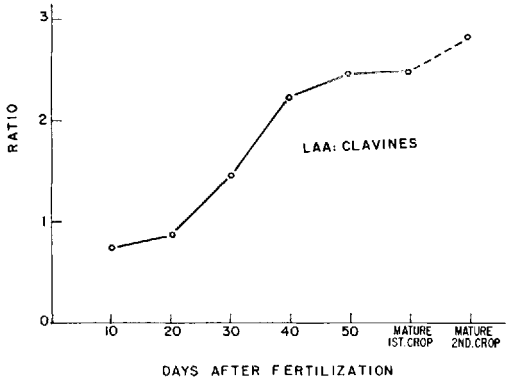

Fig. 6.—Ratio of lysergic acid amide/clavine alkaloids during increasing maturity of seeds.

metrine, on the other hand, seems to appear preferably in the middle and later period of seed development. The change of the LAA/clavine ratio (Fig. 6) would indicate a transformation of clavines (chanoclavine) into LAA during the course of increasing seed maturity. Thus, a biogenetic pathway for ergoline alkaloids in *I. violacea* analogous to that of the ergot alkaloids may be postulated: chanoclavine → (agroclavine) → (elymoclavine) → (penniclavine) → LAA; isoLAA → ergometrine → (lysergic acid methyl carbinolamide). The alkaloids shown in parentheses were found in the seeds in traces only or, in some cases, not even detected. They could, possibly, occur in greater abundance at the original site of alkaloid synthesis in the plant. The presence of mere trace amounts of lysergic acid methyl carbinolamide, which (alkaloid) was reported as one of the major components in *I. rubro - caerulea* (15) could indicate a genetic difference between this plant and *I. violacea* even though both plants are considered to be synonymous botanically. This interpretation, however, has to be used with caution since blank experiments with small amounts of carbinol showed that, under our experimental conditions, some of the applied carbinol is being decomposed.

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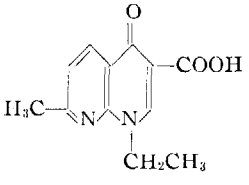
Qualitative and Quantitative Tests for Nalidixic Acid

By EDWARD F. SALIM* and IRWIN S. SHUPE†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

1-ETHYL-7-METHYL-1,8-NAPHTHYRIDIN-4-ONE-3-CARBOXYLIC ACID; $C_{12}H_{12}N_2O_3$; mol. wt. 232.24. The structural formula of nalidixic acid may be represented as:



Physical Properties.—Nalidixic acid occurs as a white to slightly yellow crystalline, odorless powder, m.p. 225–231°, U.S.P. class *Ia*. It is practically insoluble in water, slightly soluble in alcohol, soluble in chloroform, and very slightly soluble in ether. It is soluble in solutions of fixed alkali hydroxides and carbonates.

Identity Tests.—A 1 in 200,000 solution of nalidixic acid in 0.1 *N* sodium hydroxide exhibits ultraviolet absorbance maxima at about 332 and 258 mμ [absorptivity (*a*) about 111] and minima at about 276 and 236 mμ. The spectrum is shown in Fig. 1.

The infrared spectrum of a 0.5% dispersion of nalidixic acid in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 2.

Purity Tests.—Dry about 1 Gm. of nalidixic acid, accurately weighed, at 105° for 2 hr.: it loses not more than 0.5% of its weight.

Char about 1 Gm. of nalidixic acid, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.2%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of nalidixic acid in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U.S.P. heavy metals test, method I: the heavy metals limit for nalidixic acid is 20 p.p.m.

Determine the nitrogen content of nalidixic acid by the U.S.P. nitrogen determination, method II: not less than 11.77% and not more than 12.37% of nitrogen (N) is found.

Assay.—Transfer about 250 mg. of nalidixic acid, accurately weighed, to a 125-ml. conical flask, and dissolve in 30 ml. of dimethylformamide which has been previously neutralized to thymolphthalein T.S. Titrate with 0.1 *N* lithium methoxide, using a magnetic stirrer and taking precautions against the absorption of atmospheric carbon dioxide. Each milliliter of 0.1 *N* lithium methoxide is equivalent to 23.22 mg. of $C_{12}H_{12}N_2O_3$. The amount of nalidixic acid found is not less than 98% and not more than 102% of the weight of the sample taken.

DOSAGE FORMS OF NALIDIXIC ACID

Nalidixic Acid Tablets

Identity Tests.—The ultraviolet absorption spectrum of the sample solution obtained in the *Assay* exhibits maxima and minima at the same wavelengths as that of the *Standard Preparation*.

Transfer a sample of finely powdered tablets, equivalent to about 100 mg. of nalidixic acid to a glass-stoppered conical flask. Add 50 ml. of chloroform and shake for 15 min. Filter through paper and evaporate the chloroform solution on a steam bath to dryness. Dry the residue at 105° to constant weight: the resulting residue of nalidixic acid melts between 225° and 231°.

Assay.—*Standard Preparation.*—Dissolve about 100 mg. of nalidixic acid, accurately weighed, in 200.0 ml. of chloroform. Transfer 2.0 ml. of this solution to a 200-ml. volumetric flask, dilute to volume with chloroform, and mix.

Procedure.—Weigh and finely powder not less than 20 nalidixic acid tablets. Transfer an accurately weighed portion of the powder, equivalent to about 100 mg. of nalidixic acid, to a 125-ml.

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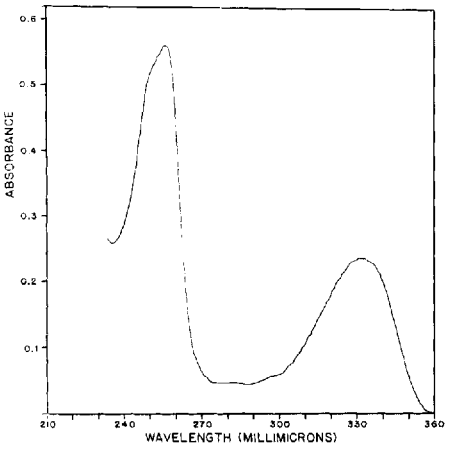

Fig. 1.—Ultraviolet absorption spectrum of nalidixic acid in 0.1 *N* sodium hydroxide (5 mcg./ml.); Beckman model DK-2A spectrophotometer.

separator containing 10 ml. of water. Completely extract the nalidixic acid with four 25-ml. portions of chloroform, filtering each portion into a 200-ml. volumetric flask, dilute to volume with chloroform, and mix. Pipet 2 ml. of this solution into a second 200-ml. volumetric flask, dilute to volume with chloroform, and mix. Concomitantly determine the absorbance of this solution and that of the *Standard Preparation* in 1-cm. cells at the wavelength of maximum absorbance at about 258 mμ with a suitable spectrophotometer, using chloroform as the blank. Calculate the quantity, in mg., of $C_{12}H_{12}N_2O_3$ in the portion of tablets taken by the formula $20\,C(A_u/A_s)$ in which C is the exact concentration, in mcg./ml., of nalidixic acid in the *Standard Preparation*, A_u is the absorbance of the solution from the tablets, and A_u is the absorbance of the solution from the tablets, and A_s is the absorbance of the *Standard Preparation*. The amount of nalidixic acid found is not less than 93% and not more than 107% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., has been used wherever feasible.

Nalidixic acid,[1] synthesized by Lesher and Gruett (1), is a systemic antibacterial agent which is highly effective against many Gram-negative bacteria and some Gram-positive organisms. It is particularly useful in genitourinary tract infections and can be used in other infections in which the causative agent is susceptible to the drug.

Quantitative Methods.—The nonaqueous titration of nalidixic acid with lithium methoxide using thymolphthalein T.S. gave an average value of 100.7 ± 0.1%.[2] The titration can be conducted using 0.1 *N* sodium methoxide with comparable results and thymol blue T.S. can be conveniently substituted as the visual indicator for either titrant.

[1] Marketed as NegGram by Winthrop Laboratories, New York, N. Y.

[2] Maximum deviation from the mean value.

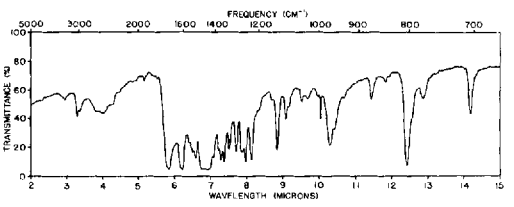

Fig. 2.—Infrared spectrum of nalidixic acid in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

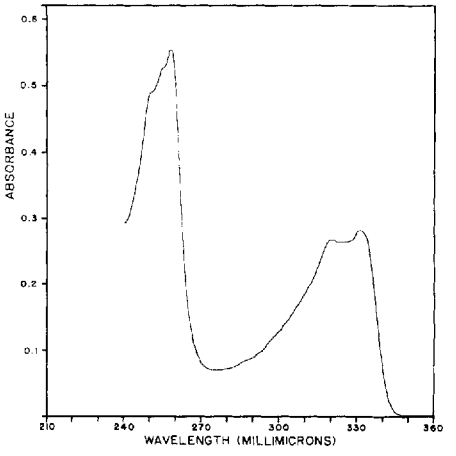

Fig. 3.—Ultraviolet absorption spectrum of nalidixic acid in chloroform (5 mcg./ml.); Beckman model DK-2A spectrophotometer.

The spectrophotometric assay for nalidixic acid tablets was designed to isolate the active ingredient prior to ultraviolet measurement. The extracted nalidixic acid is conveniently determined in chloroform solution at the absorbance maximum at either 258 or 332 mμ. The ultraviolet absorption spectrum of the *Standard Preparation* is shown in Fig. 3. Spectrophotometric analysis of commercial tablets gave an average value of 98.5 ± 0.5%[2] of the labeled amount.

An alternative method of analysis for the tablet formulations has been demonstrated by nonaqueous titration subsequent to chloroform extraction and evaporation to dryness. The residue representing about 250 mg. of nalidixic acid is dissolved in 30 ml. of dimethylformamide, previously neutralized to thymolphthalein T.S., and titrated with 0.1 *N* lithium methoxide in the manner noted for bulk nalidixic acid. An average recovery of 99.0 ± 0.4%[2] was obtained for the tablets by this procedure.

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Powder Flow Studies II

Effect of Glidants on Flow Rate and Angle of Repose

By GERALD GOLD, RONALD N. DUVALL, BLAZE T. PALERMO, and JAMES G. SLATER

Glidants have often been selected by subjective or indirect methods such as measurement of the angle of repose. As a result, several materials have been empirically classified as glidants. The recording powder flowometer described in Part I of this series was utilized to evaluate various glidants. A comparison was made between the results obtained with this instrument and those by measurement of the angle of repose. The commonly used glidants, fumed silicon dioxide, magnesium stearate, starch, and talc were studied in combination with selected materials. Many of the more widely used glidants actually decreased the flow rate. Glidants which lowered the angle of repose did not necessarily increase the flow rate and marked changes in flow rate were not always detectable by angle of repose measurement. In addition, a comparison of the angle of repose and the flow rate of various commonly used raw materials indicated that the angle of repose was not a reliable method for evaluating the flow of these materials.

THE FLOW properties of powders vary considerably from those of formulations which flow freely and continuously through a small orifice to those which are evident of noncontinuous flow under any circumstance. Poorly flowing powders or granulations present many difficulties in the pharmaceutical industry, especially in compressed tablet manufacturing, and considerable effort, therefore, has been directed toward overcoming flow problems. The most commonly used technique involves the addition of materials known as glidants in an attempt to improve the flow characteristics of the formulation. Selection of the glidant and the concentration thereof is often empirical since there is no generally accepted method for evaluating the effectiveness. As a result, glidant usage is often ascertained on a trial and error basis *via* subjective information.

One of the more objective methods, probably the most widely used, involves the measurement of the angle of repose. The powdered or granular material is allowed to fall freely through an orifice onto a flat surface to form a conical pile of the deposited material. The angle between the surface of the cone and the horizontal plane is known as the angle of repose. This numerical value is reproducible provided the conditions remain constant. Train (1), in a critical examination of four methods of determining the angle of repose with glass balls, lead shot, and silver sand, concluded that the type of method influenced the results, but that most methods of measurement would provide suitable data for comparison between samples during routine quality control tests. He did not mention relationship to flow. Although it is not clear to what physical property the angle of repose corresponds, it has often been assumed (2–4) that it relates to the flow properties of the material. A high angle reputedly indicates a poorly flowing material, while conversely a low angle indicates good flow. The magnitude of this angle is dependent on the conditions of measurement and the numerous methods by which this angle has been measured have added to the confusion. Pilpel (4), in summarizing the work of others, indicated that the value of the angle of repose depends not only on the way in which the cone is produced, but also on the nature of the powder, its preparation, particle size, and particle size distribution. In an attempt to study the problem of granulation flow in tableting as it is related to interparticle friction, Nelson (5) measured the angle of repose of a sulfathiazole granulation as a function of average particle size, the presence of lubricants, and admixed fines, but carefully avoided direct correlation with flow properties. Craik applied the supposed relation of flow to the angle of repose in an

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Previous paper: Gold, G., Duvall, R. N., and Palermo, B. T., *J. Pharm. Sci.*, **55**, 1133(1966).

investigation of the flow properties of starch powders under laboratory conditions (2) and of three very different powders under humid conditions (3).

Timed delivery through an orifice is also of value in appraising the flow of materials. A stop watch is usually used to either time a certain weight of powder flowing through the orifice or to close the orifice after a given time so that the powder flowing through in that time period can be weighed. With this method, Hammerness and Thompson (6) showed that the addition of fines increased the rate of flow to an optimum level, after which the rate began to decrease. The addition of lubricant beyond 2% did not substantially improve the flow rate. Gunsel and Lachman (7) comparatively evaluated tablet formulations by the timed delivery technique and used the angle of repose measurement to substantiate similarities in flow rates. However, their data show nearly identical flow rates for two formulations having differences in angles of repose and a marked difference in flow rate for two materials having identical angles. Munzel (8), using a modified Emix powder dispenser and weighing the amount of material discharged in 10 sec. by manually opening and closing the flap, studied the influence of glidants on the flow properties of granules. He apparently was the first to employ the term "glidant" to designate agents which added in small amounts improve flow characteristics of granulations. Flow regulators and solid flow conditioners are two other terms used synonymously with flow glidants. The report of Munzel is the source of the Remington (9) differentiation of lubricants into three groups based on their ability to carry out respective functions: glidants, antiadhesives, or antisticking agents and lubricants.

Recently, Augsburger and Shangraw (10) rated some silica-type glidants in two microcrystalline cellulose systems based on tablet weight variation data. However, this method is tedious, time consuming, and not practical at the preliminary product development stage.

In the previous report from this laboratory (11), a recording powder flowometer was described and represented a new approach to the study of powder flow. The authors believe that this technique more closely simulated and recorded flow as it occurs in a tableting operation. Although the angle of repose is controversial in the area of flow measurement, it has been widely used, and one objective of this study was to investigate the relationship between this angle and the flow rate with the recording powder flowometer. Numerous materials were evaluated in an effort to ascertain correlation, if any, between the two techniques. A second aspect of this work was to study the effect of materials generally known as glidants when added to selected powdered, crystalline, and granulated materials. The glidants used were fumed silicon

Table I.—Particle Size Analysis of Aspirin, Calcium Sulfate, and Spray-Dried Lactose

Screen	20	40	60	80	120	200	Pan
	Material on Screen, %						
Aspirin crystals	1	31	38	17	7	5	1
$CaSO_4 \cdot 2H_2O$ granules	5	49	16	9	6	8	7
S. D. lactose	0	0	0	3	16	43	38

Table II.—Effect of Magnesium Stearate, Fumed Silicon Dioxide, Cornstarch, and Talc on the Flow Rate and Angle of Repose of Aspirin Crystals, Calcium Sulfate Granules, and Spray-Dried Lactose

		ASA Crystals		$CaSO_4$ Granules		S.D. Lactose	
	% Glidant	Flow Rate	Repose Angle	Flow Rate	Repose Angle	Flow Rate	Repose Angle
Control	0	11.17	40.35	8.42	41.63	8.16	33.08
Mg stearate	0.25	12.62	36.77	8.75	41.30	8.49	32.62
	0.5	11.44	39.60	8.67	41.80	7.03	33.35
	1.0	11.48	40.73	9.14	41.40	7.21	33.90
	5.0	8.14	40.92	7.27	42.72	3.08	40.53
Fumed SiO_2	0.1	11.47	40.45	9.61	42.10	8.21	33.97
	0.25	12.08	40.17	9.27	42.32	7.63	32.42
	1.0	11.68	39.52	6.90	42.63	7.28	31.72
	5.0	5.71	40.53	3.84	43.03	1.34	35.73
Cornstarch	0.25	12.88	38.18	8.84	41.80	7.27	34.03
	1.0	12.10	37.85	8.29	42.52	6.63	33.28
	5.0	10.59	38.18	6.96	42.32	4.90	33.90
	10.0	8.93	39.33	4.83	43.15	4.11	38.62
Talc	0.25	10.24	41.42	8.26	42.02	5.78	33.77
	1.0	10.51	41.32	7.62	42.63	5.04	34.68
	3.0	9.78	42.42	7.06	42.42	3.44	36.43
	5.0	8.69	42.22	7.75	42.83	3.76	37.08

dioxide, magnesium stearate, cornstarch, and talc. Varying concentrations of these were evaluated for their effects on the angle of repose and flow rate of aspirin crystals, calcium sulfate granules, and spray-dried lactose. The numerous comparisons obtained from this study should help to clarify the status of angle of repose measurement as a measure of flowability.

EXPERIMENTAL

Materials.—The spray-dried lactose, crystalline aspirin, magnesium stearate, talc, fumed silicon dioxide,[1] and cornstarch were either U.S.P. or pharmaceutical grade materials. The calcium sulfate granulation was prepared by adding 5% gelatin solution to calcium sulfate dihydrate in a Blakeslee kitchen mixer and wet sizing through a No. 6 screen on an oscillating granulator. It was dried for 8 hr. at 140° F., and the dried granules were sized with an oscillating granulator and a No. 16 screen. Mesh analyses of the aspirin, spray-dried lactose, and calcium sulfate granulation used in the glidant studies

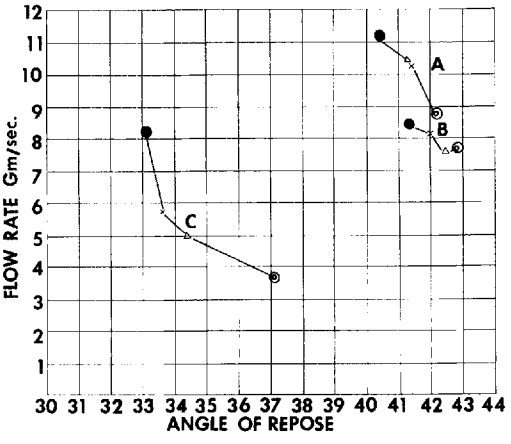

Fig. 1.—Relationship of angle of repose to flow rate for systems containing talc. Key: A, aspirin; B, calcium sulfate granules; C, S.D. lactose. Talc concentrations: ●, 0%; ×, 0.25%; △, 1.0%; ⊙, 5.0%.

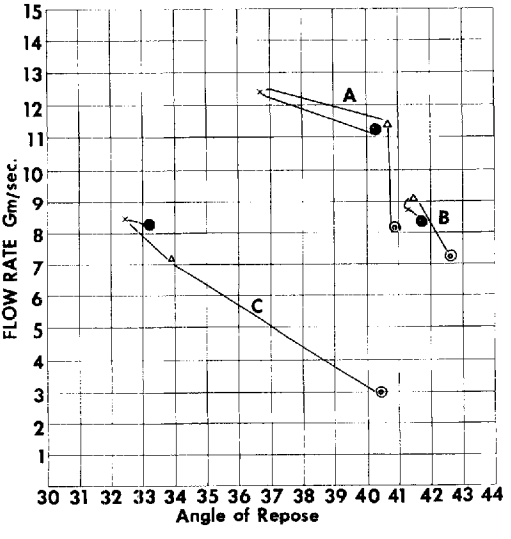

Fig. 2.—Relationship of angle of repose to flow rate for systems containing magnesium stearate. Key: A, aspirin; B, calcium sulfate granules; C, S.D. lactose. Magnesium stearate concentrations: ●, 0%; ×, 0.25%; △, 1.0%; ⊙, 5.0%.

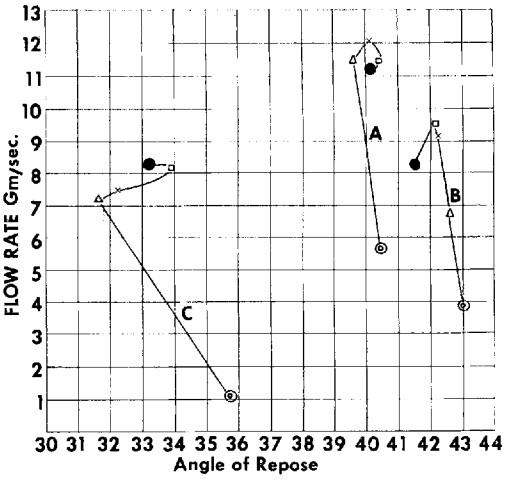

Fig. 3.—Relationship of angle of repose to flow rate for systems containing fumed silicon dioxide. Key: A, aspirin; B, calcium sulfate granules; C, S.D. lactose. Fumed silicon dioxide concentrations: ●, 0%; □, 0.1%; ×, 0.25%; △, 1.0%; ⊙, 5.0%.

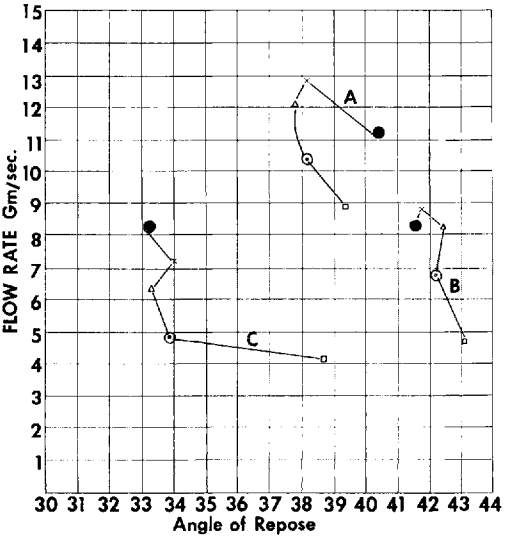

Fig. 4.—Relationship of angle of repose to flow rate for systems containing starch. Key: A, aspirin; B, calcium sulfate granules; C, S.D. lactose. Starch concentrations: ●, 0%; ×, 0.25%; △, 1%; ⊙, 5%; □, 10%.

[1] Marketed as Cab-O-Sil by the Cabot Corp., Boston, Mass.

are shown in Table I. The cornstarch was dried at 140° F. for 24 hr., and all glidants were passed through a No. 40 hand screen prior to using. Glidants were used at concentration levels as indicated in Table II.

The materials used for the flow rate and angle of repose correlation study are listed in Table III and characterized according to the U.S.P. powder fineness classification for chemicals following particle size analysis.

Particle Size Analysis.—Particle size was determined with a Ro-Tap testing sieve shaker, using U. S. standard sieves in series 20, 40, 60, 80, 120, and 200 mesh sizes. A 100-Gm. sample was tested in the shaker for 5 min.

Flow Rate Determinations.—Flow rates, expressed in Gm./sec., were determined with the flowometer on 500-Gm. samples and with a conical stainless steel hopper measuring 20 cm. top diameter by 30 cm. in length and 10 mm. orifice diameter. Each value represents the average of nine measurements. To minimize variations in ambient conditions, three measurements were made on 3 different days. The average standard deviation was 0.47 Gm./sec.

Angle of Repose Measurements.—Repose angles were measured by the fixed funnel and free standing cone procedure described by Train (1). The fixed height of the funnel orifice was 2.54 cm., and the orifice diameter was 5.0 mm. The reported repose angles represent the average of five determinations. In no case was the standard deviation greater than 0.81°, which compares favorably with the average standard deviation of 0.74° reported by Fonner *et al.* (12).

RESULTS AND DISCUSSION

The effects of varying concentrations of glidants on the flow rate and angle of repose of aspirin crystals, calcium sulfate granules, and spray-dried (S.D.) lactose are shown in Table II. The concentrations of specific glidants included those recommended or generally used. Since S.D. lactose had the slowest flow rate, it might be assumed to respond more favorably to the addition of a glidant. However, with the exception of the lowest levels of magnesium stearate and fumed silicon dioxide, the flow rate was decreased substantially. Munzel (8) reported that the flow rate of sodium chloride was reduced by the addition of glidants, and attributed this to the fact that sodium chloride itself is an excellent glidant and that its flow properties were impaired by poorer glidants. This does not apply to S.D. lactose and suggests that other factors are involved. The S.D. lactose had a particle size distribution (Table I) much finer than the other two sample materials, a factor which may influence the effectiveness of glidants. Magnesium stearate and fumed silicon dioxide at their usual concentration ranges did increase the flow rate of both calcium sulfate granules and aspirin crystals. Cornstarch, at low levels, increased the flow rate of the same two materials but decreased the flow rate at concentrations normally employed. Talc appears to be a poor glidant for these sample materials since it decreased the flow rate at all concentration levels. The talc used in this study was designated as having "good slip," a designation determined subjectively by rubbing the material between the thumb and fingers. It is evident that a glidant which is effective with a given material may prove otherwise when used with a different material. The flowometer appears to be a convenient tool to determine the best glidant for specific material more objectively. This technique also appears to be valuable to ascertain the optimum concentration of a particular glidant. In those instances where the glidant increased the flow rate, the lower concentration was usually more effective. Two exceptions were magnesium stearate with calcium sulfate granules and silicon dioxide with aspirin crystals. The indiscriminate use of glidants should be avoided since beyond their optimum concentration they usually cause a marked decrease in flow rate.

In evaluating the effect of glidants by angle of repose measurement, an increase in magnitude of the angle should be associated with a decrease in flow rate and conversely, a decrease in the angle should result in an increased flow rate. This relationship seems to exist within the three talc-containing systems which were studied, as illustrated in Fig. 1. However, the angles of repose for both the calcium sulfate and aspirin series with talc were much higher than those for the S.D. lactose–talc series, yet the former two series had much faster flow rates. Figure 2 illustrates the relationship in the three systems containing magnesium stearate. The angle of repose did correlate generally with flow rate within these systems, although again no correlation between the three series was evident, and a marked drop in flow rate for aspirin–magnesium stearate was accompanied only by a negligible change in angle of repose. Figures 3 and 4, illustrating the three systems with silicon dioxide and cornstarch, respectively, indicate many exceptions to the angle of repose-flow rate theory. In many instances a decrease in the angle of repose was not associated with an increased flow rate, and marked changes in

TABLE III.—ANGLE OF REPOSE AND FLOW RATE FOR SELECTED RAW MATERIALS

Material	Classification[a]	Angle of Repose, Degree	Flow Rate, Gm./sec.
Acetaminophen	Coarse	37.00	11.20
Ascorbic acid	Coarse	41.92	11.26
Aspirin crystals	Coarse	40.35	11.17
Aspirin crystals	Moderately coarse	43.35	7.78
Aspirin granulation	Coarse	39.60	10.79
Aspirin (10% starch)	Very coarse	40.73	11.20
Calcium sulfate, dihyd.	Coarse	41.63	8.42
Dextrose, anhyd.	Coarse	34.62	18.90
S.D. lactose	Fine	33.08	8.16
Mannitol	Very coarse	41.02	8.10
Sodium ascorbate	Moderately coarse	41.92	9.91
Sorbitol	Fine	37.42	7.09
Sugar	Fine	38.53	14.24
Tartaric acid	Coarse	39.33	16.84
Thiamine mononitrate	Moderately coarse	38.18	13.72

[a] U.S.P. XVII classification of powders by fineness.

TABLE IV.—CORRELATION COEFFICIENT AND PER CENT DEPENDENCE SHOWING RELATIONSHIP BETWEEN ANGLE OF REPOSE AND FLOW RATE

	Coefficient Correlation, r	% Dependence
Glidants with aspirin	−0.49	24.68
Glidants with calcium sulfate	−0.74	54.43
Glidants with S.D. lactose	−0.62	38.27
Selected raw materials	−0.32	10.33

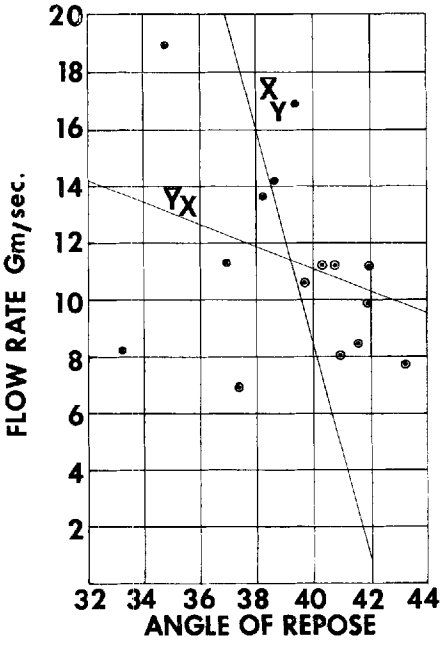

Fig. 5.—Relationship of angle of repose to flow rate for selected raw materials. Key: $\bar{X}_y$, regression line of the angle of repose on flow rate, $\bar{Y}_x$ regression line of the flow rate on the angle of repose.

the flow rate were not detectable by changes in the angle of repose.

Measurement of the angle of repose of a powder has been reported to yield information on flowability in comparison to that of other powders (2-4). Since this current work concerning the addition of various glidants to the three systems had cast considerable doubt on this relationship, the flow rate and angle of repose of 15 selected raw materials were determined. The results are shown in Table III. The materials represent wide ranges in angles of repose and flow rates. The coefficient of correlation (13) has been used to statistically measure the extent of the linear relationship between angle of repose and flow rate. A correlation coefficient close to 0 indicates a weak or nonexistent relationship, whereas a value close to +1 or −1 indicates a close relationship between the two variables. The per cent dependence (14), a measure of the percentage of the variation in flow rate related to variation in the angle of repose, has also been calculated. These statistics were used to evaluate the angle of repose–flow rate relationship for the aspirin, calcium sulfate, and S.D. lactose glidant series. Results are given in Table IV. Coefficients of correlation for the calcium sulfate and S.D. lactose systems are significant or high enough to assume some correlation between the two variables. However, the per cent dependence of these two systems are not sufficient to have high or reliable correlation. Correlation between the selected raw materials was poor. This is illustrated in Fig. 5. The difference in regression lines is indicative of the weak relationship between angle of repose and flow rate (r = −0.32). Although still not high, correlation was better within a given system. The over-all low correlation coefficients and corresponding per cent dependence values indicate that the angle of repose is not a reliable method to evaluate flow of materials.

SUMMARY

1. The effects of varying concentrations of the glidants, magnesium stearate, fumed silicon dioxide, cornstarch, and talc, on the flow rate of aspirin crystals, calcium sulfate granules, and S.D. lactose have been investigated with a recording powder flowometer. The angle of repose measurement was also used in a further attempt to evaluate flow and to investigate the relationship between this angle and flow rate.

2. The flowometer appears to be a valuable instrument to select the best glidant for a given material or granulation and to determine the optimum concentration of a particular glidant in a given system.

3. Magnesium stearate, fumed silicon dioxide, and cornstarch in low concentrations increased the flow rates of aspirin and calcium sulfate. However, effectiveness of the glidants with S.D. lactose was negligible. Talc appears to be a poor glidant for these materials since it decreased the flow rate at all concentration levels.

4. Optimum concentrations of glidants were 1% or lower. Excess of glidants should be avoided since beyond their optimum concentration they usually caused a marked decrease in flow rate.

5. Statistical tests were used to determine the relationship between angle of repose and flow rate. Low correlation was obtained within a specific system, whereas no correlation was found for selected raw materials. Consequently, angle of repose measurement does not appear to be a reliable method to evaluate flow of materials.

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Evaluation of Automatic Potentiometric Titrator in Nonaqueous Titrations

By THOMAS MEDWICK and ELY KIRSCHNER

A procedure is reported for the evaluation of the behavior of an automatic recording titrator in nonaqueous titrations and is applied to an instrument composed of commercially available units. First, the behavior of the purely instrumental features was determined and the experimental errors were compared with calculated maximum anticipated relative values. The instrument's function in this case was within expectation. Then, the response of the titrator was evaluated from titration curve properties of two compounds in acetic acid and two other compounds in isopropanol. The following maximum relative errors, with instrumental errors discounted, were observed: range, 1.5 per cent; end point potential, 4.4 per cent; analytical results, 1.0 per cent; slope of the rapidly rising portion, 11.4 per cent. On the basis of satisfactory instrumental response and reasonable titration curve errors, the relatively inexpensive unit used here is found reliable for general laboratory use. The procedure described is recommended for use in evaluating any automatic potentiometric recorder.

TITRIMETRIC analysis in various nonaqueous media enjoys success and acceptance. The biannual reviews of the literature pertaining to this work attest to this fact (1). Inspection of the official compendia (2, 3) indicates that this type of determination is useful in pharmaceutical analysis. If potentiometric end point detection is required, the use of a dependable automatic recording titrator permits convenient and rapid analyses. It is possible to build a titrator from published schematics, but this involves expense and considerable labor. Several varieties of commercial automatic recording titrators are available or, if cost is a consideration, commercial components may be simply assembled. If not already available in the laboratory, the required components are a potentiometric recorder, a suitable automatic buret, and a pH meter. No matter which of these instruments is chosen, proper evaluation of the automatic recording titrator setup should be carried out to determine performance under the specific conditions to be imposed during use.

This paper reports on a detailed evaluation procedure which examines titrimetric performance in nonaqueous media where high solution resistance makes potential measurements difficult; the titrimetric behavior of two compounds in glacial acetic acid solvent and two other compounds in isopropanol is studied. Since titration behavior yields useful acid-base information as well as purely analytical data, various features of the resultant curves are measured. In addition, the known determinate errors inherent in the various measurements are estimated in an attempt to evaluate the ability of the described instrumentation to present data reproducibly. The automatic recording potentiometric titrator used is composed of commercially available components. Wilson and Munk (4) have written about a similar titrator but did not carry out an evaluation of its performance.

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EXPERIMENTAL

Chemicals and Solutions.—All chemicals not otherwise described were reagent grade. 1,2,3-Triphenylguanidine (Eastman yellow label) was recrystallized from 95% aqueous ethanol, m.p. 145–147° uncorrected. *m*-Nitrobenzhydrazide (Eastman white label) was recrystallized from water, m.p. 152–155° uncorrected.

Benzoic acid, primary standard (J. T. Baker) 99.99% assay. *p*-Hydroxybenzoic acid (Eastman white label). Hexadecyltrimethylammonium bromide (Eastman blue label). Acetous perchloric acid, about 0.1 *N*, was prepared according to Fritz (5).

Hexadecyltrimethylammonium hydroxide, about 0.1 *M*, was prepared according to the method described by Cundiff and Markunas (6) with the exception that isopropanol was the only organic solvent used.

Apparatus.—Sargent model SR recorder, 125 mv. full scale, equipped with a 5.0-mv. range plug, a 1 in./min. chart drive motor, and wired with a switching arrangement such that it may be turned on synchronously with the constant rate buret.

Leeds and Northrup pH indicator, model 7401, equipped with a 10 or 20-ohm precision resistor (±0.05%).

Sargent model C constant-rate buret, delivering 10 ml., equipped with a Teflon stopcock and a Teflon sleeve-covered ground-glass connection, and connected to a 2-L. titrant reservoir *via* ball socket joints and glass tubing.

Magnetic stirrer, used with Teflon-coated magnetic stirring bars.

Titration vessel, for titrations requiring protection from the atmosphere, consisting of a beaker 7 cm. in height, 7.1 cm. in diameter, with a female

71/15 ground-glass joint, and a cover with a male 71/15 joint, and with four openings to admit the reference electrode, the indicating electrode, the buret tip, and a nitrogen gas tube.

For titrations in both acetic acid and isopropanol solvents, a Beckman glass electrode (40498) was used as the indicating electrode. The reference electrode used for acetic acid was a Beckman inverted sleeve calomel electrode (43462), the saturated aqueous KCl solution being replaced by 0.1 *M* acetous LiCl; for isopropanol, the same type of calomel electrode was employed but with 0.1 *M* hexadecyltrimethylammonium bromide in isopropanol replacing the saturated aqueous KCl.

Procedure.—A sample of the compound to be titrated, listed in Table I, column 1, was accurately weighed (column 2) and dissolved in 50 ml. of the proper solvent (column 3). When acetic acid solvent was used, a 400-ml. beaker was used as the titration vessel. However, when isopropanol was to be the solvent, the titration vessel was the cell previously described which provided for the use of nitrogen as an inert atmosphere. After the proper electrodes were immersed and the stirrer turned on, the solution was automatically titrated with the specified titrant (column 4) until the total volume of about 10 ml. was delivered by the buret.

The following electrical grounding arrangement was used. The negative input terminal of the recorder was grounded to its chassis and was also connected to the "ground" terminal of the pH meter. The "ground" terminal of the pH meter, the automatic constant-rate buret chassis, and the magnetic stirrer chassis were all connected to a common water pipe ground. No titration solution ground was used.

Measurement of Titration Curve Parameter.—The recorded titration curves are plots of millivoltage (ordinate) as a function of titrant volume (abscissa). For each titration curve, the following measurements as shown in Fig. 1 were made using a metric ruler: the length of chart representing the complete titration, distance X; the voltage represented by a centimeter of abscissa distance in terms of the voltage equivalent to distance Y divided by distance Y; the range, in millivolts, or distance AB; the potential at the end point, point C; the slope of the rapidly rising linear portion or the tangent of angle ADE; the end point according to the method described by Blaedel and Meloche (7). In addition, determinate errors involved in the measurement of graphical features and found in the instrumental specifications were treated according to the usual concepts of error propagation (8).

RESULTS AND DISCUSSION

An automatic recording titrator setup may be conveniently divided into two parts: (*a*) the instrumental portion composed of the automatic buret, the pH meter, and the potentiometric recorder; and (*b*) the titration cell portion containing the sample solution together with the electrodes. If the instrumental portion is found to be functioning correctly and reproducibly, then it can properly receive and represent the signal being produced by the titration cell portion. On this basis, the two sections which follow deal first with the instrumental operation and behavior, and second, discuss the entire instrument, including, of course, the titration cell portion.

Instrumental Operation and Behavior.—The components of this titrator were chosen with the aim of achieving flexibility. The recorder is one with a large chart (25 cm.), variable ranges, and variable chart speeds. However, any good potentiometric recorder may be adapted. Similarly, any good pH meter, with a recorder output, may be used. The pH meter chosen presented such advantages as stability, ease of variation of the output signal *via* precision resistors (9), and adjustability of the "zero" millivolts position. This latter advantage is extremely important when titrations are to be carried out using various solvents and different strength acidic and basic samples. The last and only component which need be obtained specifically for this titrator is the automatic constant-rate buret. The one used here was chosen since it could be cleaned easily and was adaptable to an all glass titrant delivery system.

A factor which is extremely important in obtaining instrumental stability, particularly in the titrations involving solvents of low dielectric constant, *e.g.*, acetic acid, is that of electrical grounding. If grounding is not proper then any extraneous signals will be superimposed on the desired response resulting in unsatisfactory, erratic titration curves.

Since the magnitude of a potentiometric "break" is a function of the acid or base strength of the compound being titrated, certain compounds will give rise to a smaller potentiometric break than others. In order to make the recorded titration curves as large as possible and thereby take advantage of the large recorder chart, the sensitivity of the instrument was varied. In all instances, the recorder range was kept at 5.0 mv. and the pH meter operated in the 0–1400 mv. range. However, the pH meter output was varied by using different output resistors (9). Table II, column 2, is a listing of the resistors used in each case while columns 3 and 4 show the anticipated effect of the resistor on the recorder response and instrument sensitivity. It is seen that in the study of *m*-nitrobenzhydrazide, a relatively weak base, the highest instrument sensitivity was used. The sensitivity was not increased when *p*-hydroxybenzoic acid was titrated, in spite of the weakly acidic phenol which is present, in order to titrate both acidic groups within the potential range represented by a single width of recorder chart.

It was desirable to test the reliability of the instrumental performance for every titration. The chart abscissa, in terms of centimeters of chart travelled for a complete titration, is an index of how well the automatic buret motor, the recorder chart drive motor, and the synchronous switch operate. The abscissa mean values and their standard deviation, listed in Table II, column 7, show a maximum relative standard deviation of about 0.2%. The determinate portion of this uncertainty may be ascribed to two sources. The first source, the instrumental error, arises from the automatic constant rate buret motor whose specifications list an accuracy of 0.1% in delivering the total volume (10). The recorder chart motor is not expected to contribute to the determinate error since it is activated *via* a synchronous switch and shuts off

TABLE I.—TITRATION BEHAVIOR

1	2	3	4	5	6	7	8	9
Compd.	Sample Size, meq.	Sample Solvent	Titrant	Samples, No.	Range, mv.	End point Potential, mv.	Slope, mv./ml.	Analytical Result
1,2,3-Triphenylguanidine	0.545 ± 0.009	Acetic acid	Acetous $HClO_4$	5	304, S = 4[b] (1.3%)[c]	−585, S = 7 (0.85%)	1512, S = 199 (13.2%)	N = 0.1059 S = 0.0003
m-Nitrobenzhydrazide	0.571 ± 0.009	Acetic acid	Acetous $HClO_4$	4	201, S = 6 (3.0%)	−668, S = 4 (0.30%)	306, S = 19 (6.2%)	99.8% S = 0.5
Benzoic acid	0.497 ± 0.002	Isopropanol	$HDTMA^+OH^-$ in isopropanol[a]	5	433, S = 5 (1.2%)	+286, S = 19 (5.9%)	3050, S = 457 (15.0%)	N = 0.0731 S = 0.0002
p-Hydroxybenzoic acid (as carboxylic acid)	0.322 ± 0.002	Isopropanol	$HDTMA^+OH^-$ in isopropanol	4	163.5, S = 0.7 (0.43%)	+222, S = 8 (2.7%)	314, S = 16 (5.1%)	98.0% S = 1.0
p-Hydroxybenzoic acid (as phenol)	0.322 ± 0.002	Isopropanol	$HDTMA^+OH^-$ in isopropanol	4	111, S = 2 (1.8%)	+465, S = 9 (1.5%)	183, S = 14 (7.7%)	98.5% S = 0.9

[a] $HDTMA^+OH^-$ is the abbreviation of hexadecyltrimethylammonium hydroxide. [b] In each instance, S indicates the standard deviation. [c] The figures in the parentheses are relative standard deviations.

automatically when the complete titrant volume is delivered (11). The second source of error stems from the chart measurements using a metric ruler. Distance measurements made with a metric ruler are no more accurate than about 0.02 cm. which in a distance of about 26 cm. represents 0.1% relative uncertainty. Thus, from these two sources, 0.2% total relative determinate uncertainty are calculated. This is in good agreement with the 0.2% observed uncertainty.

A second criterion for instrumental performance is the reproducibility of the ordinate, in terms of mv./cm. of chart. This axis is influenced by the reliability of the meter needle (since points on the chart are marked to indicate a particular voltage reading) as well as the ability of the recorder to reproduce the same reading. The uncertainty of the entire meter scale reading is given as 10 mv. and the visual reading error is ±2 mv. (12). Thus, in the situation where the entire scale is equivalent to 700 mv., a maximum error of ±12 mv. in 700 mv. or 1.7% relative uncertainty could be recognized. The recorder is accurate to 0.25% (13) which is added to the meter relative uncertainty to yield a total of 1.95%. Finally, the metric ruler uncertainty of 0.02 cm. in a distance of approximately 25 cm. would introduce a variation of about 0.1%. Since the ordinate is expressed as a quotient, the anticipated relative determinate error is calculated to be about 1.85%. The values presented in Table II, column 6, are seen to have a maximum relative standard deviation of about 1%. This is within the calculated maximum anticipated determinate uncertainty. It should also be mentioned that the values in column 6 are all in agreement within experimental error with the anticipated values for the instrument sensitivity noted in column 4.

Titration Behavior.—In this section the ability of the entire system to sense, transmit, and reproducibly record the course of a titration in two nonaqueous media is treated. The determinate errors attributable to instrumental and measurement factors are estimated. No determinate error is assigned to the titration cell function. The four characteristics of the titration curves are listed in Table I, columns 6–9. Some variation in the concentration dependent measurements should be expected since the sample weights (column 2) are not exactly constant. Since the dilution during titration is the same for each case, no corrections are carried out.

The range values listed in Table I, column 6, are reproducible with a maximum relative standard deviation of 3%. This value is calculated from the curve by multiplying a distance (absolute uncertainty 0.02 cm.) by the proper instrument sensitivity value, Table II, column 6. For a small distance, *e.g.*, 5 cm., a relative measurement error of 0.4% may be added to a maximum sensitivity error of 1.1% to yield a total determinate relative error of 1.5%. When the individual values presented in Table I, column 6, are examined, it is noted that three of the values are within the maximum anticipated error limits, whereas one of the remaining two is 0.3% and the second is 1.5% higher than the calculated limit. It should be noted that in the last-named case the sample size varies about 1.6%. This variation would be expected to have an effect

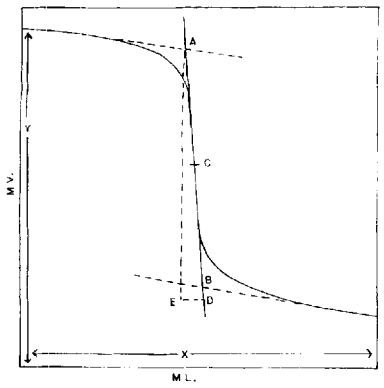

Fig. 1.—Titration curve features. Key: X, milliliters; Y, millivolts; AB, range; C, end point potential; tangent of ADE, slope.

on the range values. However, on the basis of the observed results, the titrator is able to reproduce the range value with a maximum anticipated uncertainty of 1.5% when the measurement and instrumental errors have been discounted.

The calculation of the value for the end point potential is carried out by measuring a linear distance, multiplying this distance by the recorder sensitivity, and adding the product to a reference potential on the chart. The maximum anticipated determinate error is about the same as that calculated for the range values, *viz.*, 1.5%. However, the use of a reference potential on the chart introduces an absolute uncertainty of 2 mv. in values based on this potential. In order to account for this factor, 2 mv. have been subtracted from each standard deviation (S) value reported in Table I, column 7, before the relative standard deviation is calculated. Of the 5 values reported in Table I, column 7, those for acetic acid solution are most reliable since the hydrogen-ion concentration and presumably the electrochemical potential at the end point when bases are titrated in acetic acid has been found to be independent of concentration (14). The values for 1,2,3-triphenylguanidine and *m*-nitrobenzhydrazide in acetic acid solution indicate that these potentials are reproducible within expected limits. The relative errors of the values determined in isopropanol solution are higher than the calculated limit. On the basis of the observed values, the titrator reproduces the end point potential with a maximum uncertainty of 4.4% when the measurement and instrumental errors have been discounted.

Another indication of the ability of this system to reproduce results is the analytical data which are presented in Table I, column 9. It is noted that both titrants have been standardized to values precise to about 0.3%. This is within expectations for a volumetric method. The recoveries as listed in column 9 of the other two compounds have a maximum standard deviation of 1.0%. This demonstrates that the instrument is able to yield analytical data and 1.0% maximum uncertainty.

The last criterion chosen to evaluate the behavior of the system is the slope of the rapidly rising portion of the curve. This property is also known as the sharpness index (15). The values presented in Table I, column 8, were obtained by measuring the tangent of angle ADE, Fig. 1. The calculations are carried out according to the following equation:

$$\text{tan. ADE} = \frac{(\text{AE})\ (\text{sensitivity})}{(\text{DE})}$$

The estimate of the relative errors transmitted to the final slope value was found to be a maximum of 3.6% in the case of benzoic acid. The observed relative standard deviations are larger than the estimated relative errors. On the basis of these data, the slope is reproduced with a maximum uncertainty of 11.4% (15.0–3.6) when the measurement and instrumental errors have been discounted.

The value of 11.4% which has been calculated to be the maximum uncertainty of the slope may be composed of unknown determinate as well as random errors. Blaedel and Meloche (16) discuss end point errors and particularly the random error involved in selecting the inflection point. These authors make the following statements: "In locating the inflection point, the standard error appears to be about 10% of the volume interval covered by the straight-line portion of the experimental titration curve. This figure of 10% is a rough estimate based on experience, and has no theoretical basis." The "volume interval covered by the straight-line portion" mentioned corresponds to the line DE of Fig. 1. Since DE is used in the slope calculation, its random error will be propagated according to usual concepts which dictate that the squares of the relative standard deviations

Table II.—Instrumental Performance

1 Compd.	2 Resistor at pH Meter Recorder Output, ohms ±0.05%	3 Recorder Response, Full Scale (25 cm.), mv.	4 Instrument Sensitivity Calcd., mv./cm.	5 Trials, No.	6 Instrument Sensitivity, Exptl., mv./cm.	7 Chart Travel, cm.
1,2,3-Triphenylguanidine	10	700	28.0	5	27.8, S^a = 0.3 (1.1%)[b]	26.20, S^a = 0.04 (0.15%)[b]
m-Nitrobenzhydrazide	20	350	14.0	4	13.95, S = 0.07 (0.5%)	26.16, S = 0.05 (0.19%)
Benzoic acid	10	700	28.0	5	27.9, S = 0.1 (0.4%)	26.26, S = 0.06 (0.23%)
p-Hydroxybenzoic acid	10	700	28.0	4	27.8, S = 0.2 (0.7%)	26.30, S = 0.00 (—)

[a] In each instance, S indicates the standard deviation. [b] The figures in the parentheses are relative standard deviations.

of the elements in a quotient are summed and the square root is extracted to yield the relative standard deviation of the quotient (8). When this is done with the 10% uncertainty mentioned previously, a relative random error of at least 10% is expected in the result. On this basis, only 1.4% of the original 11.4% remains unexplained. This percentage may very well be random error in AE for which no estimate is available.

During the rapidly rising portion of the curve, the sensing and recording of potentials is most difficult since the potential is changing very rapidly. Factors such as speed of recorder response, efficiency of stirring, and rate of titrant addition are all involved in this measurement. It is possible to obtain an electronic setup whereby the rate of titrant addition is slowed as a function of the rate of change of potential. This may improve the reproducibility of the slope. However, this feedback mechanism substantially increases the cost of the instrument. Commercial titrators offer this advantageous feature.

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2. The procedure consists of an instrument evaluation followed by an examination of titration curve properties. The range, end point potential, analytical results, and the slope of the rapidly rising portion of each curve are determined.

3. The maximum anticipated relative errors are calculated and compared with experimental results obtained from a relatively inexpensive titrator composed of commercially available units.

4. On the basis of satisfactory instrumental response and of reasonable titration curve errors, the titrator used here is found to be reliable for general laboratory use.

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dienyl)-1,4-benzoquinone] (8), thelephoric acid [2,3,8,9-tetrahydroxybenzobis(1,2-*b*, 4, 5-*b'*)-benzofuran-6,12-quinone] (9–14), and polyporic acid (15–21), in addition to atromentin (4, 22, 23), have been isolated from certain basidiomycetes belonging to the genera *Amanita, Cantharellus, Hydnellum, Hydnum, Lopharia, Paxillus, Polyporus,* and *Thelephora.* The *Hydnaceae* appear to be a particularly rich source of the diphenylbenzoquinone compounds with *Hydnellum, Hydnum,* and *Lopharia* species yielding atromentin, aurantiacin, polyporic acid, and thelephoric acid. *Hydnellum caeruleum* (Pers.) Karst. is known to lack anticoagulant properties (5), but the species has never been investigated chemically. When a quantity of this species became available, it was considered desirable to examine the fungus for diphenylbenzoquinone constituents.

EXPERIMENTAL AND RESULTS

Material and Extraction Procedure.—Carpophores of *H. caeruleum* were collected, cleaned, and dried in a forced-air drying oven at 48° for a minimum of 72 hr. Ninety-three grams of a 40-mesh powder of the whole mushroom was extracted exhaustively with successive portions of petroleum ether, diethyl ether, chloroform, and 95% ethanol in a Soxhlet apparatus. An orange-red crystalline precipitate (6.33 Gm.) separated from the ether solution during extraction and was removed by filtration prior to evaporation of the solvent. Gripenberg (6) had isolated aurantiacin from a similar precipitate obtained upon ether extraction of *Hydnellum aurantiacum* (Fr.) Karst. (*Hydnum aurantiacum* Batsch); consequently, this precipitate was selected as the first fraction to be examined.

Chromatographic Evaluation of the Crude Precipitate.—Chromatography was potentially the most convenient way to obtain a preliminary evaluation of the various extracts, but no chromatographic procedures have been reported for the separation of diphenylbenzoquinone compounds. Thus, a solution of the precipitate from the ether extract was examined using a variety of adsorbents and solvent systems to determine if it could be resolved readily. Maximum resolution of the components in the crude precipitate was obtained on a layer of Silica Gel G with a benzene–ethyl acetate–glacial acetic acid (75:24:1) solvent system. A mixture of 20 Gm. of Silica Gel G and 40 ml. of distilled water was shaken for 1 min. and spread on 20 × 20 cm. glass plates in a uniform 250-μ layer. The plates were allowed to stand for 10 min., heated at 105–110° for 30 min., and cooled. The crude material was applied to the chromatograms, and they were formed ascendingly using the solvent mixture. Some material remained at the point of origin; the other components separate into five chromatographic zones, two of which developed latent colors upon standing at room temperature for 24–48 hr. The actual migration of the zones on the chromatographic layers was observed to vary slightly with the quantity of material spotted and with different batches of chromatographic plates. However, the relative position of the zones was always the same, and representative R_f values are as follows: 0.33, yellow-orange; 0.24, latent lavender-brown; 0.17, yellow-brown; 0.13, latent gray; 0.10, slate gray. The ether filtrate which remained after separation of the precipitate exhibited the same qualitative composition upon chromatography. Examination of available diphenylbenzoquinones revealed that atromentin and polyporic acid remained at the point of origin under these chromatographic conditions, whereas 2,5-diphenyl-1,4-benzoquinone had an R_f value of 0.63.

Isolation of the Major Component of the Crude Precipitate.—Gripenberg (6) utilized a column of acid-washed alumina in his isolation of aurantiacin, but the results obtained with thin-layer chromatography and with preliminary columns suggested that the use of silica gel permitted more effective separation of the components of *H. caeruleum.* A mixture of silica gel (100–200 mesh) and flux calcined diatomaceous silica[1] (75:1) was suspended in benzene and transferred to an 18-mm. diameter extrusion-type chromatographic column to give a 30.5-cm. column of adsorbent. A sample of the crude precipitate (1.3 Gm.) was dissolved in a minimum volume of dioxane and adsorbed on a small quantity of the silica gel–flux calcined diatomaceous silica[1] mixture. The dioxane was evaporated at 50°, and the adsorbed material was added to the top of the chromatographic column. The column was washed successively with approximately 2000 ml. of benzene–ethyl acetate (9:1), 1850 ml. of benzene–ethyl acetate (1:1), 150 ml. of ethyl acetate, and 400 ml. of ethyl acetate–glacial acetic acid (20:1). The eluate was collected in approximately 50-ml. fractions, and 25–50-μl. portions of each fraction were examined using the thin-layer chromatographic procedure. The first 200 ml. of eluate contained no pigmented compounds, and the next 900 ml. were chromatographically homogeneous (R_f 0.33). Thin-layer chromatography revealed that the compound with R_f 0.33 was the major component of the crude precipitate; it was detected in all subsequent fractions of the eluate. The eluates containing only the one compound (R_f 0.33) were combined, the solvent was evaporated in a stream of air at room temperature, and the residue was recrystallized 3 times from dioxane. The solvent of crystallization was removed by heating in a drying pistol over phosphorus pentoxide at 146° *in vacuo* for 120 hr.

Isolation of the orange-red crystalline material using the column chromatographic procedure was tedious and relatively inefficient. Consequently, an alternate procedure which employed selective solubilization of the accompanying impurities was developed. A sample of the crude precipitate (2.5 Gm.) was stirred with successive 50-ml. portions of methanol, and the supernatant solutions were examined using thin-layer chromatography. After washing the crude precipitate with 5 portions of methanol, the remaining residue was chromatographically homogeneous (R_f 0.33). When this material was recrystallized from dioxane and dried, it was indistinguishable from the crystalline substance obtained by column chromatography.

Preliminary Identification of the Isolated Material.—Qualitative elemental analysis following sodium fusion revealed the absence of nitrogen, sulfur, and halogens in the molecule. The infrared spectrum of the compound in a potassium bromide pellet using a Beckman infrared spectrophotometer, model IR5A, strongly suggested the presence of an

[1] Marketed as Hyflo Super Cel by the Johns-Manville Corp.

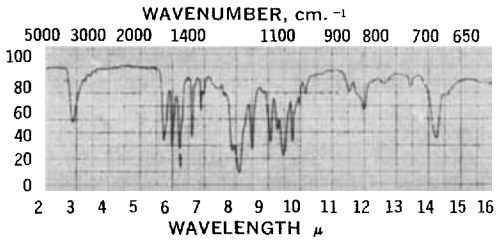

Fig. 1.—Infrared spectrum of isolated aurantiacin.

ester carbonyl group (1748 cm.$^{-1}$) and a quinone carbonyl function (1665 cm.$^{-1}$). The balance of the infrared spectrum (Fig. 1) was consistent with that anticipated for a substituted diphenylbenzoquinone compound (24), and the micro m.p., K., of 285–295°, agreed with the melting point reported for aurantiacin (6).

Visible and ultraviolet absorption spectra are useful in distinguishing some diphenylbenzoquinones (25). Such spectra were obtained by dissolving 2.0 mg. of the recrystallized compound in 10 ml. of dioxane, diluting the solution 25-fold, and recording the absorption with a Beckman spectrophotometer, model DB. The absorption maxima obtained at 240 mμ (log ϵ = 4.71) and 405 mμ (log ϵ = 3.94) were in agreement with the values 240 mμ (log ϵ = 4.63) and 405 mμ (log ϵ = 3.82) reported for aurantiacin.

Degradation of the Isolated Material.—Preliminary data suggested that the isolated material was aurantiacin, a compound which can be decomposed to atromentin and benzoic acid (6). A small sample of the crystals (100 mg.) was dissolved in 50 ml. of 2 *N* sodium hydroxide solution. The alkaline solution was acidified with concentrated hydrochloric acid and partitioned with three successive 50-ml. portions of ether. The ether solution was extracted with three 50-ml. portions of saturated sodium bicarbonate solution. Acidification of the bicarbonate solution gave a brown precipitate which was separated by filtration and washed with benzene. The mother liquor from the filtration was extracted with ether, the ether was removed by evaporation, and the residue was washed with benzene. The corresponding benzene-soluble and insoluble portions of the precipitate and residue were combined.

The benzene solution was evaporated to dryness, and the residue was sublimed under reduced pressure. Micro m.p., K., of the sublimate was 120–121°. An admixture with authentic benzoic acid showed no depression in melting point.

The benzene–insoluble material was recrystallized from methanol and dried *in vacuo* over phosphorus pentoxide at 146° for 120 hr. It decomposed without melting at about 303–305°. A dioxane solution of the material (1 mg. dissolved in 10 ml., then diluted 25-fold) had absorption maxima at 268 mμ (log ϵ = 4.51) and 384 mμ (log ϵ = 3.73); these results agreed with the values 268 mμ (log ϵ = 4.55) and 385 mμ (log ϵ = 3.66) reported for atromentin (25).

DISCUSSION AND CONCLUSIONS

Extraction and fractionation procedures were utilized to investigate the occurrence of diphenylbenzoquinone compounds in carpophores of *H. caerulum*. An orange-red crystalline compound was isolated from a precipitate that separated from the ether extract. The melting point and spectral properties (infrared, visible, and ultraviolet) of the isolated compound corresponded to those of aurantiacin. This identification was confirmed by alkaline degradation of the material to atromentin and benzoic acid.

The isolation of aurantiacin from *H. caeruleum* marks the second reported occurrence of this compound in nature and the first since its original isolation from *H. aurantiacum* (6). The genus *Hydnellum* is a reasonably well-defined taxon which is characterized by a tough fibrous or leathery context (26). Major morphologic criteria for the separation of species within the genus are the presence or absence of clamp connections in the hyphae of the pileal trama and the presence or absence of incrusting granules in the tramal hyphae. *H. aurantiacum* has incrusting granules and no clamp connections; *H. caeruleum*, the antipode, has clamp connections and no granules. Thus, morphologic considerations suggest that the two species are not as closely related as could be predicted on the basis of aurantiacin accumulation. However, there is no available evidence which permits the assignment of a greater or less phylogenetic significance to chemotaxonomic indicators than to morphologic relationships.

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Effect of Antitumor Antibiotics and Antimetabolites on Rat Diaphragm Carbohydrate Metabolism

By LEON L. GERSHBEIN

Rat hemidiaphragms have been incubated with antitumor antibiotics and antimetabolites in a phosphate–saline medium containing 120 mg. per cent glucose and the changes in oxygen uptake, hexose utilization, and glycogen turnover ascertained. Aminopterin (0.40 mg.), triethylenemelamine (0.40 mg.), and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (50 mcg.) caused a decrease in glycogen content; the latter two as well as chlorambucil and 8-azaguanine, both screened down to 10 mcg., depressed glucose utilization. Of the antibiotics, glycogenolysis occurred in the presence of tubercidin (0.50 mg.), antimycin D (0.75 mg.), streptonigrin (50 mcg.), and antimycin A (0.25 mg., suspension). Muscle glucose uptake was depressed in the presence of more physiologically significant levels of puromycin, tubercidin, streptonigrin, duazomycins A and B, and actinogan and with antimycin A (0.25 mg.); tylosin was effective in this regard at 1.00 mg. Diaphragm Q_{O_2} was depressed by 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (50 mcg.), 8-azaadenine (0.25 mg.), and 0.50 mg. each of streptonigrin, E73 base, glutinosin, psicofuranine, and actinogan and was elevated by porfiromycin (0.50 mg.).

IN RECENT studies by this laboratory, the effect of a variety of antibiotics was ascertained on the isolated rat diaphragm (1). Of these, albamycin decreased both glucose uptake and glycogen content at concentrations to 30 mcg. and at even lower levels, hexose utilization was depressed, as was also the case with chloramphenicol and kanamycin. In the present investigation, these criteria were applied to antitumor antimetabolites and antibiotics. The agents, tylosin and capreomycin, were also included as well as a cytotoxic sterol, kethoxal-bis(thiosemicarbazone), and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide. The last compound is a streptomycin antagonist, inhibiting NADH and succinate oxidation by mitochondria (2–4).

EXPERIMENTAL

Antitumor antibiotics were generously supplied by several companies, the sources and code designations of which appear in Table I. The antimetabolites of high purity originated from commercial sources.[1] The agents were dissolved in 0.85% saline and for acids and water-insoluble aza-derivatives, solution was first effected with aqueous NaOH and then further diluted to the desired concentration with saline; all mixtures were prepared fresh prior to use. For the incubation of the hemidiaphragms, the medium of Stadie and Zapp (11) was used double strength (final concentration: 0.04 *M* Na_2HPO_4, 0.005 *M* $MgCl_2.5H_2O$, and 0.08 *M* NaCl, pH 6.8–7.0; the glucose level was 120 mg. %).

The removal of hemidiaphragms from male Holtzman rats weighing 135–165 Gm., starved for 24 hr., and their processing as well as the incubation and analytical procedures were identical with those described in previous reports (12–14). By the method of paired hemidiaphragms, one tissue was incubated with 1 ml. double strength medium + 1 ml. saline–drug solution, and the other hemidiaphragm, with 1 ml. medium + 1.0 ml. saline (control); the requisite flasks without muscle were also employed in each run. Incubation was carried out under oxygen in a Warburg apparatus at 37° for 1 hr., after which time the rinsed hemidiaphragms were hydrolyzed with 30% KOH and analyzed for glycogen (15–17); glucose in the incubation fluids was determined following dilution and deproteinization (18, 19).

RESULTS AND DISCUSSION

Average differences in Q_{O_2}, glucose uptake, and glycogen content for hemidiaphragms incubated with antimetabolites and antibiotics together with the standard errors and the Fisher *t* values in the comparisons appear in Table I. Since no experimental design was followed, a statistical method was applied to the bulk data; differences in excess of $\bar{R} \pm 2.5\ \bar{R}$, $\bar{R}$ being the average range, were discarded (20). As small to slight amounts of glutinosin (0.50 mg.), porfiromycin (0.50 mg.), streptimidone (0.50 mg.), and actinomycin D (0.75 mg.) and even larger portions of chlorambucil (0.50 mg.), tubercidin, antimycin A, and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (0.050 mg.) remained undissolved, fine suspensions were used in each instance.

The mean differences were not statistically significant for the following, the values in parentheses denoting the highest levels (mg.) screened: methotrexate (0.40), thio-tepa (0.40), 6-mercaptopurine (0.25), cyclophosphamide (0.40), 5-fluorouracil (1.00), 8-azaxanthine (0.25), 6-azathymine (0.25), 6-azauracil (0.25), 7-azatryptophan (0.050), 4-azaleucine·2HCl (1.00), cycloleucine (2.00), carzinophilin (400 u.), cycloserine (1.00), mitomycin C (0.10), kethoxal-bis(thiosemicarbazone) (0.50), the cytotoxic steroid (0.050), capreomycin disulfate (1.00), actino-

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[1] Thio-tepa, triethylenemelamine (trademark, TEM), aminopterin, and methotrexate were obtained from Lederle Laboratories; the antiviral and antitumor agent, kethoxal-bis(thiosemicarbazone) [(5); derivative of β-ethoxy-α-ketobutraldehyde], the cytotoxic sterol, γ-lactone of 16-β-hydroxy-3,11-dioxopregna-4,17(20)-dien-21-oic acid (6) from the Upjohn Co.; cyclophosphamide (trademark, Cytoxan) from Mead Johnson Laboratories; capreomycin disulfate (750 mcg./mg. solids) and tylosin from Eli Lilly & Co.; chlorambucil (trademark, Leukeran) from Burroughs Wellcome & Co.; 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide, 8-azaadenine, and 8-azaguanine in addition to the antibiotic, antimycin A, from Sigma Chemical Co.; 5-fluorouracil from Roche Laboratories and 6-mercaptopurine, 6-azathymine, 6-azauracil, 7-azatryptophan, cycloserine (D-4-amino-3-isoxazolidone), the antitumor agent and methionine antagonist, cycloleucine [1-aminocyclopentanecarboxylic acid; (7–9)]; and a competitive antagonist of leucine utilization in some bacteria, 4-azaleucine hydrochloride [2-amino-3-dimethylaminopropanoic acid · 2HCl; (10)] from Nutritional Biochemicals Corp. The latter was also the source of the antibiotics, puromycin hydrochloride, carzinophilin, and mitomycin C.

TABLE I.—AVERAGE DIFFERENCES IN OXYGEN UPTAKE, GLUCOSE UTILIZATION, AND GLYCOGEN CONTENT OF HEMIDIAPHRAGMS INCUBATED WITH ANTITUMOR AGENTS[a,b]

Agent (mg.; Code No.)[c,d]	Mean Difference in Q_{O_2} μl./mg. Wet Tissue/hr.	t	Mean Glucose Difference[e] mcg./mg. Wet Tissue/hr.	t	Mean Glycogen Difference mcg./mg. Wet Tissue/hr.	t
Drugs						
Sodium aminopterin (0.40)	0.05 ± 0.057 (22)	0.81	0.11 ± 0.346 (19)	0.33	0.13 ± 0.060 (22)	2.13[f]
Triethylenemelamine (0.010)	0.00 ± 0.074 (20)	0.01	0.52 ± 0.330 (17)	1.58	0.02 ± 0.097 (20)	0.21
Triethylenemelamine (0.50)	0.02 ± 0.022 (47)	0.81	0.54 ± 0.196 (35)	2.73[g]	0.10 ± 0.044 (46)	2.39
Chlorambucil (0.010)	0.04 ± 0.026 (11)	1.50	1.71 ± 0.387 (12)	4.40[g]	0.00 ± 0.086 (11)	0.01
Chlorambucil (0.10)	0.02 ± 0.043 (24)	0.44	1.59 ± 0.272 (26)	5.80[g]	−0.04 ± 0.087 (24)	0.42
8-Azaguanine (0.010)	0.05 ± 0.056 (21)	0.89	0.86 ± 0.304 (22)	2.83[g]	−0.13 ± 0.063 (23)	2.02
8-Azaguanine (0.050)	−0.06 ± 0.053 (17)		1.38 ± 0.470 (18)	2.90[g]		
8-Azaguanine (0.25)	0.03 ± 0.026 (27)	1.30	1.50 ± 0.346 (28)	4.33[g]	−0.13 ± 0.101 (27)	1.24
8-Azaadenine (0.25)	0.07 ± 0.033 (24)	2.10[f]	0.16 ± 0.424 (23)	0.38	0.01 ± 0.041 (19)	0.23
7-Azatryptophan (0.25)	0.07 ± 0.047 (12)	1.57	0.52 ± 0.189 (11)	2.76[h]	0.04 ± 0.078 (11)	1.50
2-Heptyl-4-hydroxyquinoline-*N*-oxide (0.010)	−0.01 ± 0.044 (12)	0.20	1.11 ± 0.294 (12)	3.76[g]	−0.15 ± 0.271 (11)	0.55
2-Heptyl-4-hydroxyquinoline-*N*-oxide (0.050)	0.38 ± 0.081 (12)	4.70[g]			1.35 ± 0.200 (12)	6.70
Antibiotics						
Puromycin · 2HCl (0.020)	−0.03 ± 0.032 (16)	0.94	0.95 ± 0.374 (15)	2.55	−0.13 ± 0.084 (16)	1.52
Puromycin · 2HCl (0.25)	0.05 ± 0.066 (23)	0.70	1.08 ± 0.346 (23)	3.10[g]	0.02 ± 0.071 (22)	0.30
Tylosin (0.10; EL, 820316)	0.11 ± 0.091 (11)	1.20	−0.16 ± 0.489 (11)	0.33	0.16 ± 0.143 (11)	1.10
Tylosin (1.00)	0.07 ± 0.036 (39)	1.97	0.61 ± 0.212 (32)	2.85[g]	0.07 ± 0.053 (36)	1.40
Actinogan (5.0×10^{-4}; BL, K937)	0.01 ± 0.082 (11)	0.11	1.35 ± 0.520 (12)	2.61[g]	−0.06 ± 0.069 (11)	0.51
Actinogan (0.0010)	0.06 ± 0.052 (32)	1.18	1.33 ± 0.280 (25)	4.75[g]	0.08 ± 0.085 (32)	0.99
Actinogan (0.050)	0.03 ± 0.043 (25)	0.80	1.03 ± 0.224 (23)	4.60[g]	−0.07 ± 0.173 (24)	0.40
Actinogan (0.50)	0.06 ± 0.026(24)	2.21[f]	1.15 ± 0.346 (13)	3.33[g]	0.16 ± 0.080 (23)	2.00
Porfiromycin (0.50; U-14743)	−0.21 ± 0.074 (16)	2.87[g]	0.48 ± 0.050 (15)	1.10	0.08 ± 0.084 (13)	0.90
Tubercidin (0.050; U-10071)	0.02 ± 0.041 (24)	0.45	0.77 ± 0.255 (27)	3.00[g]	0.01 ± 0.094 (24)	0.16
Tubercidin (0.50)	−0.01 ± 0.032 (13)	0.46	0.47 ± 0.208 (14)	2.60[f]	0.10 ± 0.032 (13)	2.85[h]
Psicofuranine (0.50; U-9586)	0.32 ± 0.051 (12)	6.19[g]	0.88 ± 0.728 (8)	1.20	−0.05 ± 0.107 (12)	0.49
E73 base (0.50; PF, 2679-40-3)	0.17 ± 0.067 (9)	2.60[f]	1.16 ± 0.714 (12)	1.60	0.06 ± 0.152 (12)	0.40
Glutinosin (0.50; PF, 5910-272-8)	0.14 ± 0.040 (12)	3.60[g]	−0.35 ± 0.180 (11)	1.90	0.02 ± 0.128 (12)	0.20
Streptonigrin (0.010; PF, 1027-196-1)	−0.01 ± 0.033 (12)	0.30	0.68 ± 0.430 (11)	1.60	0.29 ± 0.140 (12)	2.10
Streptonigrin (0.050)	−0.15 ± 0.081 (11)	1.90	0.59 ± 0.910 (10)	0.60	0.46 ± 0.185 (11)	2.50[f]
Streptonigrin (0.50)	0.38 ± 0.078 (12)	4.90[g]	1.10 ± 0.360 (12)	3.10[g]	0.36 ± 0.129 (11)	2.80[h]
Mithramycin (0.010; PF, 5523-261-1A)	−0.01 ± 0.026 (10)	0.20	0.58 ± 0.208 (12)	2.80[h]		
Mithramycin (0.50)	0.10 ± 0.060 (18)	1.70	1.61 ± 0.360 (18)	4.50[g]	0.11 ± 0.110 (18)	0.99
Duazomycin A (0.010; PF, 5912-215-2)	−0.01 ± 0.032 (12)	0.40	1.23 ± 0.479 (12)	2.56[f]	−0.03 ± 0.047 (11)	0.54
Duazomycin A (0.50)	0.03 ± 0.031 (24)	1.10	0.60 ± 0.220 (24)	2.70[h]	0.02 ± 0.075 (24)	0.20
Duazomycin B (0.050; PF, 5727-249-3)			1.83 ± 0.424 (10)	4.30[g]		
Duazomycin B (0.50)	0.04 ± 0.024 (28)	1.80	1.05 ± 0.220 (26)	4.80[g]	−0.15 ± 0.085 (27)	1.70
Antimycin A (0.25; suspension)	0.09 ± 0.052 (29)	1.65	1.20 ± 0.361 (30)	3.32[g]	0.29 ± 0.073 (30)	3.98[g]

[a] The means (±S.E.) are deduced on the basis of the specified number of paired hemidiaphragms given in the parentheses. [b] A positive mean difference indicates a decrease in response in the presence of the antimetabolite or antibiotic. [c] mg. of agent per Warburg flask in a fluid volume of 2.0 ml. [d] In the codings, BL, EL, MSD, PD, PF, and U refer to Bristol Laboratories, Eli Lilly & Co., Merck Sharp & Dohme, Parke, Davis & Co., Chas. Pfizer & Co. and the Upjohn Co., respectively. [e] The extent of glucose utilization was based on the final concentration of the respective media incubated without diaphragm. [f] $p < 0.05$. [g] $p < 0.01$. [h] $p < 0.02$.

bolin sulfate (0.50, Parke Davis), streptimidone (0.50, Parke Davis), 6-diazo-5-oxo-L-norleucine (0.50, Parke Davis), azaserine (1.00, Parke Davis), pactomycin (0.50, Upjohn), streptovitacin A (0.50, Upjohn), decoyinine (0.50, Upjohn), cycloheximide (0.50, Upjohn), actinomycin P_2 (0.050, Pfizer), roseolic acid (0.50, Pfizer), and netropsin sulfate (0.50, Pfizer).

Of the tumor antimetabolites, decreases in glycogen content occurred only with aminopterin (0.40 mg.), triethylenemelamine (0.40 mg.), and even more pronounced, with 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide (0.050 mg.). The last two agents also caused depressions in glucose uptake as was also noted with chlorambucil (0.010 and 0.10 mg.) and 8-azaguanine screened down to levels of 10 mcg.; the streptomycin antagonist also markedly depressed oxygen uptake. In this regard, similar runs conducted with 8-azaxanthine, 8-azaadenine, and 6-azauracil, each at 0.25 mg., showed them to be essentially without effect, except for a minor decrease in Q_{O_2} with 8-azaadenine. At this dosage, 7-azatryptophan, an agent which can be incorporated into protein (21, 22), lowered glucose utilization, an effect which did not extend to 0.050 mg.

With the antibiotics, puromycin (0.020 and 0.25 mg.) and tubercidin (0.050 and 0.50 mg.), representing the nucleoside types, diminished glucose uptake and at the higher level of tubercidin (7-deaza-adenosine), the glycogen content underwent a definite decrease. In fact, of the antibiotics, a fall in glycogen content, in addition to tubercidin, was noted with the high dosage of actinomycin D (0.75 mg.), streptonigrin (0.050 and 0.50 mg.; almost borderline significance with 0.10 mg.), and antimycin A (0.25 mg. suspension). Glucose utilization was decreased in the presence of the last agent and streptonigrin at 0.50 mg., a level which also inhibited diaphragm respiration. It is of interest that the glutarimides, cycloheximide, streptovitacin A, and the related E73 base were without effect except that the decrease in Q_{O_2} was significant only with E73 (0.50 mg., $p < 0.05$). Respiration was also depressed with glutinosin (0.50 mg.) and psicofuranine (0.50 mg.) but was elevated at the 2% level of probability with porfiromycin (0.50 mg.). Tylosin proved a depressant of glucose uptake solely at the higher dosage (1.00 mg.).

The antitumor glycoprotein, actinogan (23), markedly depressed glucose utilization by rat diaphragm even at a level of 0.50 mcg. and with 0.50 mg., the Q_{O_2} difference was decreased at the 5% level of probability but the diminution in glycogen content was just short of statistical significance. The findings were somewhat similar with mithramycin and duazomycins A and B, each screened down to levels of 10, 10, and 50 mcg., respectively. However, Q_{O_2} and glycogen content differences were not affected at the highest dosage of each (0.50 mg.). It would be of interest to compare the effect of actinogan with peptinogan, a polypeptide fraction obtained from this antibiotic and which also possesses antitumor action (24), an experiment not included in this series.

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Attempts at Induced Agranulocytosis in Rats Using Dipyrone

By N. M. FERGUSON and ARTHUR F. NOVAK*

Dipyrone administered orally to rats at the rate of 50 and 100 mg. per day over a period of 8 weeks did not produce any abnormalities in the blood picture. In addition no visible differences could be observed in the rats receiving the drug over those of the control group. No sensitivity to the drug was observed.

AGRANULOCYTOSIS is a disease characterized by a marked change in the blood picture, particularly that of a reduction in the polynuclear leukocyte count. A great many substances have been suspected of causing agranulocytosis (1). Included in this list are industrial solvents, *e.g.*, benzene, toluene, naphtha, paints and paint removers, and heavy metals such as gold and lead and their salts. By far the greater number, however, consist of drugs. This list includes practically all of the different classes of drugs such as anti-infectives, sedatives, tranquilizers, cardiotonics, and analgesics. Since some analgesic drugs are used in large quantities for fever as well as for pain, the total number of doses prescribed annually is very great. As a result of the widespread use of these drugs even a very low incidence of agranulocytosis appears to be alarming (1).

Attempts at controlling the distribution of these drugs have resulted in their being limited to prescription use only. Since most of them are very effective in relieving pain and some in reducing fever, they often constitute the only means of producing these desired therapeutic effects.

One of the most effective analgesic drugs which is equally effective in reducing fever is dipyrone, a derivative of antipyrine. This substance has been suspected of being a causative agent in agranulocytosis (1). This tabulation indicates that prior to June 1963, eight cases of agranulocytosis were reported to the panel and an additional nine cases were cited in the literature where dipyrone was the only drug given in the 6-month period prior to the onset of the disease. Six other cases, four from direct reports and two from the literature, occurred in which dipyrone was given in conjunction with other drugs which are believed not to be associated with agranulocytosis. During the same period other cases were brought to light where dipyrone was given along with additional medication which in turn is not known to be innocent or in many cases is actually known to precipitate agranulocytosis. From July 1963 to July 1964, only two cases are reported where the only drug given during the previous 6-month period prior to the onset of the disease was dipyrone, whereas, four cases have been recorded during the same period where other drugs not known to be innocent of this reaction and four cases where drugs known to be toxic were administered. During these same periods, however, many other drug substances have been reported as being responsible for or associated with agranulocytosis and many of these in much higher incidence than dipyrone (1).

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TABLE I.—EXPERIMENTAL CONDITIONS

Group No.	Drug Received	Daily Dose, ml.	Days
1	None	0	30
2	Dipyrone[a]	0.5 (50 mg. dipyrone)	30
3	Dipyrone	1.0 (100 mg. dipyrone)	30

[a] The authors thank Savage Laboratories, Inc., Houston, Tex., for supplying dipyrone as Pyralgin Liquid used in this study.

Many attempts have been made to learn the exact mechanism of drug induced agranulocytosis. Kracke (2) found that clinical agranulocytosis could be produced in rabbits by subcutaneous injections of benzene. Lu *et al.* (3) reported that there was no reduction of blood cell count and hypoplasia of bone marrow was not observed in rabbits receiving daily administration of aminopyrine for 4 to 6 weeks even in the presence of a sensitizing agent such as busulfan.[1]

In view of the fact that dipyrone is prescribed widely for the relief of fever and pain and has been associated with the reported incidence of agranulocytosis, it was felt that further studies should be conducted with this substance. The present study, the first in a series, concerns attempts to induce agranulocytosis in rats with dipyrone.

EXPERIMENTAL

A series of 120 white rats, approximately 6 weeks old, having an average weight of 250-Gm., and equally divided as to sex, were selected for these experiments. The experimental design was planned so that the results could be analyzed statistically if this was necessary to interpret the results.

All of the rats were maintained since birth, and during the course of the entire experiment on Purina rat chow, which is a highly standardized commercial feed, fulfilling all of the nutritional requirements of rats for normal growth. Feed and water were available to the animals continuously.

The experiments were performed in an air-conditioned laboratory maintained at 72–74° F. Feed was added as required by replacing the used feed containers with clean filled containers of feed. Clean water was replaced daily. Records of feed consumption and body weight were kept for each animal.

At the outset of this investigation, the rats were separated into three groups of 20 males and 20 females each. Table I lists the experimental conditions.

Group *1* served as the control, while groups *2*

[1] Marketed as Myleran by Burroughs Wellcome & Co., Scarsdale, N. Y.

TABLE II.—AVERAGE RED BLOOD CELL COUNTS × 10^6/mm.^{3} FOR RATS RECEIVING DAILY ORAL DOSES OF DIPYRONE FOR 8 WEEKS

		Initial	Weeks 2	4	6	8
Control	Male	7.92	8.35	8.14	9.27[a]	9.16[a]
	Female	7.67	7.94	7.79	8.78[a]	8.93[a]
Dipyrone daily, 50 mg.	Male	8.10	7.88	8.54	8.61[a]	7.91[a]
	Female	8.59	8.03	7.95	8.52[a]	8.47[a]
Dipyrone daily, 100 mg.	Male	7.83	6.98	7.51	7.90[a]	8.28[a]
	Female	8.45	8.40	8.52	8.93[a]	8.76[a]

[a] Statistically similar at the 1% significance level both within the groups and also when compared to the change from initial averages.

TABLE III.—AVERAGE WHITE CELL COUNTS/mm.^{3} OF BLOOD FOR RATS RECEIVING DAILY ORAL DOSES OF DIPYRONE FOR 8 WEEKS

		Initial	Weeks 2	4	6	8
Control	Male	11,700	10,800	11,200	12,500	11,650
	Female	12,150	11,900	12,150	11,800	11,700
Dipyrone daily, 50 mg.	Male	12,000	12,100	11,350	10,700	9,950[a]
	Female	11,850	12,000	10,900	10,600	10,200
Dipyrone daily, 100 mg.	Male	11,400	10,600	9,850	9,800	9,550[a]
	Female	11,250	10,800	10,050	9,750	9,400[a]

[a] Statistically different at the 1% significance level when compared to the change from the initial averages.

TABLE IV.—DIFFERENTIAL BLOOD COUNTS × 10^3/mm.^{3} RECEIVING ORAL DOSES OF DIPYRONE FOR 8 WEEKS

	Neutrophils	Lymphocytes	Eosinophils	Monocytes	Basophils	Reticulocytes
Normal range	1.0–4.9	6.0–17.0	0.0–0.7	0.0–0.65	0.0–0.2	0.6–4.9
Control						
Male	1.2–3.2	8.0–12.2	0.2–0.4	0.2–0.6	0.0–0.2	0.7–3.8
Female	1.5–3.1	7.4–12.5	0.2–0.5	0.2–0.4	0.0–0.2	0.9–4.5
Dipyrone daily, 50 mg.						
Male	1.3–2.7	7.6–13.1	0.1–0.4	0.2–0.6	0.0–0.1	0.9–4.6[a]
Female	1.2–3.0	8.2–14.8	0.2–0.3	0.2–0.6	0.1–0.2	0.9–3.9[a]
Dipyrone daily, 100 mg.						
Male	1.2–2.3	7.7–12.6	0.2–0.5	0.2–0.5	0.1–0.2	0.8–4.2[a]
Female	1.1–2.6	7.1–13.9	0.2–0.2	0.3–0.6	0.0–0.2	0.7–4.1[a]

[a] Statistically similar at the 1% significance level when compared to the change from initial averages.

and *3* were administered daily doses of dipyrone according to the quantities listed in the above table.

Blood counts were made initially, and after every 2 weeks during the course of the experiment. Body weights were recorded and visual observations were made on each rat. None of the rats died during the course of the experiment.

PROCEDURES

Administration of Dipyrone.—The drug was administered orally each morning by employing a 1-ml. graduated pipet, and allowing the animal to drink the fluid as it was released slowly from the tip. No problems were encountered with this technique.

Blood Counts.—Since the tail is free from local circulatory change, it was used as the site of puncture. The tail was cleaned with ethanol and dried, and the tip of the tail was cut off with sterile surgical scissors. After the first few drops of blood were wiped away, the subsequent drops were obtained for red cell count, white cell count, and a blood smear for a differential count. Standard methods were employed for the counts.

RESULTS

All of the results obtained are presented in Tables II–IV. The total number of red and white blood cells are the average values. The individual variations in the population within the groups were too insignificant to have any biological importance.

DISCUSSION

The statistical treatment of the data was by the method of analyses of variance by the *F* test method. The variance of the average values of rats with dipyrone from an initial period to a period of 8 weeks was compared to that of initial averages of controls for the same period. Table IV shows the range of results for 20 rats in each series. It is to be noted that the highest and lowest values are within the normal ranges for each type of blood cell counted.

The weights and feed intake were the same for the control group as well as for the groups receiving the drug. No visual differences could be observed in the rats receiving the drug over those in the control group. Furthermore, the rats showed no sensitivity to the drug.

Four rats out of each series (*i.e.*, two males and two females) were checked at the end of 8 weeks and no abnormalities were found with respect to blood glucose, cholesterol, phospholipids, and urea nitrogen suggesting that no chemical changes resulted from the use of the drug.

SUMMARY AND CONCLUSIONS

In carefully controlled studies carried out over a period of 8 weeks, involving 120 rats, no statistically significant changes occurred in the blood picture with respect to red cell, white cell, and differential counts when varying amounts of dipyrone were administered orally.

No visual differences could be observed in the rats receiving the drug over those in the control group.

No rats died as the result of taking the drug.

No abnormalities were found with respect to blood glucose, cholesterol, phospholipids, and urea nitrogen on examining animals from each group suggesting that no chemical changes resulted from the use of the drug.

In this study using massive doses of dipyrone no indications of agranulocytosis were produced.

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Phytochemical Investigation of *Abies concolor*

By AYHAN ULUBELEN*, MARY E. CALDWELL, and JACK R. COLE

Extracts of the bark of *Abies concolor* have shown antitumor activity against the adenocarcinoma of the duodenum test system of the Cancer Chemotherapy National Service Center. One of the active materials appears to be a complex tannin. The isolation of the active fractions has been reported utilizing solvent extraction, column, paper, and thin-layer chromatography.

In a routine screen of Arizona and Mexico plants for antitumor activity, it was found that extracts of the bark of *Abies concolor* (Gordon and Glendinning) Hoopes were shown to have activity against the adenocarcinoma of the duodenum (7D1) test system of the Cancer Chemotherapy National Service Center, Bethesda, Md. The plant, also known as white fir, is a soft-wooded, resinous evergreen monoccious tree, 100–200 ft. tall, 3–6 ft. in trunk diameter. It is distributed from Wyoming west to Oregon and south into Baja California, Arizona, New Mexico, and Sonora, at elevations of 3000–10,000 ft.

The collection used in this study was obtained from upper Sabino Creek, 8000 ft. elevation, Santa-Catalina Mountains, Pima County, Ariz.[1]

EXPERIMENTAL

Preliminary Extraction.—The bark (3.5 Kg.) of *A. concolor* was extracted with chloroform–ethanol (1:1) in a Lloyd extractor for 2 days. After evaporation, this crude extract was submitted to the adenocarcinoma of the duodenum test system (7D1). The crude material showed a decrease in tumor size of approximately 86 and 84% at a dose of 400 and 200 mg./Kg., respectively (Table I).

Table I.—*In Vivo* Tumor Inhibition

	Dose, mg./Kg.	% T/C[a]
n-Hexane extract	175	32
	200	32
Brown powder	100	26
	200	11

[a] The criteria for activity is defined as being a % T/C (test/control) value of less than 42 in a satisfactory dose response test (1).

One kilogram of the crude material was extracted with 2000 ml. of *n*-hexane in a Soxhlet for 72 hr. The green *n*-hexane extract upon evaporation yielded 200 Gm. of gummy material. A brown powder was obtained in the Soxhlet. Both of the materials were active against the test system.

n-Hexane Extraction

Twenty-five grams of the green material obtained from the *n*-hexane extraction was extracted with 10 × 100 ml. of petroleum ether (36–60°). The petroleum ether was evaporated to dryness in a Rinco evaporator under reduced pressure. A light green residue (10 Gm.) was obtained.

Part A.—Five grams of this residue was chromatographed on a neutral alumina column (30 × 3 cm.) (Fisher certified reagent catalog No. A-950 Brockman, activity I, 80–200 mesh). The column

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* Present address: College of Pharmacy, University of Istanbul, Istanbul, Turkey.
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was successively washed with the following solvents until extinction: (*a*) benzene, (*b*) chloroform, (*c*) ether.

(*a*) The benzene fraction was evaporated to a small volume. A white substance was separated (45 mg.). This substance was purified by preparative thin-layer chromatography using 20 × 20 cm. plates covered with a slurry of Silica Gel G (35 Gm. in 50 ml. of water), and activated in an oven at 110° for about 2 hr. Benzene–petroleum ether (3:7) was used as the developing solvent. The compound obtained from the plates was crystallized from ethanol and had a melting point of 82–83°. The I.R. curve showed that it was a simple long chain ketone. The semicarbazone derivative was oily. The oxime was prepared. It had a melting point of 59°. The compound was identified as palmiton by comparing the melting point of the compounds and oxime derivative (2) and I.R. curves. The remaining part of the benzene fraction was evaporated to dryness. An oily compound (2.0378 Gm.) was obtained. Preparative thin-layer chromatography indicated a series of materials. These appear to be small molecular weight terpenes. Since a thorough work has been performed by Carlberg (3), and since this part had no activity, no further work has been done.

(*b*) The chloroform fraction was evaporated to dryness and yielded 2.2 Gm. of material. Again by means of the preparative thin-layer chromatography, using chloroform–petroleum ether (7:3) as the developing solvent, two main spots and a series of minor compounds were obtained. Since the two main compounds were close to each other, they were extracted from the plates with chloroform and rechromatographed with petroleum ether–ether (1:1) as the developing solvent. A better separation was achieved. The yields of the compounds were 430 mg. for compound I with an R_f value 0.5, and 700 mg. of compound II with an R_f value of 0.7. Compound I was recrystallized from methanol and had a melting point of 136–137°. The I.R. spectrophotometry and mixed melting points indicated that the compound was *p*-menthandiol-(1,4). Compound II was recrystallized from acetone and had a melting point of 70–71°. The I.R. spectrophotometry and mixed melting points indicated the compound to be docosonol-1.

(*c*) The ether fraction, when evaporated, yielded 0.5318 Gm. of material. Preparative thin-layer chromatography with chloroform–ether (99:1) as developing solvent showed four spots (R_f values were 0.2, 0.27, 0.5, 0.95). The yields were 54.2 mg., 66.8 mg., 53.4 mg., and 203.8 mg., respectively. The first spot had a melting point of 88–89°. The I.R. curve has bands at 3509, 2941, 2874, 1718, 1626, 1595, 1508, 1464, 1422, 1266, 1156, 1022, 975, 845, 808, and 720 cm.$^{-1}$. The U.V. curve has a maximum at 321, 243, and a minimum at 263 mμ. The other three compounds were simple ketones and alcohols. No further investigation of the above materials was carried on since they were inactive.

Part B.—The remaining material after the petroleum extraction (13 Gm.) was diluted to 10 times its weight with 50% aqueous potassium hydroxide (4). The mixture was left to cool and 4 times the volume of ethanol was added. The mixture was then refluxed under nitrogen atmosphere for 3 hr. At 30-min. intervals a small amount of the mixture was subjected to thin-layer chromatography using petroleum ether–ether–acetic acid (70:30:2) solvent system. This was done in order to see if the hydrolysis was completed. After the 3-hr. refluxing period the volume was increased 3 times with water. The solution was extracted with ether (10 × 100 ml.), the ether was washed with water, and dried over anhydrous sodium sulfate. The yield of the nonsaponifiable portion was 9 Gm. One gram of this material was applied to a preparative thin layer of silica gel, and chloroform–benzene–ether (6:2:3) was used as the developing solvent system. Three spots were obtained with R_f values of 0.97, 0.6, and 0.4. The material at 0.97 R_f was oily and in minimal concentration. The middle spot yielded 395 mg., which was recrystallized from alcohol and then from acetone. It had a melting point of 76–77°. Comparison of the I.R. curves and mixed melting points showed that it was lignoceryl alcohol. The third compound (117.6 mg.) was recrystallized from alcohol. It had a melting point of 138–139°. The I.R. curve comparison and mixed melting point showed that it was β-sitosterol.

After the extraction of the nonsaponifiable part with ether, the aqueous solution was acidified and extracted with ether (10 × 100 ml.). Upon evaporation 6.5 Gm. of brown material was obtained. A portion of this material (2.5 Gm.) was separated using preparative thin layers with petroleum ether–ether–acetic acid (90:10:1) as the developing system. Four fractions were obtained. R_f values were 0, 0.01, 0.04, and 0.2, and the amounts 550 mg., 1.200 Gm., 200 mg., and 280 mg., respectively. All of these fractions were subjected to further separation using reverse phase paper and thin-layer chromatography. Papers were covered with 7 and 12% of paraffin in benzene and the developing solvent was 85% aqueous methanol. Fraction 4 gave a yellow spot on a blue background when sprayed with bromophenol blue. Methyl derivatives were prepared and only one spot was obtained in the case of fraction 4 when sprayed with Sudan IV solution (5). A dimethyl polysiloxane,[2] 5% in ether, was used to impregnate the silica gel plates for the reverse phase thinlayer chromatography. Acetic acid–formic acid–water (40:40:20) was used as the mobile phase. Again fraction 4 revealed one single spot. This fraction (280 mg.) was recrystallized from alcohol. It had a melting point of 79–80°. The I.R. curve comparison and the mixed melting points revealed that it was lignoceric acid. The compounds thus far isolated from the *n*-hexane extracts do not exhibit antitumor activity in the 7D1 test system.

Alcohol Soluble Part

The material remaining after the *n*-hexane extraction was an amorphous red-brown powder which charred above 250°. Elementary analysis showed 16.9% inorganic residue and the absence of nitrogen, sulfur, and halogens. Magnesium and hydrochloric acid (6), ferric chloride (6), gelatin test (7), and Wilson's boric acid test (8) suggested that this material was a mixture of complex tannin and flavonoid substances. Attempts to purify this crude material were made using the following chromatographic substrates: silica gel, aluminum oxide (neutral),

[2] Marketed as Dow Corning 200 fluid by the Dow Corning Corp., Midland, Mich.

TABLE II.—R_f VALUES OF DELPHINIDIN AND CYANIDIN IN DIFFERENT SOLVENT SYSTEMS

No.	Solvent System	Known		Unknown	
		Cyanidin	Delphinidin		
1	H_2O–HCl–Acetic acid (10:3:30)	0.52	0.31	0.52	0.30
2	*n*-Butanol–acetic acid–H_2O (4:1:5) (upper phase)	0.91	...	0.93	...
3	Formic acid (88%)–3 *N* HCl (1:1)	0.23	0.12	0.23	0.12
4	*n*-Butanol–2 *N* HCl (1:1) (upper phase)	0.62	...	0.62	...

magnesium trisilicate, and polycaprolactam pulver "S".[3]

The first three columns adsorbed the material. When polycaprolactam pulver "S" is mixed with the same amount of diatomaceous earth[4] and packed into a 35 × 3 cm. column, a yellow substance was obtained from ethanol elutions. This was a flavonoid substance. Column chromatography with polycaprolactam powder utilizing preparative thin-layer chromatography yielded the same material, but no antitumor activity was demonstrated by this material. Solvent purification was then employed. Fifty grams of the crude material was dissolved in alcohol and upon the addition of water a precipitate appeared (6.5 Gm.). This precipitate was washed with ether. One gram of a yellow flavonoid substance was obtained. The U.V. spectrum of the flavonoid showed maxima at 285 and 206 mμ and a minimum at 260 mμ. The ether and water-insoluble portion was active against adenocarcinoma of the duodenum test system (7D1), 11% T/C at 200 mg./Kg., 26% T/C at 100-mg. doses. This part gave long streaking blue spots when applied on paper with solvent systems such as (*a*) *n*-butanol–acetic acid–water (4:1:5), (*b*) 50% acetic acid, and (*c*) 2% acetic acid and when sprayed with ferric–ferricyanide, a long pink spot with *p*-toluene sulfonic acid, two violet-pink spots with vanillin-HCl, and no sugar spots with aniline phthalate. The I.R. curve revealed a large hydroxyl band at 3400, and phenyl group at 1595, 1505, and 855 $cm.^{-1}$. The U.V. spectra showed adsorption maxima at 280 and 206 mμ and a minimum at 260 mμ. This compound, when boiled with hydrochloric acid, caused the solution to turn a light pink color and had only 2.5% inorganic material. The compound was water and ether insoluble, nonhydrolyzable, and the classification tests indicated the presence of a phlobaphene. Further purification was accomplished by dissolving the material in alcohol and precipitation by the addition of ether. Column chromatographic separation or hydrolysis with HCl resulted in a loss of activity. The water-soluble portion was extracted with isoamyl alcohol. A slightly brown-red solution was obtained. This also gave streaking spots with the same solvent systems that were used on the phlobaphenes, and the same results were obtained when sprayed with the reagents as indicated above. This compound had less activity, 49% T/C at a dose of 100 mg./Kg. No residue was obtained upon burning. The I.R. curve was very similar to that of the phlobaphene. The U.V. curve gave maxima at 281 and 206 mμ and a minimum at 260 mμ. Being water soluble and ether insoluble, the differential classification reactions indicated above showed that this was a complex tannin. In the three compounds separated the U.V. curve did not show any shift when 1.2 ml. of a 1% $AlCl_3$ in alcohol solution was added, which shows the absence of 3-hydroxy flavones or flavonones with *o*-hydroxy carbonyl groups. When the alcoholic solution was made 0.006 *N* alkaline with KOH, the flavonoid spectra had a bathochromic shift of 20 mμ, phlobaphenes 22 mμ, and the complex tannin 14 mμ. The maximum 280–281 mμ indicated a flavan nucleus, and this is corroborated by the absence of a shift in the spectra with $AlCl_3$ and a definite shift in the spectra of the alkaline solution.

Boiling the complex tannin with hydrochloric acid for approximately 2 hr. changed the color to a bright red. Paper chromatography of this hydrolyzed compound using several solvent systems indicated the presence of cyanidin and delphinidin (Table II).

After hydrolysis, the compound did not yield any sugar spots on paper. Drastic hydrolysis of anthocyanidins isolated using the same method as reported previously (9) yielded protocatechuic acid, phloroglucinol, and gallic acid.

SUMMARY

A. concolor has been investigated to determine the chemical agents responsible for its activity against adenocarcinoma of the duodenum. A complex tannin and phlobaphene fractions appear to be the active compounds in the plant. In addition to the above, palmiton, *p*-menthandiol-(1,4), docosonol-1, lignoceryl alcohol, β-sitosterol, and lignoceric acid were separated and identified by using column and paper chromatographic techniques.

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[3] Marketed as Perlon Powder by Farbwerke Hoeschst AG, Bobingern, Germany.

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Evaluation of Oral Dilution as a First Aid Measure in Poisoning

By METTA LOU HENDERSON, ALBERT L. PICCHIONI, and LINCOLN CHIN

Dilution with large volumes of water is a widely recommended first aid measure for the treatment of poisoning from ingested chemical agents. In view of the lack of experimental work to support its clinical application and of possible adverse effects that may attend its use, further investigation was undertaken to determine the influence of oral dilution on blood levels of sodium pentobarbital, quinine hydrochloride, and aspirin. The results indicate that oral dilution with large volumes of water increases the rate and degree of gastrointestinal absorption of pentobarbital and quinine, although it has no significant effect on the absorption of aspirin. In view of the possibility of enhanced gastrointestinal absorption of certain chemicals caused by oral dilution, it is suggested that this procedure not be employed as a first aid treatment for ingested systemic poisons.

DILUTION with large volumes of water or other fluids is a widely recommended first aid measure for the treatment of poisoning from ingested chemical agents (1–4). This procedure is based on the belief that dilution will slow absorption of a chemical from the gastrointestinal tract by increasing the amount of fluid that must be absorbed for a given amount of poison (4). However, no experimental evidence has been presented to support this view. Indeed, a study by Ferguson (5) showed that oral median lethal doses (LD_{50}s) of a number of drugs in rats vary inversely with the volume of water in which a drug is administered. In contrast to the above view, the observations of Ferguson would seem to imply that dilution of an ingested chemical may enhance rather than retard its absorption from the gastrointestinal tract.

In view of the lack of experimental work to justify the use of oral dilution in poisoning, and of the possible adverse effects that may attend its use, further investigation of this recommended procedure seems warranted.

The present investigation was designed to determine the influence of oral dilution with water on blood levels of certain chemicals, since the blood concentration of a chemical compound is related to its rate of absorption from the gastrointestinal tract. Sodium pentobarbital, quinine hydrochloride, and aspirin were selected as representative chemical compounds for this project. Barbiturates, in general, are absorbed from the stomach (6, 7) and small intestine (6–9). Quinine is predominantly absorbed from the small intestine (10, 11). Aspirin is considered to be readily absorbed from both the stomach and small intestine (7, 12, 13). The results obtained constitute the basis of this report.

EXPERIMENTAL

Female Sprague-Dawley rats weighing 170–295 Gm. were fasted for 24 hr. and divided into control and test groups of five to nine animals. They were allowed free access to water except during the last hour prior to testing. All drugs were given by oral intubation in a volume of 2 ml./Kg.; 1 min. later the control animals were administered a small volume of water (1 ml./Kg.) by gavage and the test animals were administered a large volume of water (20 ml./Kg.). At each time period after drug treatment, as indicated below, a control group and a test group of animals were anesthetized with ether and blood samples collected from the abdominal aorta for analyses. The difference in the concentrations of each drug in the blood of test and control animals at each time period was statistically compared by means of Student's t test (14).

Pentobarbital.—Control and test animals were administered sodium pentobarbital,[1] 25 mg./Kg., calculated as the base. Blood samples were collected 10, 20, 40, 80, 160, and 320 min. after drug treatment and extracted and analyzed for pentobarbital by the method of Goldbaum (15) as modified in this laboratory (16).

Quinine.—Control and test animals were administered quinine hydrochloride, 100 mg./Kg., calculated as the base. Plasma samples were collected 15, 60, and 120 min. after drug treatment and extracted and analyzed for quinine by the fluorimetric procedure described by Brodie and Udenfriend (17); measurements of fluorescence were made with an Aminco-Bowman spectrophotofluorometer.

Aspirin.—Control and test animals were administered aspirin, 200 mg./Kg., as an aqueous suspension.[2] Plasma samples were collected 5, 15, and 60 min. after drug treatment and analyzed for salicylate by the method of Trinder (19). At the 5-min. time period, the plasma samples were incubated in a water bath at 37° for 2 hr. to ensure that all of the aspirin was hydrolyzed to salicylic acid (20).[3]

RESULTS

Pentobarbital.—The results of the pentobarbital study are presented in Fig. 1. Ten minutes after the administration of pentobarbital the blood concentration of the drug in test animals was 33% higher than in control animals, but the difference is not statistically significant ($p > 0.05$). At the 20- and 40-min. time periods, the pentobarbital levels of test animals were 90 and 78% greater than those of corresponding control animals, respectively. These elevated blood levels of pentobarbital are significantly higher than the control levels ($p < 0.01$). By the 80-, 160-, and 320-min. time periods

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[1] Sodium pentobarbital was supplied through the courtesy of Abbott Laboratories, Chicago, Ill.

[2] The aspirin was triturated in a mortar and sifted through a 100-mesh sieve in order to provide a more uniform suspension and to reduce variability in absorption (18).

[3] Preliminary studies in this laboratory demonstrated that aspirin was completely hydrolyzed to salicylic acid in plasma collected 15 and 60 min. after drug treatment; hence, plasma was not incubated at these two time periods.

the blood pentobarbital concentrations of the test animals were similar to those of the corresponding control animals ($p > 0.05$).

Quinine.—The results of the quinine study are presented in Fig. 2. At the 15-, 60-, and 120-min. time periods the plasma concentrations of quinine in the test animals were 138, 72, and 62.5% higher than those of the corresponding control animals. These values are statistically significant ($p < 0.05$).

Aspirin.—The results of the aspirin study are presented in Fig. 3. The plasma levels of aspirin in test animals did not differ significantly from those of the corresponding control animals during the 5-, 15-, and 60-min. time periods ($p > 0.05$).

DISCUSSION

The results of the present study indicate that oral dilution with large volumes of water increases the rate and degree of gastrointestinal absorption of pentobarbital and quinine, although it has no significant effect on the absorption of aspirin.

Some factors which influence the absorption of drugs from the gastrointestinal tract include: (*a*) degree of ionization of the drug, (*b*) lipoid solubility of the nonionized form of the drug, (*c*) gastric emptying time, (*d*) concentration of the drug in the digestive system, (*e*) solubility of the drug in the digestive system, and (*f*) size of the area over which the drug is spread (6–10, 21–24). Oral dilution with a large volume of water would affect factors (*c*), (*d*), and (*f*).

Since barbiturates are absorbed from the intestine (6–9) as well as from the stomach (6, 7) by nonionized diffusion (8), gastric emptying time probably has only a slight influence on the absorption rate of pentobarbital. The large volume of water administered to the test rats would decrease the concentration of pentobarbital in the gastrointestinal tract and tend to reduce the rate of absorption. However, oral dilution causes a marked increase in surface area over which absorption may take place; this factor undoubtedly exerts a more significant effect on gastrointestinal absorption of pentobarbital than does the factor of concentration of the drug in the digestive system.

Quinine is poorly absorbed from the stomach (7, 10), but it is readily absorbed from the small intestine (10, 11). Hence, hastening the passage of the drug from the stomach into the intestine by oral dilution would tend to allow intestinal absorption to start sooner in the test animals than in the control animals. However, the higher plasma quinine levels in the test animals are most likely due to the increase in surface area for absorption in the intestine.

Aspirin is readily absorbed from the stomach and small intestine (7, 12, 13). Martin (12) states that gastrointestinal absorption is very rapid and complete. In the present study, since the plasma salicylate concentration of the test rats is no higher than that of the control rats, it could be postulated that aspirin is absorbed so rapidly and completely that oral dilution has no significant effect on gastrointestinal absorption of this drug.

Dreisbach (4), who has recommended oral dilution as a first aid treatment in poisoning, warns that the volume of fluid given should not exceed the capacity of the stomach, otherwise the noxious material may be forced into the intestine and absorption would be increased. However, due to the increased surface area for absorption in the stomach following oral dilution, absorption is likely to be enhanced even in the absence of gastric emptying.

Although the present investigation involves only three chemical agents, it appears logical to conclude that oral dilution would generally be ineffective in retarding the gastrointestinal absorption of ingested chemicals. The act of dilution with water cannot occur without a concomitant increase in volume and a consequent increase in surface area from which absorption of a chemical can take place. In view of the possibility that oral dilution may enhance gastrointestinal absorption of chemicals, it is suggested that this procedure not be employed as a first aid treatment for ingested systemic poisons.

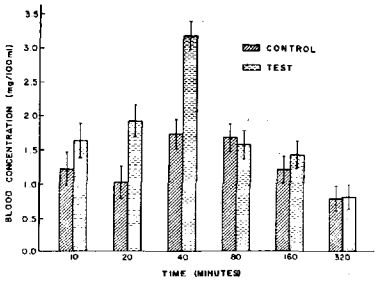

Fig. 1.—The effect of oral dilution on blood pentobarbital concentration. Bracketed lines represent 95% confidence limits. Key: control, 1 ml. water/Kg.; test, 20 ml. water/Kg.

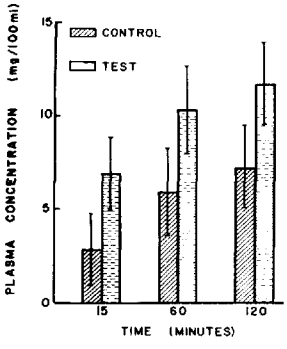

Fig. 2.—The effect of oral dilution on plasma quinine concentration. Bracketed lines represent 95% confidence limits. Key: control, 1 ml. water/Kg.; test, 20 ml. water/Kg.

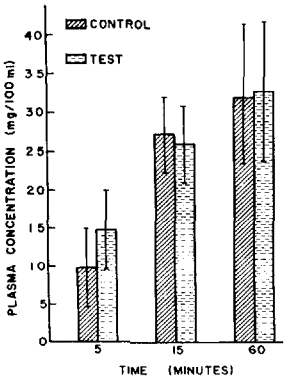

Fig. 3.—The effect of oral dilution on plasma aspirin concentration. Bracketed lines represent 95% confidence limits. Key: control, 1 ml. water/Kg.; test, 20 ml. water/Kg.

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By D. L. SIMMONS, H. S. L. WOO, C. M. KOORENGEVEL, and P. SEERS

A two-dimensional thin-layer chromatography (TLC) procedure on microcrystalline cellulose is presented for the quantitative determination of anhydrotetracycline and epianhydrotetracycline in degraded tetracycline tablets. Initial development is performed with 0.1 *M* EDTA–0.1 per cent ammonium chloride solution to separate the anhydrotetracyclines (R_f 0.34–0.38) from the tetracycline (R_f 0.72) and methanol-soluble excipients. Anhydrotetracycline (R_f 1.0) and epianhydrotetracycline (R_f 0.52) are then resolved by developing the chromatogram with chloroform which has been saturated with the same EDTA–ammonium chloride solution. A complete assay for these anhydro compounds can be performed in less than 2 hr.

POOR RESOLUTION and/or excessive development time has characterized previous chromatographic attempts to separate tetracycline (TC) and its major degradation products, anhydrotetracycline (ATC) and 4-epianhydrotetracycline (EATC). The latter was recently incriminated in the reversible renal dysfunction (Fanconi-type syndrome) caused by the ingestion of degraded TC products (1–7).

In a qualitative examination of TC and its anhydro derivatives by radial chromatography, using water-saturated butanol on silica gel, Rustici and Ferappi (8) obtained the following similar R_f values after 2 hr. of development: TC (0.36), ATC (0.50), and EATC (0.40). When Kelly and Buyske (9) employed paper which had been impregnated with 0.1 *M* EDTA solution and the solvent system *n*-butanol–ammonium hydroxide–water (4:1:5), they obtained the following 16 hr. R_f values: TC (0.39), ATC (0.62), and EATC (0.40). In a recent paper (10) the authors reported the quantitative analysis of ATC from TC test mixtures by TLC on microcrystalline cellulose. This method required only 20 min. development with 0.1% ammonium chloride solution to give R_f values of 0.38 and 0.72 for the ATC and TC, respectively. Subsequent experiments revealed that mixtures of EATC, ATC, and TC are partially resolved by the same chromatographic system, but overlapping of the ATC (0.38) and EATC (0.34) occurred. In order to completely resolve the two anhydro-compounds, two-dimensional chromatography was attempted. The findings of Kelly (11) that ATC and EATC can be separated by partition chromatography employing buffered 0.1 *M* EDTA solution (pH 7.8) as stationary phase and buffer saturated chloroform as moving phase, prompted the authors to utilize these findings in their search for a suitable second solvent system. By developing the chromatogram with 0.1 *M* EDTA (disodium salt)–0.1% ammonium chloride solution (pH 4.5), followed by chloroform which had been saturated with the same EDTA–ammonium chloride solution, complete resolution of the ATC (1.0) and EATC (0.52) occurred. On altering the pH of the aqueous phase between 3.5 and 8.0, R_f values for ATC (1.0) and TC (0.17) were observed to remain constant in the second development;

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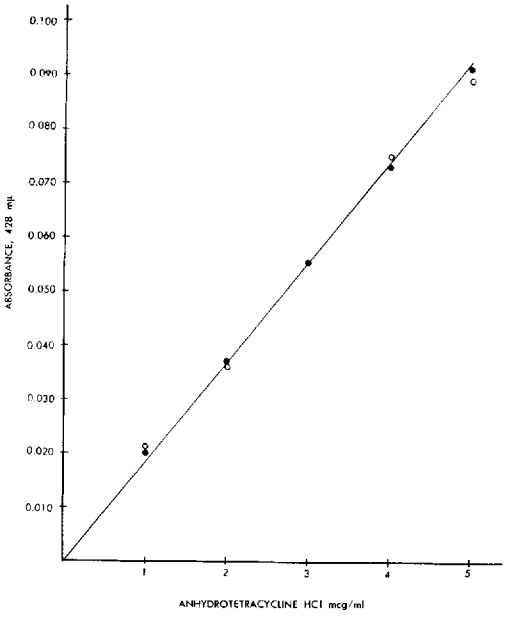

Fig. 1.—Absorbance of ATC as a function of concentration. Key: ●, direct dilutions; ○, dilutions after TLC.

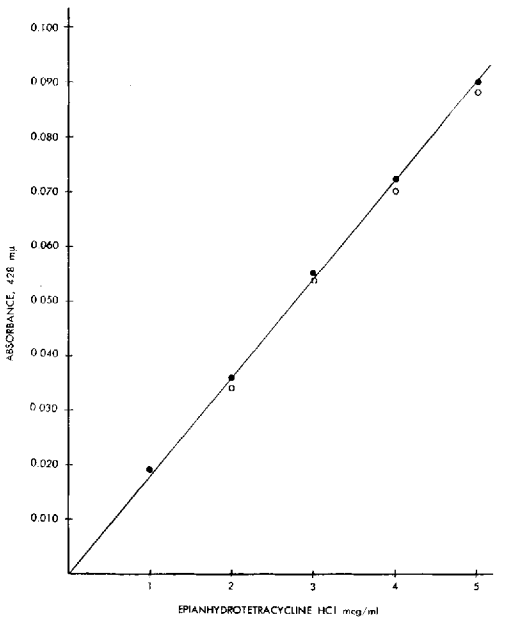

Fig. 2.—Absorbance of EATC as a function of concentration. Key: ●, direct dilutions; ○, dilutions after TLC.

TABLE I.—ANALYSIS[a] OF TETRACYCLINE TABLET TEST MIXTURES

ATC, mg./Tablet			EATC, mg./Tablet			Spot Load, ml.	Dilutions of TLC Extracts, ml.
Added	Found	Recovery, %	Added	Found	Recovery, %		
0	1.08 ± 0.06		0	0.28 ± 0.02		0.2	5
0.5	1.54 ± 0.11	97.4	0.5	0.76 ± 0.13	97.4	0.2	5
1.0	1.99 ± 0.16	99.0	1.0	1.35 ± 0.08	105.5	0.1	5
5.0	6.09 ± 0.10	100.2	5.0	5.16 ± 0.12	97.8	0.1	10
10.0	10.95 ± 0.08	98.8	10.0	10.32 ± 0.24	100.4	0.05	10

[a] ± = standard deviation. The average of five determinations. The blank value for EATC (0.23 mg.) was obtained from the difference between total anhydro content and ATC only.

on the other hand, the R_f value for EATC varied between 0.60 and 0.30. In contrast to Kelly's procedure (11) the results demonstrate that precise pH requirements are not necessary for the system to be operative. Such a large difference between the R_f values for ATC and EATC led to their easy isolation and determination.

EXPERIMENTAL

Preparation of Anhydrotetracycline Hydrochloride.—A modified procedure of McCormick *et al.* (12) was used. Tetracycline hydrochloride was heated in methanol–concentrated hydrochloric acid (1:4) on a steam bath for 30 min. The solution was cooled and diluted with ice water; the resulting precipitate was collected by filtration. Purification was achieved by dissolving the precipitate in hot methanol–concentrated hydrochloric acid (30:1), treating the solution with charcoal, and filtering it through Celite. Crystallization occurred when the filtrate was cooled, and a small amount of concentrated hydrochloric acid was added. By repeating this procedure 6 times, a product was obtained with the melting point and ultraviolet absorption spectrum (0.1 *N* sodium hydroxide solution) of pure anhydrotetracycline hydrochloride.

Preparation of Epianhydrotetracycline Hydrochloride.—By employing the ammonium salt of epi-tetracycline (12) in the foregoing procedure instead of tetracycline, a pure sample of epianhydrotetracycline hydrochloride was prepared.

Preparation of Standards.—Standard solutions (2 mg./ml.) of ATC and EATC in methanol were prepared and aliquots (5, 10, . . . 25 μl.) were removed and diluted to volume with methanol in 10-ml. volumetric flasks. Dilution absorbances were determined at 428 mμ on a Beckman DU spectrophotometer. The results are illustrated in Figs. 1 and 2.

Identical aliquots of the two foregoing standard solutions were subjected to the two-dimensional chromatographic procedures described below for TC tablet test mixtures. The methanolic TLC extracts were diluted to volume with 10 ml. of methanol and dilution absorbances were determined spectrophotometrically at 428 mμ. Standard

TABLE II.—ANALYSIS[a] OF DEGRADED TETRACYCLINE TABLETS

Sample	Spot Load, Vol., ml.	ATC, mg./Tablet	EATC, mg./Tablet
A	0.02	16.23 ± 0.48	15.28 ± 0.66
B	0.04	8.10 ± 0.29	5.89 ± 0.37

[a] ± standard deviation. The average of five determinations.

curves, comparing dilutions after TLC with direct dilutions, are illustrated for ATC and EATC in Figs. 1 and 2, respectively.

Procedure.—*TC Tablet Test Mixtures.*—TC tablets[1] each containing 250 mg. of tetracycline hydrochloride were crushed by mortar and pestle. The resulting powder was deliberately contaminated with known quantities of ATC and EATC. Powder equivalent to one tablet was transferred to a 3-ml. sintered-glass funnel and vacuum extracted with approximately 20 ml. of hot methanol. The extract volumes were adjusted to 25 ml. in volumetric flasks and appropriate aliquots spotted for two-dimensional chromatography (13) on microcrystalline cellulose plates (10). The chromatograms were developed for 20 min. in a chamber containing 0.1 *M* EDTA—0.1% ammonium chloride solution (pH 4.5). Development was followed by drying the plates under ambient conditions for 25 min. The plates were then placed for two-dimensional chromatography in a second chamber which contained chloroform saturated with the EDTA–ammonium chloride solution as used previously. Development for 20 min. was then permitted. R_f values of 1.0 and 0.60 were obtained for ATC and EATC, respectively. After removing and thoroughly drying the plates, the visible spots were scraped into 3-ml. sintered-glass funnels and vacuum extracted with hot methanol. Care was taken to ensure that complete drying of the microcrystalline cellulose occurred prior to extraction with methanol. When the adsorbent was too wet, EDTA was found to be extracted and caused characteristic increases (0.008) in absorbance at 428 mμ. These results were confirmed by deliberate addition of EDTA solution to test samples. A drying period of not less than 5 min. under a gentle stream of air[2] proved to be satisfactory.

[1] Marketed as Tetrosol Tablets by Frank W. Horner Ltd., Montreal, Quebec, Canada (July 1965).

[2] Oster "airjet" hair dryer, John Oster Manufacturing Co., Milwaukee, Wis.

As previously described by the authors (10), quantitative isolation and determination of anhydrotetracycline from microcrystalline cellulose is only possible in the 10–40 mcg./spot range. To keep within this range when working with samples containing unknown quantities of anhydro- compounds, the authors used the separation of the initial plate load as an indication of the quantities involved in the analysis. Suitable absorbance readings for ATC and EATC solutions were obtained by diluting the extracts of the plate scrapings with appropriate quantities of methanol. The spot load volumes and TLC extract dilutions which were used experimentally are indicated in Table I for easy reference.

Degraded Tetracycline Tablets.—Having established the validity of the foregoing procedures in the analysis of TC samples to which anhydrotetracyclines had been added, the authors applied the procedure to the analysis of degraded TC tablets. Sample *A* had been subjected to a 6-week thermal degradation study at 70° (14) followed by a storage period of 4 years at room temperature in screw-cap vials. Sample *B* had been stored in plug-cap glass vials, under tropical conditions for 4 years. The results are given in Table II.

DISCUSSION

A simple, rapid, and quantitative analysis for ATC and EATC has been performed by two-dimensional TLC on microcrystalline cellulose. The procedure is not affected by tablet excipients, and permits easy separation and quantitative recovery of minute (0.5 mg.) quantities of the harmful impurity, EATC. The method can be adopted for routine control quantitative or qualitative analysis of TC raw materials and solid dosage forms.

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New Synthesis of Thiphenamil Hydrochloride

By A. GARNETT RICHARDSON

A new synthesis of thiphenamil hydrochloride, 2-(diethylaminoethyl)diphenylthioacetate hydrochloride, a direct-acting smooth muscle antispasmodic with no appreciable anticholinergic or muscarinic activity, is described. The synthesis is carried out under mild conditions and is much simpler than any other known method of preparing this drug.

THIPHENAMIL HYDROCHLORIDE [2-(diethylaminoethyl)diphenylthioacetate hydrochloride][1] was prepared in two steps from diphenylacetyl chloride by reacting the latter with ethylene sulfide, followed by treatment of the resulting 2-(chloroethyl)diphenylthioacetate with diethylamine. Ethylene sulfide is not commercially available, but it can be prepared easily from ethylene carbonate and potassium thiocyanate by the method of Searles and Lutz (1). Ethylene sulfide polymerizes readily, so it is advisable to prepare it immediately before use.

EXPERIMENTAL

2-(Chloroethyl)diphenylthioacetate.—A solution of 231 Gm. (1 mole) of diphenylacetyl chloride in 225 ml. of benzene was mixed with 63 Gm. (1.05 moles) of ethylene sulfide in a suitable vessel fitted with an air-tight closure. The air above the surface of the liquid was swept out with nitrogen, the vessel sealed, and the mixture allowed to stand at room temperature for 7 days. At the end of this period the liquid was filtered with decolorizing carbon and the solvent evaporated from the filtrate under reduced pressure below 40°. The yield of white, crystalline product was 270 Gm. (93%) melting at 45°–47°. 2-(Chloroethyl)diphenylthioacetate was originally prepared by this author (2) from potassium diphenylthioacetate and ethylene chlorobromide.

2-(Diethylaminoethyl)diphenylthioacetate Hydrochloride.—The 2-(chloroethyl)diphenylthioacetate from the preceding reaction was dissolved in 146 Gm. (2 moles) of diethylamine, and the mixture was allowed to stand in a sealed vessel at room temperature for 7 days. At the end of this period the unreacted diethylamine was removed by evaporation under reduced pressure below 30°. The residual material was stirred with 600 Gm. of cracked ice and water in which 30 Gm. of sodium carbonate had been dissolved. The basic ester precipitated as a white, waxy solid. This was collected on a filter and washed thoroughly with cold water. The pure basic ester melts at 35°, but in this case the product was not purified.

The crude material was suspended in approximately twice its volume of water containing the calculated quantity of hydrogen chloride (77 ml. of hydrochloric acid, sp. gr. 1.19 in 600 ml. of water for 0.93 mole). When all of the base had dissolved the pH of the solution was adjusted to 5.0 with additional hydrochloric acid, or with sodium hydroxide. Five grams of decolorizing carbon was added and the mixture filtered with suction at 60°. The product crystallized as the monohydrate when the filtrate was slowly cooled. The cooling was carried almost to the freezing point and the crystals collected on a filter. The anhydrous salt was obtained by placing the monohydrate in a hot-air drier at 60° and raising the temperature at the rate of 5°/hr. to 105°. The yield of fine, white powder was 244 Gm. (72%) melting at 127°–129°.

DISCUSSION

This synthesis of thiphenamil hydrochloride precludes the use of high reaction temperatures and to a great extent the use of flammable solvents as well. These are advantages in a manufacturing process. In addition, this procedure offers a considerable saving in labor and materials compared to other known methods for the preparation of this compound.

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[1] Marketed as Trocinate by the Wm. P. Poythress & Co., Richmond, Va.

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Effect of Chronic Nortriptyline Pretreatment on the Acute Toxicity of Various Medicinal Agents in Rats

By D. B. MEYERS, D. O. KANYUCK, and R. C. ANDERSON

A method using rats that can be of value in predicting the effects of chronic treatment with psychotherapeutics on the acute toxicity of other medicinal agents is described. Results obtained by this method showed that nortriptyline, like amitriptyline, enhanced the toxicity of most cerebral depressants in an additive manner. Both of the antidepressive compounds were shown to markedly potentiate the toxicity of physostigmine and to a lesser degree pilocarpine. Ephedrine toxicity was significantly antagonized by thymoleptic pretreatment. The test failed to verify reports that the tricyclic antidepressives dangerously potentiate the toxicity of alcohol or monoamine oxidase inhibitors. Results indicated that a variety of medicinal agents can be used in the nortriptyline treated patient without any problem of significant drug interaction.

NUMEROUS but sometimes contradictory reports of clinical problems resulting from the interaction of antidepressive agents with various other drugs have appeared in recent months. The potentiation of toxicity of thymoleptics by ethyl alcohol has been reported by some workers but not confirmed by others (1, 2). Various articles have cited the hazards of concurrent use of the tricyclic antidepressives and monoamine oxidase inhibitors while others have minimized the danger and cited evidence of the value of such combinations (3–8). More isolated cases in which a thymoleptic agent has been suspected of potentiating the toxicity of barbiturates, chlordiazepoxide, amphetamine, digitoxin, meperidine, or isopropylarterenol have been reported.

In view of the controversial nature of many of these observations this study was designed to evaluate the effect of chronic pretreatment of rats with nortriptyline HCl upon the acute toxicity of a variety of medicinal agents. It was felt that information gained from a systematic animal study might be of value in predicting the safety or hazards of administering other drugs to patients receiving thymoleptic treatment.

Lethality rather than more subtle pharmacological effects was chosen as the criterion for measuring interaction as it clearly predicts the actual hazard of drug therapy. It also provided a well-defined end point free of operator bias.

EXPERIMENTAL

Materials.—The rats used in this study were Harlan strain males weighing about 250 Gm. Animals of this size were 100–120 days of age and were considered sexually mature.

The nortriptyline HCl and amitriptyline HCl used to pretreat the rats were medicinal grade compounds obtained from the manufacturers. Both were incorporated as the dry powder into the standard rat diet by means of a Hobart mixer.

All of the neuro-pharmacologic agents used to challenge the pretreated rats were standard medicinal preparations. Most of them were soluble enough so that they could be injected intraperitoneally in aqueous solutions of suitable concentration. Acetylsalicylic acid, meprobamate, and isocarboxazid were given by the same route suspended in a 3% acacia vehicle.

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Method.—Rats were divided into groups so that one group received a standard control diet and a second group the same diet containing 0.04% nortriptyline HCl. For comparison purposes a third group was placed on a diet containing 0.04% amitriptyline HCl. The 0.04% drug concentration was great enough to produce overt signs of thymoleptic activity without undue debilitation of the animals. The rats were caged separately with food and water available *ad libitum*. After remaining on their respective diets for 11–15 days, five animals from each group were used for each interaction test. The compounds used to challenge the thymoleptic pretreated animal for a test of interaction included a variety of neuro-pharmacological agents with established clinical uses. In each interaction test predetermined doses of the challenging agent were given intraperitoneally every 30 min. to the control and thymoleptic pretreated rats until death ensued. A cumulative lethal dose (CLD) for the challenging agent was computed for the individual animals by multiplying the amount of drug per dose (mg./Kg.) by the number of doses required to produce death. An index of interaction (I.I.) was then established by dividing the geometric mean of the CLD values for the control animals by the geometric mean of the CLD's in each of the thymoleptic pretreated groups. Thus, an I.I. significantly larger than 1.00 indicated a synergistic interaction, while an index significantly less than 1.00 was considered evidence of antagonism.

RESULTS AND DISCUSSION

Table I shows the effects of pretreatment of rats with nortriptyline HCl and amitriptyline HCl on the acute toxicity of several compounds that have primary cerebral depressant activity.

Thymoleptic pretreatment augmented the lethal action of most of the cerebral depressants in rats. This observation was in accord with previous findings that many depressants increase nortriptyline toxicity in mice, and lends support to the statement that the newer antidepressives increase barbiturate toxicity in man (5). The increase in depressant toxicity may be explained in part by the fact that the tricyclic antidepressives possess a measurable CNS depressant component of activity (9). Contrary to some reports, this study failed to reveal any significant effect on ethyl alcohol toxicity by either amitriptyline or nortriptyline.

Table II illustrates the influence of nortriptyline and amitriptyline on the toxicity of various types of CNS stimulants.

TABLE I.—EFFECT OF THYMOLEPTIC PRETREATMENT ON THE ACUTE TOXICITY OF CEREBRAL DEPRESSANTS IN RATS

	Interaction Indices	
	Nortriptyline	Amitriptyline
Sodium pentobarbital	1.38[a]	1.40[a]
Sodium phenobarbital	1.08	1.11
Sodium secobarbital	1.54[b]	...
Methapyrilene HCl	1.67[b]	1.21
Meprobamate	1.07	1.33[a]
Chlordiazepoxide HCl	1.30[b]	...
Ethyl alcohol	.95	1.10

[a] $p = 0.1$. [b] $p = 0.05$.

TABLE II.—EFFECT OF THYMOLEPTIC PRETREATMENT OF RATS ON TOXICITY OF CNS STIMULANTS

	Interaction Indices	
	Nortriptyline	Amitriptyline
Pentylenetetrazol	0.94	1.01
Desoxyephedrine HCl	0.81	1.12
Isocarboxazid	0.92	0.90
Tranylcypromine sulfate	1.21	...
Procaine HCl	0.94	0.98
Methylphenidate HCl	1.01	...

TABLE III.—EFFECT OF THYMOLEPTIC PRETREATMENT OF RATS ON THE ACUTE TOXICITY OF AUTONOMIC AGENTS

	Interaction Indices	
	Nortriptyline	Amitriptyline
Ephedrine HCl	0.49[b]	0.64[a]
Isoproterenol HCl	0.83	0.96
Atropine sulfate	0.84	0.81[a]
Scopolamine HBr	1.00	0.87
Physostigmine sulfate	>2.72[b]	>3.60[b]
Pilocarpine HCl	1.68[b]	1.16[a]

[a] $p = 0.1$. [b] $p = 0.01$.

TABLE IV.—INFLUENCE OF THYMOLEPTIC PRETREATMENT OF RATS ON THE ACUTE TOXICITY OF VARIOUS OTHER AGENTS

	Interaction Indices	
	Nortriptyline	Amitriptyline
Meperidine HCl	0.92	1.06
Dextropropoxyphene HCl	0.82	0.68[a]
Acetylsalicylic acid	1.05	1.01
Chlorpromazine HCl	0.96	1.07
Scopolamine HBr	1.00	0.87
Nortriptyline HCl	1.15	...
Quinidine sulfate	0.92	0.95

[a] $p = 0.1$.

Although the intensity of the stimulant-induced convulsions was somewhat moderated in the pretreated animals, there was no significant interaction between the thymoleptics and any of the stimulants tested. Neither type of monoamine oxidase inhibitor, isocarboxazid or tranylcypromine, proved to be significantly more toxic in the pretreated animals.

The influence of pretreatment with the thymoleptics on the toxicity of various types of agents that actively affect the autonomic nervous system is shown in Table III.

Marked influences of the antidepressive pretreatment on the toxicity of some of the drugs which act upon the autonomic system were apparent. In general the toxicity of the adrenergic stimulants and cholinergic blockers was reduced. The most significant reductions occurred with ephedrine and atropine, both of which possess a measurable component of central stimulation. The most dramatic potentiation of toxicity was seen with physostigmine which killed the thymoleptic pretreated rats with intraperitoneal cumulative doses as low as 0.9 mg./Kg. Evidence that this potentiation of toxicity might be applicable to human medicine was found in a medical report which described an abnormal response to neostigmine in a patient who was concurrently receiving therapeutic doses of nortriptyline HCl (10). Pilocarpine toxicity was also increased in the pretreated animals but not in the same magnitude as was physostigmine.

The effect of the cyclazid antidepressives on the acute toxicity of other drugs with neuro-pharmacological properties is shown in Table IV.

The only compound in this group to show an interaction was dextropropoxyphene HCl. The significant reduction in toxicity can be accounted for by the fact that dextropropoxyphene when administered intraperitoneally or intravenously exhibits a stimulating action in rodents which contributes greatly to its lethality. It is probable that this component of action was antagonized by the thymoleptics. The result obtained with nortriptyline HCl indicated that neither tolerance nor sensitivity to the compound itself developed during the pretreatment period.

CONCLUSION

The data collected by the method described show that nortriptyline, like amitriptyline pretreatment, enhances the toxicity of most cerebral depressants in rats. Quantitatively, the results indicate that the increase is probably in the order of an additive effect.

The test confirmed that the concurrent use of physostigmine and the cyclazid antidepressives may produce dangerous results. The data indicate that the physostigmine–thymoleptic interaction should be described as marked potentiation. The increase in pilocarpine toxicity may also be regarded as potentiation but of a much milder degree.

The test failed to supply confirming evidence to clinical reports of the potentiation of the toxicity of alcohol by the antidepressive compounds. Nor did it substantiate the validity of the controversial warning that the concurrent use of thymoleptics and monoamine oxidase inhibitors should be strictly avoided.

The experimental results indicated that a wide variety of medicinal agents could be used following thymoleptic pretreatment without danger of drug interaction.

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Absorption, Metabolism, and Excretion of Salicylic Phenolic Glucuronide in Rats

By GERHARD LEVY, LEONARD WEINTRAUB*, TAI MATSUZAWA, and STANLEY R. OLES*

Salicylic phenolic glucuronide is excreted rapidly by apparent first-order kinetics ($t_{1/2}$ about 0.7 hr.) after intravenous administration to adult rats. Only a small fraction of an injected dose is hydrolyzed *in vivo* to salicylic acid. The glucuronide is apparently not absorbed significantly as such from the gastrointestinal tract. Part of an orally administered dose is absorbed apparently from the lower region of the intestinal tract after hydrolysis to salicylic acid.

THE PHARMACOKINETICS of elimination of salicylic acid and its major metabolite, salicyluric acid, have been studied in some detail in man and rats (1–4). Much less is known about the kinetics of elimination of salicylic phenolic glucuronide, another important metabolite of salicylic acid.[1] This glucuronide has been prepared in pure form and (since technical problems did not permit its intravenous administration to man) its absorption, metabolism, and excretion have been studied in rats. A previous study has shown that the pharmacokinetics of salicylate elimination in man and rats are quite similar (4).

EXPERIMENTAL

Synthesis of Salicylic Phenolic Glucuronide.—Methyl (tri-*O*-acetyl-α-D-glucopyranosyl bromide) uronate was prepared from glucuronolactone by the procedure of Bollenback *et al.* (6). From it, *o*-carboxyphenyl-β-D-glucopyranosiduronic acid (salicylic phenolic glucuronide) was obtained by methods described by Lunsford and Murphey (7), m.p. 144.5–146°. (Lit. 145–146°.) $[\alpha]_D^{27} = -70.8°$ (C, 6,H_2O). [Lit. $[\alpha]_D^{23} = -75.9°$ (C,6,H_2O).]

Anal.—Calcd. for $C_{13}H_{14}O_9$: C, 49.69; H, 4.49. Found: C, 49.64, 49.73; H, 4.87, 4.72.

No free salicylic acid was detected by a thin-layer chromatographic method capable of detecting 0.5% of this compound in the glucuronide.

Animal Study.—Male Sprague-Dawley rats, weighing 250–400 Gm., were fasted for 24 hr. prior to drug administration but had unrestricted access to drinking water at all times. The drug was dissolved in 1.5 ml. distilled water and injected intravenously into the femoral vein, or administered orally by stomach tube as 5 ml. aqueous solution. The rats were confined in plastic animal holders for the first 24 hr. in order to permit frequent urine and feces collections. The animals were then transferred to individual metabolic cages for another 24 hr. Food and water were freely available to the rats throughout the 48-hr. period of urine and feces collection.

Assay Methods.—Salicylic acid and its metabolites were determined in the urine by a modification of the methods of Smith *et al.* (8). Feces was homogenized with distilled water and assayed similarly.

RESULTS

The urinary excretion kinetics of salicylic phenolic glucuronide (SPG) as a function of time after intravenous injection are shown in Fig. 1. Excretion followed apparent first-order kinetics, with a half-life of about 0.7 hr. The slow initial excretion of SPG in one of the animals appears to be due to accidental extravascular injection of part of the dose. About 82% of the injected amount was recovered in the urine; of this, about 85% was excreted as SPG (Table I). A much smaller fraction of the dose (about 36%) was recovered in the urine after oral administration and the fraction of urinary salicylate excreted as SPG was also much smaller than after intravenous injection (Table I). No measurable amounts of salicylic acid or its metabolites were present in the feces after intravenous administration of SPG. The time course of urinary excretion of total salicylate after oral administration of SPG indicates an appreciable delay in absorption (Table II).

DISCUSSION

Man excretes SPG relatively rapidly by a combination of glomerular filtration and tubular secretion (9). The rapid excretion of intravenously administered SPG by rats is consistent with these observations.[2] The renal excretion kinetics of SPG are not affected by urine pH and urine flow rate (9); this probably accounts for the lack of significant variation between animals in the excretion half-life of SPG. The excretion of SPG mainly as such after intravenous administration suggests that β-glucuronidase mediated hydrolysis in the tissues is too slow to reduce appreciably the net formation rate of SPG after salicylic acid administration. Inhibitors of β-glucuronidase, such as glucuronolactone, are therefore unlikely to enhance salicylate elimination. This conclusion is consistent with the results of a recent study (4). The lack of biliary excretion of SPG, suggested by the absence of detectable amounts of salicylates in the feces and by the absence of secondary maxima in the excretion rate *versus* time curve after intravenous injection of SPG, is in agreement with the observations of Williams *et al.* (10). These workers recovered just

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[1] Only a small fraction of salicylic acid is biotransformed to salicylic acyl glucuronide in man (5).

[2] The rapid excretion of SPG after intravenous injection indicates also that the SPG found in the urine is not the product of conjugation of free salicylic acid derived from the *in vivo* hydrolysis of injected SPG. Excretion of SPG by the latter mechanism is rate-limited by the formation process and would proceed at a considerably lower rate (4).

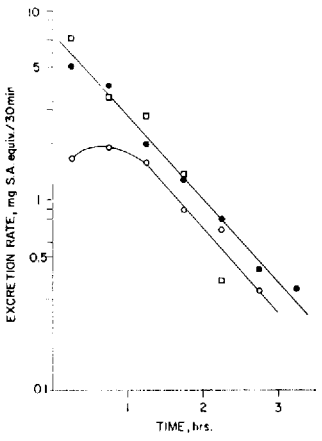

Fig. 1.—Urinary excretion rate of salicylic phenolic glucuronide in three rats as a function of time after intravenous injection of 120 mg./Kg. body weight. The slow initial excretion of SPG in one of the animals (○) appears to be due to accidental extravascular injection of part of the dose.

TABLE I.—URINARY RECOVERY OF SALICYLATE AFTER INTRAVENOUS AND ORAL ADMINISTRATION OF SALICYLIC PHENOLIC GLUCURONIDE TO RATS

Rt.	Animal	Dose, mg./Kg.	Total Urinary Recovery, % of Dose	% of Recovered Drug Excreted as Glucuronide
i.v.	*1*	120	86	86
i.v.	*2*	120	75	79
i.v.	*3*	120	84	89
Oral	*4*	600	41	22
Oral	*5*	600	28	6
Oral	*6*	600	39	29

1.5% of the dose (and only in the form of free salicylic acid) in the bile of rats within 24 hr. after i.p. injection of 50 mg. salicylic acid/Kg. body weight.

Vogt *et al.* (11) have shown that appreciable β-glucuronidase activity is located in the large intestine of the rat, but that no significant activity is present in the small intestine. They observed that desacetylbisacodyl, when administered as the glucuronide, is absorbed only in the large intestine, after enzymic hydrolysis. There is strong evidence

TABLE II.—TIME COURSE OF URINARY EXCRETION OF TOTAL SALICYLATE AFTER ORAL ADMINISTRATION OF SALICYLIC PHENOLIC GLUCURONIDE TO RATS[a]

Time, hr.	Amt. Excreted, mg. Salicylic Acid Equiv. Rat *4*	Rat *5*	Rat *6*
0–3	5.8	1.9	24.1[b]
3–5	2.0	1.2	
5–24	21.8	15.4	
24–48	2.8	4.9	12.1

[a] The administered amounts of SPG, expressed as mg. salicylic acid equivalent, were: rat *4*, 79.16 mg.; rat *5*, 83.55 mg.; rat *6*, 92.35 mg. [b] Only a single urine collection was made in rat *6* during the first 24 hr.

that other glucuronides also are not absorbed as such (10, 11). The approximately 4-hr. delay in absorption observed by Vogt *et al.* is similar to the delay in salicylate absorption after oral administration of SPG as judged by the time course of urinary excretion of total salicylates (Table II). The composition of urinary metabolites after oral administration of SPG (Table I) is similar to that which would be expected after administration of free salicylate (4). The very small SPG fraction in rat *5* appears to be due to the particularly slow absorption of salicylate in this animal and the resulting lack of saturation of the salicylurate formation process (4). The delayed and incomplete absorption of salicylate after oral administration of SPG, and the difference in the composition of urinary metabolites of SPG after oral and intravenous administration, respectively, suggest strongly that SPG is absorbed from the gastrointestinal tract of the rat only after hydrolysis to salicylic acid.

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Determination of Acetyl Values

Sir:

The determination of acetyl values of acetylated fatty acids as described in the "National Formulary," 12th ed. (1), was adapted from the derivation by Cook (2). This procedure is also very useful where hydroxyl groups exist together with esters. Cook's derivation provides a mathematical correction for the molecular weight difference between an original fatty material and the acetylated product and requires the determination of the saponification values of both materials.

The equation employed by the N.F. substitutes the acid value of the unacetylated free fatty acid for the saponification value of the original fatty substance in the Cook formula. The acid value of a free fatty acid is equivalent to its saponification value since there are no esters present. The N.F. equation, however, is incorrect in the denominator term. Since the correct denominator is a function of the saponification value *of the original fatty substance* (not the acetylated product), the proper denominator should be based on the acid value of the free fatty acids. The correct equation is

$$A = S - F/1 - 0.00075\ F$$

where A is the acetyl value of the acetylated fatty acids, S is the saponification value of the acetylated fatty acids, and F is the acid value of the original fatty acids. The existing official formula gives values which are 10 to 15% high for usual monohydroxylated fatty acids. Di- and trihydroxylated fatty acids show progressively higher deviations. The corrected formula above is in exact agreement with theoretical values in all cases. The term "A" is incorrectly defined in the monograph as the acetyl value of the *free* acid. This term must be the acetyl value of the *acetylated* fatty acid which would be in agreement with the N.F. definition of acetyl value and with the Cook formula derivation.

In the specific case of fatty acids, it is also possible to further simplify the calculations and the analytical procedure since all saponifiable groups are hydrolyzed prior to acetylation. The absence of interfering mixed anhydrides referred to by Warth (3) is assured since the acetylated product is purified by twice boiling in water. After saponification of the fatty material and acetylation of the fatty acids, it is only necessary to determine the ester value of the acetylated product as described in the N.F. In this case, ester value and acetyl value are identical. If acetylated fats, oils, waxes, or similar substances containing ester groups other than acetyl are used, the official methodology must be employed.

Results obtained using the official procedure and ester value determinations are compared to theoretical acetyl values in Table I for acetylricinoleic acid and cholesterol acetate.

TABLE I.—ACETYL VALUES

Compd.	Theoretical	Official Method[a]	As Ester Value
Acetylricinoleic acid	164.76	163.13	162.41
Cholesterol acetate	130.86	136.26	123.65

[a] Corrected formula.

Since the procedure described in the N.F. is very reliable and applicable to a wide variety of fatty materials, the scope of the monograph could be expanded to include these substances. The formula for calculation of results and the definition of the term "A" should be corrected as indicated.

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Apparent Renal Tubular Secretion of Riboflavin in Man

Sir:

The authors recently have shown that riboflavin is absorbed from the gastrointestinal tract of man by a specialized transport process rather than by passive diffusion (1). Data have now become available from the study of Stripp (2) which permit determination of the mechanism of renal excretion of this vitamin. Figure 1 is a plot of riboflavin excretion rate *versus* time during and after administration of 84 mg. riboflavin to a human by slow intravenous infusion. The data were obtained from a photographic enlargement of Fig. 6 of Stripp's paper (2). The postinfusion excretion rate declined exponentially as a function of time, with a half-life of 1.2 hr. This value is in excellent agreement with the 1.1 hr. average value observed after oral administration of riboflavin (1).

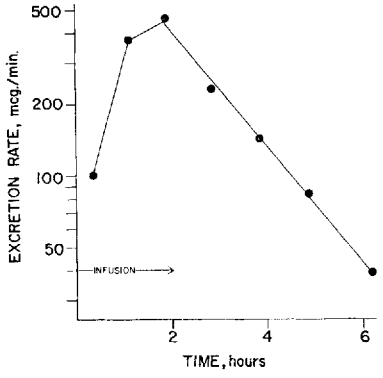

Fig. 1.—Excretion rate of riboflavin as a function of time during and after intravenous infusion of 84 mg. of riboflavin (data from Fig. 6 of *Reference 2*).

Apparent volumes of distribution for riboflavin have been calculated from Stripp's urinary excretion and blood level data (2) by three standard pharmacokinetic methods (see *References 3* and *4*) and are listed in Table I. There was good agreement between the results obtained by these different procedures. Renal clearance values were also calculated by three different methods and are listed in Table II. The calculations are based on riboflavin plasma concentrations which have not been corrected for protein binding (5) and are therefore minimum values which, had the extent of protein binding been known and taken into account, would have been even higher. However, even these minimum values are about twice as large as the glomerular filtration rate of 131 ± 22 ml./min. in normal man (6). This indicates that the renal excretion of riboflavin involves not only glomerular filtration but also renal tubular secretion. It is of interest that at least two other B vitamins, thiamine and pantothenic acid, are actively secreted by the renal tubules (7, 8).

TABLE I.—ESTIMATION OF THE APPARENT VOLUME OF DISTRIBUTION OF RIBOFLAVIN

Method of Calculation[a]	Determinations, No.	Vol. of Distribution, L. Mean	Range
$\frac{A}{C}$	5	31	29–33
$\frac{dU/dt}{kfC}$	6	26	23–30
$\frac{D}{k \text{ area}}$	1	26	...

[a] A = amount in the body; C = plasma concentration; dU/dt = urinary excretion rate; k = apparent first-order elimination rate constant; f = fraction excreted unchanged; D = dose; area = area under the plasma level *vs.* time curve.

TABLE II.—ESTIMATION OF THE MINIMUM RENAL CLEARANCE OF RIBOFLAVIN

Method of Calculation[a]	Determinations, No.	Min. Renal Clearance,[b] ml./min. Mean	Range
$\frac{Akf}{C}$	5	290	270–310
$\frac{dU/dt}{C}$	6	240	220–280
$\frac{Df}{\text{area}}$	1	240	...

[a] Abbreviations as in Table I. [b] Not corrected for plasma protein binding.

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New Method of Solid-State Dispersion for Increasing Dissolution Rates

Sir:

The authors have recently reported on the potential value of solid-state dispersion, *viz.*, solid solutions, in enhancing the dissolution rate and gastrointestinal absorption of drugs (1–4). Although this approach has been quite successful in modifying the dissolution characteristics of griseofulvin (3) and chloramphenicol (4), the application of solid solutions formed by fusion technique, for enhancing dissolution, is somewhat limited. The method has been found inapplicable to a number of drugs which are unstable at or near their melting points, or which fail to crystallize from the mixed melt.

Therefore, other methods have been sought to obtain solid-state dispersions of insoluble drugs in water-soluble matrices. In 1965, Tachibana and Nakamura (5) reported a method for preparing aqueous dispersions of β-carotene by using water-soluble polymers.

An earlier report by Stone (6) described a similar technique for the preparation of water dispersible antibiotics. This preliminary communication concerns the use of the dispersion method to obtain physically modified forms of a drug, which are much more rapidly soluble than the pure compound. Although the mechanism of the dispersion has not yet been rigorously established, the striking findings and the apparent general applicability of the method warrant consideration.

The authors' initial studies were concerned with dispersions of griseofulvin in polyvinylpyrrolidone (PVP). The drug was crystallized from a 1% solution in chloroform containing 0, 5, 10, and 20% PVP. In each case the solvent was evaporated at 37° until a clear film, essentially free of solvent, remained. The film was further dried to constant weight, and the material was then sized using a Syntron shaker. The 40–50 mesh particles were collected for dissolution rate studies.

Sekiguchi *et al.* (7) have reported that griseofulvin forms a 1:1 solvate with chloroform. In the present work, the existence of the solvate was confirmed; but it was found to be quite unstable and decomposed rapidly under our drying condition. In order to determine the dissolution rate of the solvate, a sample was carefully prepared. Analysis of the material immediately before dissolution rate studies indicated that it contained about 23% chloroform corresponding to a 1:1 solvate.

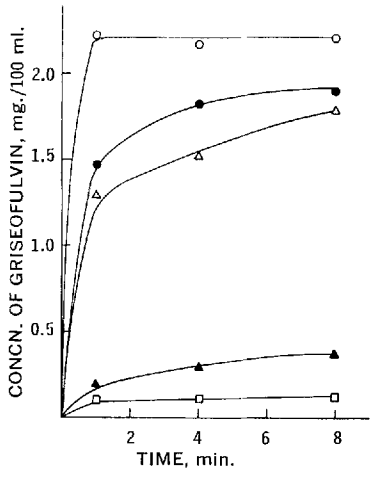

Fig. 1.—Dissolution rate of micronized griseofulvin (▲); griseofulvin chloroformate (□); griseofulvin–PVP, 1:5 (△); griseofulvin–PVP, 1:10 (●); and griseofulvin–PVP, 1:20 (○).

TABLE I.—DISSOLUTION STUDIES OF GRISEOFULVIN

Sample	Relative Dissolution Rate 1 min.	4 min.
Micronized griseofulvin	1.0	1.0
Griseofulvin–chloroform solvate	0.5	0.4
Griseofulvin–PVP (1:5)	6.1	5.1
Griseofulvin–PVP (1:10)	7.2	6.1
Griseofulvin–PVP (1:20)	11.0	7.3

Particulate dissolution rate was determined in the following manner. Each sample, containing 10 mg. of griseofulvin, was added to a 600-ml. beaker containing 300 ml. of distilled water at 37°. Stirring was provided by an overhead stirrer operating at 150 r.p.m. In order to overcome the "nonwetting" character of the micronized griseofulvin[1] (included in the study to provide a basis of comparison) the dissolution medium contained 0.02% polysorbate 80. The wetting agent was used in each dissolution study, although it proved inconsequential with the PVP–griseofulvin samples. After the addition of the drug, 5-ml. samples of the dissolution medium were taken periodically, rapidly filtered through a Millipore filter (0.45 μ), and assayed for griseofulvin using a Beckman DB recording spectrophotometer.

The results of this investigation are shown in Fig. 1. Table I provides a comparison of the dis-

[1] The griseofulvin used in this investigation was generously supplied by the Schering Corp.

solution rate of the various samples with the dissolution rate of micronized griseofulvin. The solid-state dispersion of griseofulvin in PVP results in a five- to tenfold increase in the dissolution rate of the drug. In the absence of wetting agent in the dissolution medium the enhancement is still greater.

Although some complexation seems to exist between griseofulvin and PVP (on the basis of preliminary solubility studies), the authors do not believe that this imposes a restriction on the utility of this dispersion technique for a large number of water-insoluble drugs. Based on our initial observations, it is suggested that griseofulvin is dispersed molecularly in the polymer film and forms a solid solution with PVP in the film. It is believed that this approach to the modification of drug properties may be of broad import in the area of biopharmaceutics and may find significant therapeutic application.

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The data contained in this volume are a creditable compilation but should be augmented for extensive and accurate identification. Improvement, is required in the form of definitive identification tests for many substances, and wherever feasible the tables should be expanded to include entries for nonofficial drugs. A worthwhile addition would be classical tabulations by functional groups which could be used in conjunction with accrued preliminary data for rapid identification of many compounds.

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Review Article

Powders: Particle–Particle Interactions

By EVERETT N. HIESTAND

BECAUSE nearly all medicaments exist as powders at some stage in their manufacture, it is not considered necessary to justify an interest in powders.

Many difficulties are associated with the study of the powdered state of solids. Even today technological advances have not produced satisfactory quantitative methods for describing their properties. However, it is possible to define more precisely the problems associated with characterizing powders and to discuss in a qualitative manner the factors that determine their physical properties. Because much of the work pertinent to the subject is scattered throughout the literature and has been done outside of the pharmaceutical field, it seems worthwhile to attempt to organize this material into a review article.

The emphasis of this discussion will be on the properties of solids that determine the magnitude of the forces acting between the particles when they are in contact. These are predominately surface properties. If the particles have been compressed enough to produce plastic deformation or crushing of the particle, the mechanical properties become very important also. Since compressed tablets compose the largest volume of pharmaceutical products sold, it is tempting to extend the discussion to include the compression of particles into aggregates. However, in the interest of brevity, the scope of this review will be limited to particulate solids under compression forces of a much smaller nature than used in tableting. For example, forces such as might be exerted by the weight of the powder bed on the particles at the bottom of the bed or the forces imposed by an auger in moving the material. The theoretical discussion will describe the properties of solid surfaces. The discussion of experimental methods will emphasize methods of measuring the cohesion and adhesion of powder particles.

Definition of the Problem.—The bulk density of a powder bed is not uniform. Therefore, the physical properties of the bed are not uniform either. The properties of a powder bed depend on the cumulative effect of the previous history of all the portions of the bulk being considered. Isolated regions of shear, vibration, or compaction may have produced high bulk density regions. These may remain intact in subsequent flow of the powder or may fragment into macroscopic regions mixed throughout the less dense bed. The forces acting on the top of a bed may be quite different from those at the bottom of the bed. Therefore, a thermodynamic treatment of a powder bed as a homogeneous mass is not possible. This discussion will try to develop, qualitatively, the factors that contribute to the interaction of solids. The following are considered the main topics of interest.

(*a*) The interaction between two particles is

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dependent upon the surface to interfacial energy change that occurs when the solids come into contact. The surface energy of a solid rarely is homogeneous. Therefore, the energy change at the true areas of contact will not be a single function of the true area of contact but will vary with the nature of the exact portions of the two solid surfaces in contact.

(*b*) The area of true contact between individual particles is dependent on the particle shape, size distribution, roughness, the compressive force at the interface, the shear to which the sample has been subjected, and the mechanical properties of the particles. Also, the number of nearest neighbors, *i.e.*, the coordination number, will be a function of these same factors.

(*c*) Most organic solids are insulators. Unless their surface is made conducting by contaminants such as surfactants or water (especially under high humidity conditions), the solids will charge on contact. The resulting electrostatic attraction may be large.

GENERAL BULK PROPERTIES OF SOLIDS

When the forces acting between molecules are strong enough to overcome the translational motion of the molecules and to compensate for necessary entropy changes, the molecules associate as a solid mass. If the association is in a predominately orderly manner, the solid is said to be crystalline. The crystalline solid is not necessarily the permanent, fixed arrangement often implied in elementary texts. Entropy *versus* temperature curves may provide evidence of nonisothermal changes in addition to the isothermal changes that occur when there is a change in crystal structure (1). Globular solids are said to be the antipode of liquid crystals (2). In globular solids, rotational freedom apparently occurs at lower temperature than the translational freedom of the molecules, whereas in liquid crystals, translational motion occurs prior to rotational freedom. These phenomena suggest that the properties of all crystalline solids are not the same and that the physical properties are not a single entity for a given solid substance but may be strongly temperature-dependent.

The order within the crystal always is much less than perfect. Solids often consist of numerous intimately associated grains. Crystal lattice continuity does not exist across these grain boundaries. Within the grains, numerous crystal dislocations, both screw and edge type, exist (3). The mechanical properties of solids are determined by the freedom of these dislocations to move. If they move readily, the solid will be plastic. If they cannot move, the solid will be brittle (4). The mechanical strength of brittle solids will vary with the presence or absence of surface cracks (5). Also, the mechanical properties are influenced by the surface energy. The mechanical properties of solids are a principal factor in determining the true area of contact between solid materials under pressure.

The mechanical properties of organic solids have had only limited attention and our knowledge of the role of dislocations must, for the most part, be extrapolated from the studies on metals, semiconductors, and a few inorganic materials. However, dislocations in organic crystals do exist (6).

Some grain boundaries and dislocations extend to the surface of crystals and provide one source of heterogeneity on the surface. Adsorption of impurities result in additional sources of surface heterogeneity.

Because our primary interest is the forces of interaction between two surfaces brought into contact, the bulk properties will not be discussed in greater detail. However, it is necessary that one recognize that both the bulk and surface properties of a given substance may vary significantly and particle–particle interactions may be influenced by these variations. Furthermore, plastic flow is known to occur in many organic solids. Globular solids are sometimes called "plastic crystals." Some nonglobular solids may be tableted directly. This would not be possible unless plastic flow occurred to relieve elastic stresses.

THE "ADSORPTION" THEORY OF PARTICLE–PARTICLE INTERACTIONS

The Minimum Work of Fracture.—The minimum work required for the separation of two surfaces and, therefore, the energy bonding them together is equal to the difference in free energy after separation and the free energy before separation, *i.e.*, for materials C and D, the work of adhesion, W_a, is given by:

$$W_a = A\ (\gamma_C + \gamma_D - \gamma_{CD})$$

where

A = the area produced by the separation,
γ_C = the free energy of the surface per unit area of the solid C in air,
γ_D = the free energy of the surface per unit area of the solid D in air, and
γ_{CD} = the free energy of the C–D interface per unit area.

Similarly, for the fracture of a single material, for

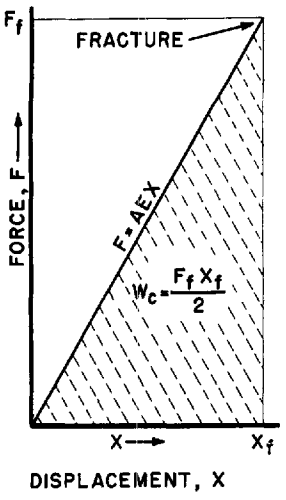

Fig. 1.—The minimum work of fracture of an ideal elastic body.

example, substance C, the work of cohesion, W_c, is given by:

$$W_c = 2A\ \gamma_C$$

If substance C obeys Hooke's law until it fractures, then the stress-strain diagram is as shown in Fig. 1. The equation for the line is $F = AEX$, where E is Young's modulus, F is the tensile force, and X is the displacement. The minimum work done in fracturing the solid, *i.e.*, the work of cohesion, is the area under the line, $W_c = F_f X_f/2$; where F_f is F at fracture and X_f is the displacement at fracture.

Eliminating X_f from the work equation gives $W_c = F_f^2/2AE$. Replacing W_c with the surface energy from our earlier equation, one obtains on rearrangement:[1]

$$F_f = 2A\ \sqrt{\gamma_C E}$$

The analogous equation for W_a is:

$$F_f = 2A\ \sqrt{\frac{E_C + E_D}{E_C E_D}\ (\gamma_C + \gamma_D - \gamma_{CD})}$$

The notion that the cohesion of solids is related to the surface energy is not new. Bradley (8), in 1932, discussed the role of dispersion forces in surface energy and in the cohesion of solids. Morgan (9) and Rumpf (10) have considered the strength of attraction between solids in a similar way. Recently Krupp and Sperling (11) have developed a model for the interaction of powder particles that involves the surface energy.

[1] Note the similarity of this equation to the fundamental equation of the Griffith crack theory of the strength of brittle solids (5, 7) which is:

$$F = K\sqrt{\frac{\gamma E}{C}}$$

where K is a proportionality constant and $2C$ is the crack length.

The Surface Energy of a Solid.—The argument in the preceding paragraphs has demonstrated that the surface energy of a solid is an important term in the strength of attraction between two substances. Therefore, the factors contributing to surface energy must be explored if even a qualitative concept is to be obtained. Part of the information may be inferred from our knowledge of liquids. First, let us consider the differences between solid surfaces and liquid surfaces.

Obviously, the relative immobility at room temperatures of the molecules of a solid account for the differences between the solid and liquid. For example, the chemical potential is the same in both the surface and bulk of a liquid phase, even when more than one component is present. However, for the solid, the surface usually is impure and the surface layer cannot equilibrate with the bulk in any reasonable length of time. Therefore, the chemical potential of a component on the surface may be quite different from that in the bulk.

The surface layer of a liquid has a lower density of molecules than the bulk. Since these molecules are free to move in the surface plane, they tend to undergo a two-dimensional "condensation," which results in a tension force in the surface plane. Because of random fluctuations, this process is never completed; the "condensed" molecules disappear into the bulk and are replaced randomly by other molecules. Gurney (12) discusses the thermodynamics of surfaces and concludes that equilibrium can exist only when the surface molecules are under a tension stress.

The tension component in the surface of liquids depends on the mobility of the surface layer. A solid surface cannot produce a tension component unless its surface molecules are mobile also. However, the unbalance of forces acting at the surface does result in a significant surface energy. Often the terms surface tension and specific surface free energy of a solid are carefully defined mathematically but the molecular model is not discussed. A recent review of the surface tension of solids (13) is of interest and illustrates this point. In this review, reference will be made only to the specific free energy of solids.

Attraction Forces Between Molecules at Interfaces.—There is an oft-stated observation that oil repels water. This useful though inexact statement could induce careless thinking. Only differences in magnitude of attraction are involved, not repulsion. All electrically neutral materials attract one another by dispersion

forces except when the spacing between them is so small that electron orbitals overlap. Attraction forces of greater magnitude than the dispersion forces often exist. The only repulsion force known that acts over a long distance is the electrostatic repulsion of like charges. Water is attracted to paraffin. A small droplet of water will cling to the underside of a paraffin surface. However, when the size of the drop is increased, enough gravitational force exists to overcome the attraction of the water for the paraffin and it falls off. Each water molecule has greater attraction for the other water molecules than for the paraffin; therefore, the entire drop falls away.

The origin of the attractive forces between two different solids in contact should not be a mystery to anyone since they are the same forces that act to hold all solids together. The one whose magnitude is most commonly underestimated is the London–van der Waals' force or the dispersion forces. Too often, the dispersion forces are described as "those weak interactions such as between hydrogen molecules, which explains it being a gas." However, these same forces cause many materials to be a solid at room temperature, *e.g.*, the longer chain aliphaptic hydrocarbons. Unfortunately, theoretical treatments such as those given by Rowlinson (14), Debye (15), and de Boer (16) often do not leave the reader with an intuitive feel for their magnitude. Consequently, we shall look at some experimental evidence that indicates the relative importance of various forces.

Solubility Parameters.—The development of the solubility parameter concept provided considerable insight into the interactions between unlike molecules. However, the primary reason for including the solubility parameter in this discussion is to show the relationship of surface tension to the cohesion energy density. The term "cohesive energy density" is the square of the solubility parameter δ. Solubility parameters are intended only for "regular solutions" (17–20), *i.e.*, nonpolar substances. This parameter has been identified with the heat of vaporization:

$$\delta = \left(\frac{\Delta E}{V}\right)^{1/2}$$

where ΔE is the energy of vaporization to a gas at zero pressure and V is the molal volume of the liquid. Also, $\delta \simeq 1.2\, a^{1/2}/V$, where a is van der Waals' gas constant.

Note that all the above mathematical relations are measures of the cohesion of the individual liquids. Hildebrand gives for mixing components 1 and 2:

$$\frac{\Delta H_m}{V_m \phi_1 \phi_2} = (\delta_1 - \delta_2)^2$$

where ΔH_m is the heat of mixing, V_m is the total volume of the mixture, ϕ_1 and ϕ_2 are the volume fractions of the respective component in the mixture.

Thus, the square of the differences in the square roots of the cohesion energy densities gives the heat of mixing if corrected for volume effects. Solubility results when the difference $\delta_1 - \delta_2$ is small, *i.e.*, the heat of mixing does not dominate the entropy contribution to the free energy change of the solution process.

Another interesting relationship is $\delta = 4.1\,(\gamma/V^{1/3})^{0.43}$,[2] where γ is the surface tension. The experimental verification of this relationship is evidence that the surface tension results from the attraction between molecules. Each of these equations express the interaction of molecules with each other.

Interfacial Tension Method of Evaluating Interactions.—A more recent approach to describing the forces between unlike molecules is given by Fowkes (21–23). He has obtained, by a systematic use of interfacial tension values, a measure of the contribution made by dispersion forces and the other types of forces acting at interfaces. His model makes use of the fact that the surface tension in air is a measure of the attraction due to all intermolecular forces in the liquid. Also, he assumes that the dispersion forces at the interface between two substances produce an interaction between unlike molecules. The interaction from dispersion forces is equal to the square root of the product of the dispersion component for each. He writes two equations—one for the contribution of each material to the interfacial tension. Since each equation describes only one liquid at the interface, let us call it the 1/2-interface equation. If the surface tension of C in air is γ_C and of D is γ_D, and provided only dispersion forces produce an interaction between C and D, the 1/2-interface equation for component C is:

$$\gamma_{C^{1/2}} = \gamma_C - \sqrt{\gamma_C{}^d \gamma_D{}^d}$$

where $\gamma_D{}^d$ and $\gamma_D{}^d$ are the respective contributions to surface tension produced by the dispersion forces.

For component D the 1/2-interface equation is:

$$\gamma_{D^{1/2}} = \gamma_D - \sqrt{\gamma_C{}^d \gamma_D{}^d}$$

The sum of the two yield the interfacial tension between C and D:

[2] This is an empirical relationship that gives more accurate results than the dimensionally homogeneous form $\delta \propto (\gamma/V^{1/3})^{0.5}$.

$$\gamma_{CD} = \gamma_C + \gamma_D - 2\sqrt{\gamma_C{}^d\gamma_D{}^d}$$

Since γ_{CD}, γ_C, and γ_D each can be determined by an independent experiment, it is necessary to know only either $\gamma_C{}^d$ or $\gamma_D{}^d$ before the other can be calculated. In the special case of a liquid such as an aliphatic hydrocarbon whose molecular interactions are due only to dispersion forces, $\gamma = \gamma^d$. For example, the surface tension of benzene is about 29 dynes/cm. and is due entirely to dispersion forces. Furthermore, in contact with mercury, only dispersion forces act between benzene and mercury. If benzene is called C, then $\gamma_C = \gamma_C{}^d = 28.85$ dynes/cm. Mercury is liquid D, so $\gamma_D = 484$ dynes/cm. γ_{CD} is measured and is 363 dynes/cm. Substituting into the equation and calculating $\gamma_D{}^d$, one obtains $\gamma_D{}^d = 194$ dynes/cm. Then one may calculate $\gamma_{C1/2}$ which turns out to be about -47 dynes/cm. The negative sign results because the mercury attracts the benzene stronger than the benzene attracts itself. Similarly, $\gamma_{D1/2} = 408$. Fowkes finds as average values for the mercury–air interface about 284 dynes/cm. is due to the metallic bond and 200 dynes/cm. from the dispersion forces of the mercury.

Similarly, Fowkes shows that the dispersion forces for water account for 21.8 dynes/cm. of its 72.8 dynes/cm. total surface tension. This value comes from interfacial tension data taken at a water–straight chain hydrocarbon interface. However, for the water–benzene case the assumption of only dispersion force interactions between water and benzene is not valid. About 13–16 dynes/cm. come from other forces (perhaps π bonds).

The metallic bond of mercury and the hydrogen bond in water should not interact at the water–mercury interface. However, it was questioned whether the permanent dipole of water might induce an image dipole in the mercury to produce a net attraction. Theory predicts this to be negligible and the measurements made by Fowkes confirm the prediction.

Fowkes has extended his studies to solid–liquid interfaces. Table I lists a few values for the dispersion forces of solids.

TABLE I.—γ^d VALUES FOR SOLIDS[a]

Solid	γ^d(ergs/cm.^{2})
Paraffin wax	25.5
Polytetrafluoroethylene	19.5
Polyethylene	35.0
Graphite	120–132
Copper	60.0
Lead	99.0
Silica	78.0

[a] Data from *Reference 22.*

TABLE II.—γ_c VALUES FOR POLYMERIC SOLIDS[a]

Solid	γ_c(dynes/Cm.)
Polyhexafluoropropylene	16.2
Polytrifluoroethylene	22.0
Polyethylene	31.0
Polymethyl methacrylate	39.0
Polyhexamethylene adipamide	46.0

[a] Data from *Reference 25.*

Critical Surface Tension.—Zisman and coworkers (24–26) have used the equilibrium contact angle method to assign an average surface energy value to the solid surface. A homologous series of liquids is placed on the surface of the solid and their respective contact angles are observed. A plot is made of cos θ *versus* γ_{lv} where γ_{lv} is the liquid surface tension of liquid in equilibrium with its saturated vapor in air. This plot is extrapolated to zero contact angle, and the value of the surface tension at this point noted. This surface tension at zero contact angle is called the critical surface tension, γ_c. The critical surface tension is assumed equal to the specific surface energy of the solid (23).

The technique sounds easy, but only when homologous liquids are used does one obtain a straight line. Otherwise, scatter and/or curvature exists. The experimental problems are many because of the difference between advancing and receding contact angles that often are observed. Also, contamination and roughness of the surfaces may vary and influence the results. Nevertheless, the critical surface tension has been identified within a narrow range on some solids. The γ_c values of some of these are given in Table II.

Friction and Adhesion.—In this discussion, the consideration of the forces between molecules has progressed from studies of liquid–liquid to solid–liquid interfaces. Studies of friction and adhesion of solids focuses the attention directly on the solid–solid interface. Here, for the first time, one must consider the important problem of determining the area of true contact. Since the true contact area is unknown in nearly all cases, it is most difficult to evaluate solid–solid interactions on a quantitative basis.

Friction sometimes is defined as the shear force required to break the adhesion "bonds" that form when solids are in contact, and the adhesional force is the tensile strength of the same bonds. This is the position taken in the classic work of Bowden and Tabor (27–30). They claim that the friction manifestation of adhesion is readily observed with large objects because all the "bonds" are sheared simultaneously in a friction measurement. However,

the tensile strength manifestation of adhesion usually is not observed because the elastic energy stored in the touching asperities is released gradually on separation, *i.e.*, the asperities have different lengths so that the separation does not occur simultaneously.

The classical, empirical laws of friction state that the frictional resistance to movement is independent of the apparent geometrical area of contact. It depends directly on the normal load. In the modern concept, if all unit areas of true contact have equal shear strength, then the true area of contact must be identical for a given normal load independent of the apparent geometrical contact area. Actually, exceptions to this law are readily found. However, the rarity of exceptions to the classical laws is remarkable when one considers the vast variety of surfaces that may be in contact.

The importance of the true area of contact has led to a study of adhesion and friction under very special conditions. Some of these illustrate the importance of the mechanical properties of solids and account for the author's brief treatment of the mechanical properties in an earlier part of the discussion.

A steel ball pressed into freshly scraped indium exhibits very large adhesive forces. When removed, the steel surface is covered with indium showing adhesion to be stronger than the cohesion of the indium (27). Obviously, the choice of indium is because of the low yield value that must be overcome to produce plastic flow. In plastic flow the elastic energy is reduced to a very small value, and the area of intimate contact becomes very large. Hence, the mechanical properties of indium make it possible to observe the adhesional force in a tension measurement.

The importance of a clean metallic surface, as in the freshly scraped indium above, is demonstrated in another experiment. Two clean iron surfaces, cleaned by outgassing under vacuum at high temperature, were pressed together with an initial load of only a few grams. Yet, over 500 Gm. was required to pull them apart. The metallic junction area was estimated and found to have the bulk strength of iron (29). Stepwise addition of traces of oxygen reduced the cohesion stepwise. Even H_2 and He will prevent gross seizure. The surface contamination apparently prevents the cold welding of the metals. Metallic bonding is much stronger than dispersion forces and the adsorbed contaminants prevent the stronger metallic bond from forming. The contaminants, thereby, reduce the energy of interaction.

The influence of a monolayer of lauric acid on the adhesion between two metallic surfaces has been measured (28). The monolayer was spread on the plastic surface of indium. The indentation produced by the steel ball pressed into the indium surface caused an increase of surface area so that some uncoated area of indium was formed at the indium–steel interface. The direct contact of metallic surfaces at the uncoated areas permitted metallic bonding to occur and produced high adhesion, in proportion to the area of metal–metal contact area.

Friction studies have not been limited to metals. Bowden (30) has reviewed adhesion and friction for various materials. Polymers represent a plastic material that deforms readily under load. He concludes that organic polymers under light loads exhibit friction forces that follow the same variation with load as the area of contact. Also, it appears that many polymers adhere strongly to metals and the shearing plane is in the polymer and not at the interface. Thus, transfer or wear occurs. With polytetrafluoroethylene (Teflon), the adhesion is small, slip occurs at the interface, and wear is minimal.

Also, it is of interest to consider elastic materials. Bowden (30) discusses the friction of diamond on diamond. Because diamond is a highly elastic material, the real area of contact is not expected to be proportional to the load but to the load to the 2/3 power. As expected, the friction coefficient decreases as the load increases. Removing gas films from diamond causes the friction coefficient to increase markedly, apparently due to the stronger forces of interaction at uncontaminated diamond interfaces. Estimates of the true area of contact indicate that for clean diamond sliding over clean diamond *in vacuo*, the effective shear strength of the regions of true contact is comparable to the bulk strength of diamond.

Another interesting series of studies on the interaction between solid surfaces involve molecularly flat surfaces of mica. Freshly cleaved but uncontaminated mica surfaces can be put back together with very little loss in strength. However, if the sheets are separated and coated with a monomolecular layer of a fluorinated fatty acid and then placed together, the work required to separate them is reduced. The reduction is believed to be in proportion to the surface energy, *i.e.*, to the work necessary to create the new area of solid–air interface. The shear strength of freshly cleaved mica surfaces in contact was very high and the surface damage from sliding was great. However, monomolecular layers of cal-

cium stearate placed on the sheets reduced the shear strength markedly and left the surface undamaged by the shearing process. Obviously, the shearing plane remained in the interface when contaminants reduced the energy of interaction. These studies are mentioned by Bowden (30). Much of the work is that of Bailey, some of which is reported in *References 31* and *32*.

In a later section of this review, it will become evident that much of the experimental effort to study powders has been by friction measurements. Hence, the above information from some of the general studies of friction will be useful later. Furthermore, it is one of the few fields of study in which an extensive literature of solid–solid adhesion exists. Of course, adhesives, soldering, and welding also contribute some pertinent experimental observations.

All of these studies show the significance of the surface energy and the importance of the kinds of bonds that act across the interface. Obviously, the metal–metal bond is much stronger than the van der Waals' forces for the organic monomolecular layers. Similarly, the carbon–carbon bond in diamond is much stronger than between the contaminants. The mica experiments produce similar results.

Adhesives.—Adhesion in relation to friction has been discussed. Studies in the field of adhesives confirm all of the observations that have been discussed, but also point out one additional factor that has not been considered—*viz.*, the electrostatic term due to charge transfer at an interface.

There are said to be three theories of adhesives, *viz.*, the adsorption theory (33), the electrostatic theory (34, 35), and the diffusion theory (36). All of these have two factors in common: (*a*) a very large area of true contact must be established between the adhesive and the solid; and (*b*) to assure that the air is displaced from the interface, a low contact angle is needed. The differences among the theories are in the emphasis placed on the origin of the bond strength. The adsorption theory considers the bond strength to be determined by the changes in interfacial energy necessary to remove the adhesive from the solid (provided the adhesive itself is not split at an even lower energy). The electrostatic theory assumes that an electric double layer is established in the fluid adhesive and removal requires doing work to separate this double layer. The diffusional theory assumes that the macromolecules of the adhesive diffuse into the solid surface sufficiently to add an entanglement factor to the strength of the bond. Probably all three factors are, at least in some cases, important. However, the diffusional aspect could be important for solid–solid interfaces only when contact exists for extended periods or when a solid has a very mobile surface layer.

Usually, adhesives are applied as a liquid phase in order to develop the high true area of contact. The solid–solid interactions exist after the adhesive "sets." This may be a drying by evaporational or diffusional loss of "solvent" or a chemical change such as occurs in the epoxy resins. A strong bond remains only when the setting occurs without excessive loss of true area of contact and without the development of significant elastic stresses resulting from dimensional changes of the adhesive. Again the important roles of a high true area of contact and of the mechanical properties are demonstrated.

Wear.—The process of wear has been studied in some detail (37). Although there are several classifications of types of wear, only the adhesional wear process is considered pertinent to this discussion. Adhesional wear occurs when surfaces slide over each other, and adhesion of small regions develop because of intimate contact. The adhesion is strong enough to pull fragments out from one of the solids. The size of wear particles is found to be remarkably uniform. Archard (38) has described a model for wear for which corresponding equations have been derived. Rabinowicz (39) gives the following equation for the condition of loose particle formation:

$$d = 60 \times 10^3 \frac{W_{ab}}{p}$$

where d is the diameter of the particle, W_{ab} is the work of adhesion, and p is the hardness of the surface yielding the particle. Note this is not a rate equation but only relates the size of the particles to the properties of the solid when wear occurs. Nonmetals and metals have been studied and reasonable correlation exists. One very important difference between wear and friction arises because only a few of the adhesional bonds produce wear, but all adhesional bonds contribute to friction.

Since W_{ab} appears in this equation, there is additional evidence that the surface energy is the important property determining solid–solid interactions. Although an oversimplification, boundary lubrication may be considered the technology of reducing wear by reducing W_{ab} to such a small magnitude that the wear rate is reduced to a negligible or very small value.

The "adsorption" theory of the particle–particle interaction of powders is only an ex-

tension of the "adsorption or adhesion" theories of friction, wear, and adhesives. Our interest in the forces of interaction between molecules at surfaces has been shown to be germane to a study of solid particles in contact.

Adsorption and Heats of Immersion.—Now let us look at evidence that suggests a nearly universal, heterogeneous nature for solid surfaces. We have learned already that grain boundaries and dislocations extend to the surface so that some heterogeneity results from these high energy regions. The best available evidence for the heterogeneity of a surface is found as before in reactions involving the interaction of the solid with individual molecules either from the gaseous or the liquid state. Adsorption and the related catalysis studies are of much interest.

Champion and Halsey (40) have shown that the formation of multilayers on a *homogeneous surface* must give rise to "stepped" adsorption isotherms. Apparently, the steps correspond to the formation of a new layer, each 1 molecule thick. However, the adsorption isotherms usually observed are not stepped; instead, they start off at high slope and gradually, but in a continuous manner, change to a curve of low slope. This suggests that most solid surfaces are heterogeneous, as expected. Classically, adsorption isotherms have been considered as belonging to one of five types (41). All of these show the gradual curvature characteristic of a heterogeneous surface.

Zettlemoyer and co-workers (42, 43) have used differential heats of immersion studies to characterize the heterogeneity of surfaces of solids. The derivative of the heat of immersion curve provides a differential heat curve, and the differential of the latter curve yields, when inverted, an approximate site energy distribution (43, 44). These workers have shown that the van der Waals' interaction energy between a polar adsorbent and a polar adsorbate consists of three parts, *viz.*, a nonpolar dispersion force, E_D; the force of interaction of the electrostatic field of the surface and the dipole moment of the adsorbate molecules, E_0; and a force term arising from the polarization of the adsorbate by the surface, E_I. For alcohol on rutile, the distribution is about 68% E_0, 6% E_I, and 26% E_D; for hydrocarbons on the same surface, it is 67% E_D and 33% E_I, and for polar or nonpolar liquids on graphon, it is 100% E_D (45). These results predate the data of Fowkes. Both show the importance of the dispersion forces.

Studies of catalysts have led to a more detailed understanding of chemisorption. A brief review of this type of reaction has been given by Dowden (46). Another interesting review is by Emmett (47). Both articles discuss the formation of bonds with surface atoms. Some may be electron donors and others electron acceptors. Active centers of catalysts often are classified as acid or basic. Apparently it is not clear whether a proton switching or an electron-pair switching mechanism is involved, *i.e.*, the Brönsted or the Lewis mechanisms.

Chemical interactions between particulate solids have been studied also. Specific solid–solid surface interactions of pharmaceuticals have been reported by Lach and co-workers (48).

Electrostatic Attraction.—It appears that solids are rarely separated without also separating a charge. The charge transfer becomes evident at separation of the surfaces. Richards (49) reports that by wringing together optically flat pieces of glass a charge is developed that is independent of the amount of frictional work but is proportional to the area of contact. As long as the plates remained in contact, an X-ray beam would not diminish the charge. Jefimenko (50) demonstrated that only contact and not friction is essential to electrification. A glass rod dipped into mercury or a paraffin rod dipped into water produced the same characteristic charge for the respective couple independent of the amount of work done. The kinetics of charging are not a factor in the last case because high true area of contact exists between a liquid and a solid. These experiments have established that charge transfer may result at areas of true contact between solids. Work must be done to separate the transferred charge and this is a part of the energy causing solid surfaces to attract. The amount of the charge and the direction of charge transfer will depend on the specific properties of the surfaces in contact.

Kunkel (51) dispersed dusts of homogeneous composition from containers lined with the same material. In all such cases, the total dust cloud was essentially neutral but practically all the particles were charged. If homogeneous clouds are blown so that they make and break contact with a different solid surface, the separation of the heterogeneous interface may impose an asymmetry on the relative members of positive and negative particles. The average charge increases somewhat more slowly than the surface of the particles, so particle size is important.

Nash *et al.* (52) observed the charges on powders after they passed through a copper funnel. They observed that the fraction of the sample that was charged could be altered by treating them with different surface-active agents. However, they did not distinguish between ag-

glomerates that were composed of equal numbers of oppositely charged particles and truly neutral particles or agglomerates.

Kordecki *et al.* (53) used centrifugal force to expel particles from solid surfaces. They found that glass spheres were much more difficult to remove from a Teflon surface when in a very dry atmosphere than when in a 50% relative humidity atmosphere even in the presence of a weak α-emitting radioactive source. However, the radiation from radium-D, a more powerful source, reduced the measured adhesion in a 10% relative humidity environment. They do not claim that the electrostatic component was completely removed from the adhesion by the ionization of the atmosphere. Richards' (49) work would suggest that it would not be completely removed.

Deryagin and Zimon (54) have observed the charges on individual particles torn from a flat surface. They found that both the adhesion and charge increased with residence time on the surface. [Deryagin has contributed, also, to the electrostatic theory of adhesives (55, 56).] These experiments demonstrate the importance of the electrostatic effect in the attraction between solids.

To discuss the kinetics of charging one really discusses the accumulation of charge by repeated contacts of one solid with another. This is the common way of observing friction and accounts for the term "frictional electricity." If one of the solids is a metal grounded to earth, the charge on the metal may be neutralized as soon as the separation reduces the inductive effect. Ciborowski and Wlodarski (57) observed the effect of grounding a conductor by repeatedly passing particles of a fluidized bed over a surface. They observed the charge on the powder by placing in the fluidized powder a small metal electrode connected to an electrometer. They state, "The highest electrode potentials, of a range of several thousand volts, were observed in equipment (a) when, apart from the electrode, other metal elements were also introduced into the fluidized bed and connected to earth. If there were no such elements, or else when they were not connected to earth, the electrode potential was generally considerably lower, of a range of a hundred volts at the utmost." Their equipment (a) used a glass tube to contain the fluidized bed. The grounded metal behaved as a very large body that did not change charge. All the difference in charge resulted from charge changes on the particles. Because of the grounded metal probe, the charge transfer could continue until all the powder particles were highly charged. Also, agglomeration of insulating particles was observed. This suggested that the particles were not all highly charged with the same sign. Many particles stuck to the metal probes and to the walls of the container. Furthermore, a lowering of electrode potential was produced by applying a grounded conductor to the glass wall of the equipment.

Another interesting experiment with powders was reported by Gill and Alfrey (58). Cubes of ebonite or a volume of sand slid down a grounded metal plate into a cup connected to an electrometer. Approximately 1 cm. above this plane, an insulated, parallel metal plate was positioned, and a source of potential was placed between the two plates. When the top plate had no charge, the particles became negative as they slid over the plate. When the top plate was positive the magnitude of the negative charge on the particle increased. When the top plate was negative the charge on the particles decreased; if sufficiently negative, the particles had a reversed sign of charge. The following equation fits their data:

$$Q = AX - Q_0$$

where

Q = the charge for a potential on the plate of X volts,
Q_0 = the charge when sliding in zero field,
A = a constant, and
X = taken as positive when the upper plate is at positive potential with respect to the lower one.

This experiment suggests that the charge transfer will occur until some definite potential difference exists between the particles and the surface. Perhaps most important is the observation that the particles could be made to have zero charge with respect to ground when the potential difference between the plates was chosen correctly.

The mechanism of charging of metals and semiconductors is adequately treated by the band theory of solids. Perfect insulators would not charge in this manner. However, it is not clear to what extent the charging of insulators is dependent on the electrons associated with surface states. Harper (59, 60) states that the actual transfer of ions may account for the charging of insulators. For additional discussion of the mechanism of charging, several articles about static electrification may be consulted: Montgomery (61), Loeb (62), Rose and Ward (63). Skinner (64) has treated the case of insulators from the thermodynamic viewpoint.

By now it should be clear that when a solid is in contact with another solid a difference in charge

will develop. The only sure cure for the accumulation of charge is to make the nonconductor a conductor. High humidity often provides enough surface conductivity to reduce the accumulation of charge significantly. Also, antistatic agents may be used on the surface. These function by increasing the surface conductivity. Surfactants (detergents) are commonly used for this purpose. Several patents have been obtained on combinations involving zinc soaps (65). Another approach has been demonstrated by the work of Gill and Alfrey (58) discussed earlier. This requires the presence of a highly charged surface and could result in some hazardous conditions if used indiscriminately.

Grinding and Agglomeration.—*Agglomeration During Grinding.*—Ball mills of either the rotating or vibrating type seem to be superior to air mills for producing very fine particles. Also, they produce, at least in some cases, an interesting reversal of grinding, *i.e.*, they produce compact aggregates. Khodakov and Rebinder (66) have studied the disintegration of quartz in various media. They describe their mill as a laboratory eccentric vibromill but do not elaborate further. However, in a separate study they describe a vibromill operating at 50 c.p.s. with amplitudes of 3 to 5 mm., and mention steel balls in the grinding. Perhaps it is the same mill. They found that quartz ground in air did not show the very large specific surface area of quartz ground in water (determined by nitrogen adsorption). In air the surface area reached a maximum and further grinding produced agglomeration. The agglomerates were so compact that the bonding between particles reduced the surface available to nitrogen gas. After 16 min. of grinding the specific surface was 6.5 $M.^2/Gm.$ and had decreased to 5.9 $M.^2/Gm.$ after 32 min. of grinding. When ground in water, the specific surface was 42 $M.^2/Gm.$ after 16 min. and 73 $M.^2/Gm.$ after 32 min. Evidence that grinding in air produced a high degree of agglomeration was obtained when water was added to the quartz ground in air. A very sharp increase of specific area was obtained with only 40 sec. of additional grinding after adding water. Furthermore, powder obtained by grinding in water when dried and then reground in air produced a decrease in specific surface during the air grinding.

Another interesting case, described by Gregg (67), shows the change with grinding time of specific surface (by N_2 adsorption) and the change in dissolution rate of kaolin in acid solution. The dissolution rate continues to increase even when the specific surface is decreasing by agglomeration. Apparently a high energy kaolin is produced. The evidence that a new state of kaolin is produced by grinding is obtained by thermogravimetric analysis. After many hours of grinding, most of the solid has changed to a new form, the form that dissolves more rapidly.

In Khodakov and Rebinder's work with quartz, other liquids, such as benzene, acetone, and alcohol, influenced the grinding, but only a slight improvement over air grinding was observed. This is somewhat surprising since the heats of wetting for all the liquids are similar to that for water. (If the Rebinder effect, adsorptional-strength-lowering, were the only determining factor, the specific surface values for grinding in these media should be nearly the same.) The product of grinding in these liquids was similar to the air-ground material, *i.e.*, very short periods of additional grinding in water yielded higher specific surfaces. Dried, water-ground materials, reground in the other liquids, agglomerated to give decreased surface areas. Only small amounts of liquids were required to produce significant effects on the amount of "amorphous" material produced. These authors studied, in addition to quartz, calcite, corundum, talc, rutile, alumina, silicate glass, quartz glass, and cement. In most cases the results were similar. Of course, the effects of hydration of cement prevented a complete study of it.

The influence of the grinding medium on the agglomeration process demonstrates that the bonding of solid particles involves a surface phenomenon. The unavailability of the "internal" surface of the agglomerates to nitrogen is evidence that the bonding involves a high true area of contact between the individual particles of the agglomerate.

Disturbed Surface Layers Produced Mechanically.—In the preceding paragraphs, the surface material produced by grinding has been referred to as high energy material and "amorphous" material. Khodakov (68) has reviewed the influence of fine grinding on the properties of solids. Rieck and Koopmans (69) have investigated wet ground quartz particles of 3 μM. diameter.[3] They have concluded that the quartz particles are coated by a disturbed layer that is about 0.4-μM. in thickness.

Bacon (70) used line width of X-ray data to follow the change in crystallinity of graphite during grinding in a mill for a period exceeding 60 hr. The deterioration of crystallinity continued throughout this period. Gundermann (71) reports that materials, such as sugar and

[3] 1 μM. = 1×10^{-6} M.

cellulose, also exhibit amorphization of the surface on prolonged grinding. He reports that the heat of solution of sugar appears to be increased by grinding; in fact, it changed from negative to positive.

The above evidence suggests that powder surfaces may have unusual properties because of a disturbed layer. Consequently, the electrostatic properties, the surface energy, and the mechanical properties may be different from the bulk properties of the same material.

Capillary Condensation Between Particles.—Moisture may influence the force of interaction between solid particles in at least three ways—namely, (*a*) it may adsorb on the surface and influence the surface energy, (*b*) it may alter the surface conductivity and, therefore, the electrostatic charging of the particles, and (*c*) it may condense in the capillary regions contiguous to the true areas of contact. Previous sections of this paper have discussed the first two. Now, we shall consider the third.

Capillary condensation occurs at high relative humidities, usually in excess of 60%. Only small amounts of water need be involved. The phenomenon of adhesion produced by capillary condensation is the same as wet granulation using very small amounts of water. Small liquid bridges are formed between particles. The case of the wet granule has been treated quantitatively and will be used here to describe the attraction between powder particles produced by capillary condensation.

Rumpf (10, 72) considers three classes of wet granules. They are: (*a*) the case where the hollow spaces between particles are only partially filled with liquid, the liquid being held by capillary forces as "lens" at the point of particle–particle contact; (*b*) the internal voids are completely filled with liquid but the external or surface layer voids of the granule are not completely filled; and (*c*) the liquid completely envelopes the solid, and only the surface tension of the drop holds the particle together (this is really a drop of a suspension, not a granule). Newitt and Conway-Jones (73) also use three classifications. Corresponding to class (*a*) according to Rumpf is a state Newitt and Conway-Jones call the *pendular* state. When the moisture content is high enough that the liquid forms a continuous network throughout the internal surface of the granule, but air spaces still exist inside the granule, these authors call it the *funicular* state. It is a condition intermediate between the (*a*) and (*b*) classes of Rumpf. The third state, according to Newitt and Conway-Jones, is called the *capillary* state. This corresponds to Rumpf's class (*b*).

The strength of a wet granule depends on the surface tension of the liquid phase and the contact angle of the liquid with the solid. The following is essentially the Newitt–Conway-Jones treatment. Consider an ideal case of two hard identical spheres of radius r in contact. About the point of contact, a small droplet of liquid would form, in the plane tangent to the two spheres, a circle of radius b. The case is simplified further by neglecting the force of gravity and by assuming the contact angle of the liquid on the solid is zero. For a small amount of liquid between the two spheres, the liquid would form a concave surface approximating the arc of a circle of radius c if viewed in a plane passing through the centers of the two spheres. The tensile force, f_1, of attraction between the two spheres produced by the surface tension at this concave surface would be:

$$f_1 = 2 \pi b \gamma$$

where γ is the surface tension. Also, the surface tension is causing the pressure inside the liquid phase to be reduced so that there is a hydrostatic "suction" pressure holding the particles together. This is given by the Laplace equation for two curvature capillary pressure and is:

$$f_2 = \pi b^2 \gamma \left(\frac{1}{c} - \frac{1}{b}\right)$$

The total force holding the particles together is:

$$f_1 + f_2 = 2 \pi b \gamma + \pi b^2 \gamma \left(\frac{1}{c} - \frac{1}{b}\right)$$

To obtain the strength of a granule it is necessary to correct this for the number of particle–particle contacts, *i.e.*, the packing. However, the above will suffice to explain the origin of the granule strength under a static condition. In powder beds, it explains the attraction produced by capillary condensation between each pair of particles. Mason and Clark (74) have eliminated the influence of gravity by studying liquid bridges between particles dispersed in liquid vehicles. They report that the maximum values agree with the calculations of Fisher (75).

A dynamic term may be added to f_2. If the liquid between the particles has a high viscosity, separation of the particles must overcome the resistance to deformation of the viscous liquid. This produces a change of the "suction" pressure that increases with the rate of particle separation. Consequently, viscous liquids add a shock resistance to the strength of the liquid "bond."

Among the classes of granules discussed by Rumpf and by Newitt *et al.*, the capillary state is the strongest. However, the amount of water condensed into the capillary regions surrounding the contact regions of powders usually would be

much less than that required to produce a high-strength granule.

Even small amounts of water deposited from the atmosphere by capillary condensation may increase the adhesion throughout the bed. If some solubility of the powder occurs in the liquid, caking may result, especially when alternating high–low humidity cycles exist. Ettle (76) used a penetrometer to detect caking in stored fertilizer. Whynes and Dee (77) have used a procedure adapted from the study of soil stability. It measures the crushing strength of a cylinder of the preconditioned powder held in a rubber sleeve.

As the liquid evaporates from the capillary region, any dissolved materials deposit at the points of particle–particle contact. If no material were dissolved in the liquid phase there would be no increase in dry strength produced by the wet–dry cycle. Newitt *et al.* report that solutions of ammonium sulfate were used to granulate fine sand. They state, "... as total dryness is approached, the strength may exceed some fifty times the original moist strength."

THE TRUE AREA OF CONTACT BETWEEN SOLIDS

This section will be limited to a discussion of the areas of true contact established between macroscopic solid objects because the author is unaware of any work done to measure the true area of contact between small particles. Worthwhile perspective on the problem is gained by considering the evidence available for the macroscopic case.

The importance of the true area of contact is obvious from the previous discussion. The surface interactions are nearly all from short-range forces. Electrostatic charging occurs at actual contact points and elastic and plastic displacement require true contact. All of these are important factors in determining the tensile strength between particles in contact.

Again the source of much of the information is from the studies of friction and wear. Earlier in this review mention has been made of the extensive contributions of Bowden and Tabor (27–29). In addition to their work, some recent reviews of studies of the true areas of contact between solids (78, 79) have been translated from the Russian language. None of the experimental methods used are free from error. However, many of the results are worthy of consideration. Among the methods used are (*a*) changes in electrical conductivity with pressure, (*b*) transfer of very thin layers of paint (both luminous phosphors and radioactive isotopes have been used with this method), and (*c*) optical methods, suitable for use only when one or both solids are transparent. These depend on light reflection or transmission, respectively, at points of contact and light scattering at regions of no contact. The optical method permits contact area measurements to be made during sliding. Theoretical calculations based on specific models have been made. These models include: (*a*) elastic contact involving hemispherical asperities in contact with a plane, (*b*) elastic contact of two surfaces involving an assembly of rods, (*c*) contacts between surfaces with randomly distributed asperities, and (*d*) elastic-plastic contacts of asperities with a rigid plane and without work hardening of the solid.

The following general conclusions are based on a large amount of experimental data and are taken from *Reference 79*.

(*a*) "The actual contact area is determined to a large degree by the original microgeometry of the compressible bodies."

(*b*) "The microgeometric shape of the surface varies during compression."

(*c*) "The contact area is directly proportional to a power smaller than one of the applied load."

(*d*) "The contact area depends on the physicomechanical properties of the surface layer of the compressed body."

(*e*) "The area of a single contact zone is almost completely independent of the applied load."

(*f*) "The deformation of a compressed surface is elastic."

A series of plots of the real area of contact *versus* the applied pressure is given in *Reference 78* for different metals. Only a slight curvature is apparent in most of the plots. Therefore, the deviation from linearity, referred to in (*c*) above, is not large for these specific materials. From simultaneous measurements of both the static frictional force and the true area of contact, the real specific frictional force in Kg./cm.^{2} and the real pressure in Kg./cm.^{2} were obtained. The results for polymethyl methacrylate and for silver chloride (apparently against a very smooth glass surface) are given. The specific frictional force was not constant. For both cases, the force appeared to increase with increase in pressure. The increase was more rapid with polymethyl methacrylate than with silver chloride. The author states that this is consistent with the known property of work hardening of this polymeric solid.

If true areas of contact are established through strong asperities, then plastic flow may occur in the bulk of the solid below the asperities. This would not lead to establishing high areas of true contact; but it would result in the storage of

some energy in the elastic displacement of the asperities. This would be necessary to exceed the yield value and produce plastic flow. Consequently, the cohesion of such a solid would be very small.

Bowden and Tabor (83) have discussed the persistence of work-hardened asperities. They have made gross indentations in bulk copper without producing extensive deformation of preformed microscopic work-hardened ridges. Herring and Galt (81) have pointed out that whiskers (growth around a single screw dislocation) of tin are many times stronger than the bulk metal. Beams *et al.* (82) have demonstrated the very large strength of thin films. The explanation is believed to be that plastic flow does not occur where sections are so small that crystal dislocations are either not present or where they cannot move to generate new dislocations.

Since none of the reported studies involve typical pharmaceutical, organic solids, it is possible only to speculate on whether these results are applicable. Probably the mechanisms are the same, but the relative occurrence or importance of various mechanisms may be different. There are important unknowns concerning the true nature of the surface regions of an organic solid. How rough is the surface of a powder particle? What is the distribution of asperities? Are the asperities whiskers? How readily do dislocations move in complex organic crystalline solids? Only further study will resolve these problems.

It is common knowledge that many powders become "sticky," *i.e.*, exhibit high cohesional forces, when the size range includes many particles of less than 10 μ diameter. However, other materials may not become "sticky" even when in a much smaller size range. Possibly the structure of the surface varies; those powders with smooth enough surfaces to readily produce high true areas of contact, even at low contact pressure, would be expected to be sticky. Those with a very rough surface and a low true contact area would be less sticky. Electron photomicrographs such as those made of polyethylene glycol 6000[4] by Nash *et al.* (52, 80) suggest that some solids may have relatively flat surfaces. Nevertheless, the resolution of these photomicrographs is not sufficient to provide evidence of the degree of roughness at molecular levels.

EXPERIMENTAL EVALUATION OF COHESION AND ADHESION OF POWDER PARTICLES

Numerous other reviews have been written that discuss both the methods and the results of measuring cohesion and adhesion of powders. Therefore, the author has chosen to treat this section relatively briefly and leave it to the reader to consult some of the other reviews or the original articles for more details. Other reports summarizing some of the studies are Böhme *et al.* (84), Lowes (85), Morgan (9), Patat and Schmid (86), and Brown (87).

Angle of Repose and the Angle of Internal Friction.—The earlier discussion of friction has indicated the significance of friction measurements in relation to particle–particle interactions. Numerous methods have been employed to obtain friction values. The simplest of these is the angle of repose. Train (88) has compared the results from four different methods and reports that the value obtained depends on the method used. The four methods he compared are: (*a*) fixed funnel, free standing cone, (*b*) fixed bed cone, (*c*) tilting box, and (*d*) revolving cylinder. Zenz and Othmer (89, 90) conclude that there are two different types of angles of repose. One is obtained when a pile is formed and the other when a hollow cone is formed by draining through an orifice. (These authors define other related angular properties of solids, *viz.*, angle of internal friction, angle of wall friction, angle of rupture, and angle of slide.) Taylor (91) points out that the angle of repose represents, at best, a crude approximation to the angle of internal friction. The angle of repose differs from the angle of internal friction because it is determined by the least stable particles. The particles on the surface can be stable only when the least stable grains are in equilibrium. The internal friction depends on the average condition for all grains. Perhaps internal friction would correlate more closely with flow properties. Dalla Valle (92) equates the tangent of the angle of repose to the coefficient of friction. However, general application of this formula may lead to serious error because the derivation assumes no cohesion between particles. When cohesion is present the normal force, σ, on a pile would be:

$$\sigma = mg \cos \theta + C$$

where m is mass, g is gravitational constant, θ is the angle of repose, and C is the cohesion force. The tangential force, τ, is:

$$\tau = mg \sin \theta$$

and the friction equation is:

$$\tau = \mu\sigma$$

or

$$mg \sin \theta = \mu (mg \cos \theta + C)$$

In this equation, μ and C are both unknowns

[4] Marketed as Carbowax 6000 by Union Carbide Corp., New York, N. Y.

and cannot be evaluated from an angle of repose measurement. Usually, C is ignored, and μ is calculated as if C equals zero.

In this laboratory, the arbitrary assignment of $C = 0$ has been overcome by adding a centrifugal component to the pile. This is accomplished by forming the pile on a rotating horizontal disk. It is called the "spinning disk" technique. The corresponding equations are:

$$\sigma = mg \cos \phi_r + C - mr\omega^2 \sin \phi_r$$
$$\tau = mg \sin \phi_r + mr\omega^2 \cos \phi_r$$

and the friction equation is:

$$g \sin \phi_r + r\omega^2 \cos \phi_r = \mu \left(g \cos \phi_r + \frac{C}{m} - r\omega^2 \sin \phi_r\right)$$

where ϕ_r is the slope of the profile of the pile at radius, r, *i.e.*, the angle between the horizontal and a tangent to the surface of the pile at radius, r, $\omega = 2\pi$ (rev./sec.).

Values of ϕ_r for various r values are obtained by sifting as much powder as possible onto an 11-cm. diameter disk covered with sandpaper. A small lip on the edge of the disk helps during the starting of the pile. The completed pile is photographed on transparency film and then projected. From the projected profile of the pile, corresponding values of r and ϕ_r are obtained by drawing tangents to the surface of the pile. By plotting the corresponding values of τ/m *versus* $\frac{\sigma}{m} - \frac{C}{m}$ and then calculating the least squares line, it is determined whether $C = 0$, *i.e.*, it is determined whether the plot goes through the origin. The slope of the line is μ. Often the plot is not linear when $C > 0$; and the method fails. Attempts to use this method to obtain both μ and C/m when $C > 0$ usually have been unsuccessful. However, the fact that one obtains an indication whether C is or is not zero is an important improvement over a simple angle of repose measurement. The accuracy and precision of a value obtained from a least squares line was found far superior to the single point method of an oridinary angle of repose pile. Of course, constant humidity conditions are essential for precision of any method.

Figure 2 shows the apparatus and Fig. 3 dramatically shows the cohesion existing in a pile of sticky powder.[5] To form this pile the powder was sifted gently onto the disk, no compaction was used to make the pile stick. The relative humidity was 50%. The surface of the pile was

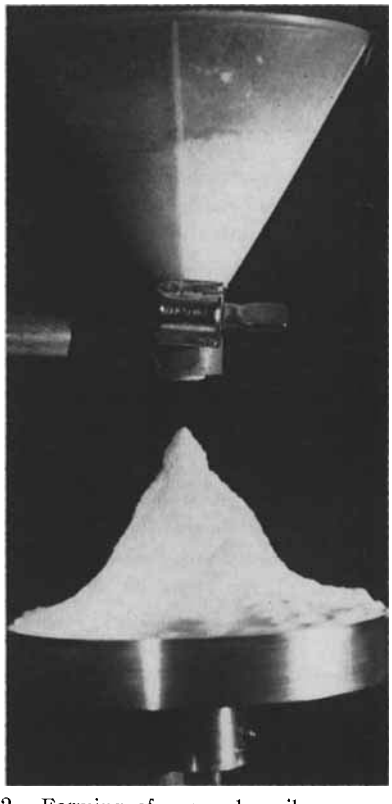

Fig. 2.—Forming of a powder pile on a spinning disk. Powder is lidocaine base.

exposed to radiation from a polonium source to remove the accumulated charge. (Of course, this did not remove the electrostatic component of adhesion at points of true contact.) The pile was inverted immediately after forming; only the tip fell off.

Variations of the angle of repose method have been used to obtain cohesion values. This is useful only when the powder bed behaves as a plastic body. A preformed bed may be tilted very slowly until a shear plane develops and a mass of the powder slides off. The angle of

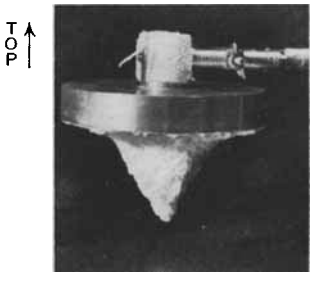

Fig. 3.—Lidocaine base pile formed in 50% R.H. air has sufficient cohesion and adhesion to remain intact when inverted.

[5] Lidocaine. Marketed as Xylocaine Base by Astra Pharmaceutical Products, Inc., Worcester, Mass.

slide observed is assumed to be the angle of internal friction. Hayashi and Minami (93) varied the length of a tilted box filled with powder to obtain variable forces and angles so that the cohesion could be calculated. Lowes (85) and Lowes and Perry (94) describe a similar method based on the tilting of a preformed cone until a shear plane develops.

Studies in which the cohesion was not evaluated are very numerous. Possibly the angle of repose is the most common method of obtaining an index of flow, in spite of its limitations. A few of these reports will be mentioned here to provide a starting point for the reader interested in pursuing the subject further. A complete list is prohibitively long.

Studies of the influence of particle size and size distribution on the angle of repose have been reported by Krishna and Rao (95), Train (88), Nelson (96), Pilpel (97), and Nakajima *et al.* (98). The influence of additives has been studied by Craik (99), Nelson (96), Awada *et al.* (100), and Nash *et al.* (52, 80). Awada *et al.* report the use of glass beads as a diluent to sticky powders. They estimate the angle of repose for the pure powder by varying the composition and extrapolating to 100%. The influence of moisture content has been reported by Fowler and Wyatt (101), Lowes and Perry (94), and Craik and Miller (102). Fonner *et al.* (103) attribute to surface roughness of granules a strong influence on the angle of repose. Other reports and discussions of interest are those of Dawes (104) and Carr (105). In general, these studies indicate that the presence of fines increase the angle of repose. Diluents alter the flow properties; diluents that are themselves free flowing usually decrease the angle of repose and vice versa of the mixture. It is clear that the angle of repose increases at high relative humidities, presumably as a result of capillary condensation in the regions of true contact.

Tilted Plane Method.—If small amounts of powder are placed on a flat surface and then the surface is tilted at an ever-increasing angle until the powder slides off, the process may be described by the same equations as before. The friction equation used is:

$$mg \sin \theta = \mu (mg \cos \theta + A)$$

A is the adhesion of the powder to the plate when the shear plane is between the powder and the plate, *i.e.*, the powder slides off as a single clump. Cremer *et al.* (106) obtained plots of τ *versus* σ by varying the size of the powder layer to change the mass. She used the equation in the form:

$$mg \sin \theta = \mu\, mg \cos \theta + H$$

Her H values correspond to μA above. Böhme *et al.* (84) discusses the work of Patat and Schmid (86) in which they concluded that the complicated nature of the sliding process prevents this from being a satisfactory method. For example, it sometimes produced negative values for H. (The same complication was observed by the author with sticky powders when using the spinning disk.) Zimon (107) reports good results with the method but points out that it is not a true measure of adhesion of individual particles because of the mechanism of sliding. Many others have used it, *viz.*, Pecht (108), Batel (109), Krishna and Rao (95), and Hayashi and Minami (93). Pecht, Batel, and Patat and Schmid used a vacuum arrangement so that the influence of different atmospheric conditions could be explored. Pecht studied relatively narrow size distributions and found that sometimes the adhesion decreased with increase in size distribution.

The above method is designed to measure friction and adhesion to the surface. However, it is not always possible to maintain the shear plane at the powder–substrate interface. Sometimes the particles may adhere to the substrate and the shear plane develops in the powder bed. Also, the particles may move as individuals. In either case the method fails.

The Shear Cell Method.—The intentional development of a shear plane in a powder bed provides another method of using the friction equations to measure cohesion. Dawes (104) used two types of shear cells. One of these assured that the shear plane was in the powder by having vertical vanes on both the upper and lower plate. The powder bed was just thick enough to prevent the vanes from striking each other. His alternate cell used only sandpaper on the bottom of the top plate. He compared the values of σ and τ for both methods; and he found, for a given powder, that the values obtained in the two cells were slightly different. In his study both μ and C were somewhat lower when the sandpaper-covered plate was used.

Jenike (110, 111) has used a shear cell that is packed with powder. The cell is split so that the shear plane is in the powder. Since the powder is packed into the cell, the true area of contact between powder particles and the test results may be influenced by the packing. Jenike has used data from his apparatus to design hoppers. Ashton *et al.* (112) have attempted to control the bulk density in a Jenike-type cell and have obtained a series of isodensity curves for various powders. These authors find that the equation $(\tau/C')^n = \frac{\sigma}{T} + 1$ describes the "yield loci" at

constant bulk density of many powders; n is a constant for a given powder of a given particle size and size distribution. It is independent of bulk density and usually is between 1 and 2. C' is equivalent to μC in the earlier equations. T is the tensile strength of the powder measured in a special apparatus described by Ashton *et al.* (113); it is a modification of an apparatus described by Dawes (114). The cohesion apparatus will be discussed under *Tensile Strength of Powder Beds*. Apparently the tensile strength measured in this manner is always less than the value of C obtained by extrapolation of the shear cell data, *i.e.*, the τ *versus* $\sigma - C$ plots.

The results of Ashton *et al.* show the effect of the bulk density changes. These results confirm many of the general conclusions stated in the sections dealing with the theoretical considerations.

The Jenike method of describing powder flow and of hopper design using the shear cell data is discussed by Williams (115). Additional discussion of the shear cell and its uses can be found in several papers in a single issue of *Rheologica Acta* (116).

Other workers have used a shear cell method in evaluating powders. Taneya (117)[6] has compared the results of the shear cell technique with another based on a modified Couette viscometer (discussed below). The two methods do not always produce the same results. Okada and Abe (118) report results using an apparatus they attribute to Deryagin and Lasarev (119).

A simplified apparatus is described by Nash *et al.* (52, 80). This simple shear cell has been used in these laboratories. The force required to shear a thin layer of powder between two emery paper-coated surfaces is observed. The author has modified the apparatus to permit the top plate to be suspended on a long thread from a balance so that normal loads less than the weight of the top element may be applied. A laboratory jack is used to raise the powder bed gently up and under the top plate, stopping at the null point of the balance. Because of the simplicity of the apparatus, it is readily used inside a constant humidity chamber.

It is difficult to assess the merits of the various shear cell designs and methods of using them. However, the choice of the simple cell described by Nash *et al.* is not determined only by its convenience in use. Because it uses a very thin layer of powder, it was felt that the sample density will be more uniform throughout; also, that its bulk density will be determined by the load applied instead of by the method of prepacking of the cell. Tests indicate that the bed thickness used, $^1/_8$ in., is sufficient that the magnitude of τ is not affected by changes in thickness. The powder bed is formed by gentle sifting to minimize the packing. However, the screen size through which the powder passes and previous handling may determine the agglomerate size and influence the density of beds of a cohesive powder. Certainly these factors can influence the results.

Certain powders do not yield straight line plots of τ *versus* $\sigma - C$. Often these same powders form nearly spherical compact aggregates when handled, often called pilling. The author's experience has been that these are very cohesive powders of fine particle size. Also, qualitatively a low plastic yield value for beds of these powders has been observed. Under the heavier loads, *e.g.*, about 20 Gm./cm.^{2}, they form a sufficiently strong wafer that it must be peeled off the sandpaper. Also, a few cohesive powders yield negative values of C. Hence, the assumed model on which the simple friction law is based is not sufficient to describe all powders.

Several investigators have used coaxial cylinders in an arrangement similar to a Couette viscometer to measure the friction coefficient and the cohesion. Matheson *et al.* (120) used a Stormer type viscometer with a flat paddle as the moving element. It was suspended in a fluidized bed. They classified the flow into three types, *viz.*, (*a*) cohesive, (*b*) aggregative with good fluidization, and (*c*) slugging. Type (*a*) was observed with small particles, usually less than 40 μ diameter. Slugging occurred with high air velocities. Meaningful results were obtained only with type (*b*) flow. Quantitative values for the cohesion are not possible with this arrangement. Benarie (121), Taneya (122), and Kuno and Kurihara (123) have used the "viscometer" arrangement to produce a cylindrical shear region in a powder bed. This permits calculation of μ and C if the radius of the effective cylinder can be determined. It was found that this experimental approach is limited to relatively noncohesive powders. The powders must flow freely into any voids formed by the motion in the shear plane. Cohesive powders that have a low bulk density first undergo forced packing in the vicinity of the shear plane and then form a cylindrical void region. The shear region becomes an air gap and no measurement is obtained. Taneya and also Benarie report surprisingly large cohesion values for the coarse particles they studied. Possibly this technique is a more sensitive method than the direct tensile strength test procedure to be described next.

[6] Refers to description of apparatus in Taneya, S., and Sone, T., *Oyo Butsuri*, 31, 286(1962).

Tensile Strength of Powder Beds.—Reference has been made previously to the Tideswell-Tallyfield apparatus described by Dawes (114). An improved model is described by Thouzeau and Taylor (124); also, a similar apparatus used by Ashton *et al.* (113) already has been mentioned. These apparatus are split containers with one-half fixed and the other movable. They are arranged so that the powder bed breaks into two halves either by tilting the apparatus so the movable half moves away (104) or by using the apparatus in a horizontal position and applying a force on the movable half of the cell (113) to pull it away and break the powder bed. Obviously, this method cannot be used unless the powder is cohesive enough to provide a clean break. Otherwise, the powder would slide into the space between the two halves of the cell as they separate.

The true area of the break plane is not easily calculated since the fracture results in an irregular surface. Furthermore, the concentration of stresses at local regions usually cause tensile strength tests of brittle solids to measure a low cohesion value. It seems reasonable to assume that in this test the powders would behave similar to a brittle solid. However, McKee (125) has measured very weak, sintered compacts in a modified Tideswell apparatus, and he claims that the Griffith crack mechanism is not a significant factor in the breaking mechanism of these compacts.

Farley and Valentin (126) have combined data from the equipment described by Ashton *et al.* with data from a Jenike-type shear cell. The observed cohesion is less than the intercept value, C, from the shear cell data. Eisner *et al.* (127) have studied the increase in tensile strength of powder beds on exposure to humid air. Increases continued for up to 8 hr. Thouzeau and Taylor (124) observed differences in raw materials supposedly supplied from the same source. In both studies (124, 127), a decreased cohesion value was observed after the powder had been "waterproofed," *i.e.*, coated to alter its surface energy. Shotton and Harb (128) studied various starches and observed various patterns of change in cohesion with changes in moisture content of the powder.

Another device for evaluating the cohesion of powders has been described by Nash *et al.* (52, 80). Two coaxial cylinders of the same diameter are placed end to end. Powder is placed in the cylinders; and it is compressed at low load. The force required to separate the cylinders and to fracture the powder bed is observed. For a given compression load, the bulk tensile strength is an exponential function of the length of the column in the cylinders. By using different amounts of powder to change the column length and then plotting the observed cohesion values on semilog paper, an extrapolation to zero column height may be made. This zero height value varies with compression load. A plot of zero column length cohesion values *versus* the respective compressive loads yields an approximately linear relationship over the range studied by these investigators.

So far the methods considered for evaluating powders have been concerned only with a powder bed, not the individual particles. Another very interesting body of experimental data has been obtained on individual particles. The normal force required to pull a particle off a surface is a tensile strength test of the individual particle "bond" to the surface. These studies are most interesting. They reveal a very broad range of forces for similar particles and the maximum force between a particle and a solid surface is surprisingly large.

The Centrifugal Method.—Kordecki *et al.* (53, 129) used a centrifuge in studies of the adhesion of particles to a flat surface. Determinations were made of the size distribution of the particles initially sprinkled on a slide and of the size distributions of those remaining after subjection in discrete steps to successively higher fields of force. The maximum acceleration applied was in excess of 8 g. At maximum acceleration, nearly all of the largest particles and a significant fraction of the smallest particles were removed.

Böhme *et al.* (84, 130–132) also have used the ultracentrifuge, one of them capable of producing forces in excess of 10^6 g. Because they used a very narrow size range of particles, they plotted their results differently. They compared the per cent of particles adhering *versus* the applied force (dynes) and found that the variation of force with particle size was small. Apparently the larger acceleration required for small particles is a consequence of their small mass. Also, these authors have gathered some data on the influence of surface composition and texture on the adhesion of particles to the surface. Their results are consistent with the concepts developed in the early paragraphs of this review. Krupp and Sperling (11) have developed a theory of the adhesion of small particles using the concepts of deformability of solids, surface roughness, and surface energy.

Zimon and Volkova (133) have used the centrifuge method to study the effect of surface roughness. Their conclusions are in agreement

with others, *i.e.*, the adhesion is highest on very smooth surfaces. Zimon (134) has studied the influence of capillary condensation on the adhesion of individual particles. The observed adhesion force was smaller than his calculated values.

Deryagin *et al.* (135) have used centrifugal fields providing up to 300,000 g. They did not succeed in removing all 5-μ diameter glass particles from a polished steel rotor. They used, also, an impact method but give no details. They state that they completely removed glass particles using an impact. The experiments were performed at relatively high velocities of the bullet.

The Vibrating Plate Method.—Deryagin and Zimon (54) used ultrasonic vibration of a metal support surface to measure the force required to remove particles from the metal. Accelerations up to 24,000 g were possible with this unit. Because the forces required to hold individual mono-sized particles covers such a broad range, the representative force used in making comparisons was chosen as the force required to remove 50% of the particles. They found that to remove small particles a larger force was required than for large particles. In this paper, these authors report also the electrical charge observed on particles being blown off the surface of a solid. A broad range of charges was observed. The charge on the particles increased with time in a manner similar to the rate of the increase in adhesional force with time. Consequently, these authors propose that the electrostatic charging process makes an important contribution to the adhesional force.

Impingement and/or Dispersibility of Powders in an Air Stream.—Both the dispersibility in an air stream and the adhesion on impingement of powder in an air stream are used to evaluate the stickiness of powder. Dawes *et al.* (104, 136, 137) have studied the dispersibility of a powder bed in an air blast. In general, two types of behavior are observed. Noncohesive powders allow a gradual erosion process to occur. Cohesive powders denude suddenly by the tearing away of large chunks.

Pecht (138) measured the loss in an air stream of powder from the surface of a granular substrate. From this he developed a relative stickiness factor. Jordan (139) used the air velocities required to remove various percentages of the particles from a plate for making comparisons. Larsen (140) and Corn and Silverman (141) have studied removal by air streams of solid particles from filters. The relationship between adhesion and adherence on impingement has been discussed by Jordan (139). Adherence on impingement has been studied both in connection with the air filtration problem and in connection with the impactor as a sampler of dust in air.

The cascade-impactor is used in the particle size analysis of aerosols. It depends on the size selective impingement (with adhesion) of particles from successively higher velocity air streams. Several factors that influence collection efficiency in impactors have been discussed by Stern *et al.* (142). Aerosol sampling has been reviewed briefly by Whitby and Liu (143).

In addition to the relatively sophisticated approaches, some very empirical methods have been used to provide indexes of dispersibility. Drop tests have been described by Neumann (144) and by Carr (105).

The Weight Method.—Böhme *et al.* (84) reviewed the older efforts to measure directly by weighing the adhesion of relatively large particles or fibers. A more recent study is reported by Corn (145) in which the adhesional force of micron-sized particles to solid surfaces were measured using a microbalance. The pendulum technique was used by Howe *et al.* (146) for measuring adhesion of a large bead (0.5 mm. or larger) to a surface measures adhesion directly but is not suitable for very small particles.

SUMMARY

It would be encouraging if from all of the studies considered in this review one could summarize the results in a simple quantitative manner. However, the only generalizations possible seem to be statements of the variations observed. The observed cohesion varies with the experimental method used to measure it. Some powder beds seem to exhibit negligible cohesion. Yet, individual particles may require accelerations approaching 1,000,000 g to remove them. At low humidities, the electrostatic component may be large. Theoretical considerations of the electrostatic charging of insulators is not sufficiently developed to be of any help in predicting the behavior of most organic powders. Surface energy and topography are seldom known. Mechanical properties of individual particles vary and have been explored insufficiently to permit any quantitative consideration.

In this review the author has attempted to discuss experimental evidence that indicates the types of forces that act between solids in contact. That powder particles attract is not surprising. Also, the relationship of the mechanical properties and topography to the areas of true con-

tact have been considered. The final section reviewed the methods used by various investigators to obtain either numerical values or rank order classifications of the forces of attraction between solids, powders, and/or particles. The complexity of the problem of developing a quantitative, universal theory for the interaction of powder particles with solids makes such a result appear to be remote indeed. However, from a qualitative understanding, it often is possible to determine a systematic approach to our formulation problems; some call it guided empiricism.

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Factors Influencing the Surface Activity of Chlorpromazine at the Air–Solution Interface

Effect of Inorganic and Organic Electrolytes

By RASHMIKANT M. PATEL and GEORGE ZOGRAFI

In a previous study it was observed that adsorption of chlorpromazine at the air-solution interface is influenced significantly by anionic buffer components. In view of this, the effect of a large number of inorganic and organic ions has been considered. Marked inhibitory effects were noted in the presence of organic cations *e.g.*, the tetraalkylammonium ions; the greater the chain length, the greater the inhibition. Inhibition was also noted in the presence of sodium methanesulfonate. On the other hand, bromide, iodide, propanesulfonate, benzenesulfonate, and naphthalenesulfonate ions all produced increased surface activity when compared to the system containing NaCl. The inhibitory effects appear related to factors influencing the structure of water, while the effect of anions appears due to interfacial ion-pair formation.

RECENT studies in this laboratory have been concerned with the possible relationship between surface activity at various interfaces and pharmacological activity of the phenothiazine drugs (1–3). The rationale for such studies is based upon many reports of phenothiazine involvement in metabolic processes controlled by the presence of biological membrane interfaces (4).

In a recent study (3) the surface activity of various phenothiazine derivatives was compared at the air–solution interface and found to reflect the relative nonpolarity and pharmacological activity of each compound. In addition, a significant effect due to the presence of buffer ingredients was noted at pH 5.0 and ionic strength 0.1. Phthalate, citrate, and succinate buffers markedly increased surface activity, while an acetate buffer decreased the tendency for surface pressure development. In contrast to acetate, studies with a phenylacetate buffer also showed a marked increase in surface activity (5). Dilution of all buffers, while maintaining pH and ionic strength constant, tended to restore surface activity to the value expected of the protonated form.[1]

It was suggested at that time (3) that increases in surface activity in the presence of the various buffer ingredients were due to specific interactions between the cationic drugs and the anionic buffer ingredients. The decrease in surface activity due to the acetate–acetic acid system suggested the possibility that the thermodynamic activity of these drugs was decreased by some change in water structure (18) or by a competing process at the interface. Since such factors could play an important role in determining the properties of these drugs at biological interfaces, the authors decided to examine more closely those factors influencing the surface. This first study is concerned with the influence of some inorganic and organic ions. The latter are comparable to the buffer ingredients utilized in the authors' earlier study; but, in addition, they are completely ionized at all pH values.

EXPERIMENTAL

Materials.—The hydrochloride, hydrobromide, and hydroiodide salts of chlorpromazine were obtained from the Smith Kline & French Laboratories, Philadelphia, Pa. They were all recrystallized twice from reagent grade isopropyl alcohol. All inorganic salts were reagent grade and, with the exception of NH_4Cl, were dried before use by heating to 200°. The various tetraalkylammonium chlorides were obtained from Eastman Chemicals. The tetramethyl derivative was recrystallized from isopropyl alcohol, the tetraethyl and tetrapropyl derivatives from acetone, and the tetrabutyl derivative from benzene. Sodium-1-propanesulfonate, sodium benzenesulfonate, and sodium-2-naphthalenesulfonate were obtained from Eastman and recrystallized from absolute methanol. Sodium methanesulfonate was pre-

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[1] Since the pKa values of all drugs studied are above 9.0 (6, 7), it is assumed that the drug is essentially 100% dissociated at pH 5.0. Therefore, the point of comparison for the protonated form, uninfluenced by buffer, is a solution of the hydrochloride brought to pH 2.0 and ionic strength 0.1 with HCl and NaCl.

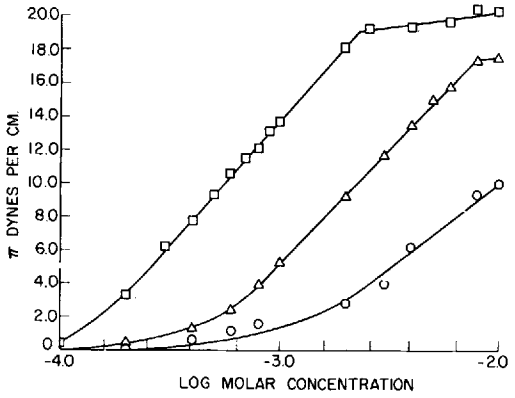

Fig. 1.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25°. Key: O, no salt; ▽, 0.1 *M* NaCl; □, 0.5 *M* NaCl.

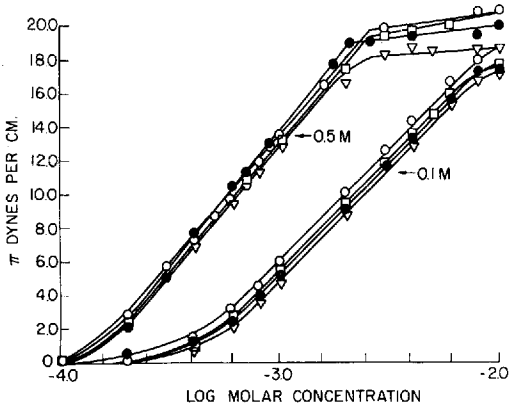

Fig. 2.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of various inorganic electrolytes. Key: O, LiCl; ●, NaCl; □, KCl; ▽, NH_4Cl.

pared by adding an equivalent amount of a sodium hydroxide solution to methanesulfonic acid (Eastman). The reaction mixture was treated with activated charcoal. All recrystallized materials were powdered and dried under vacuum at 60°.

Methods.—Surface tension measurements of all solutions were made at 25° utilizing the drop-volume apparatus, described previously (3, 8). Details for measuring the volume of drops and for calculating surface tension have been reported earlier (8). Values reported here are generally accurate to ±0.3 dyne/cm. Due to the possibility of chlorpromazine photodecomposition, all solutions were prepared just prior to measurement and were kept from any contact with light. The buffer utilized to maintain pH 5.0 was a 0.01 *M* acetate–acetic acid system, which did not exhibit any surface pressure beyond that of a blank solution and did not have any effect on the expected surface activity of the protonated drug. Unless otherwise stated, in all studies chlorpromazine hydrochloride was used as the drug. All pH measurements were made with a Beckman research pH meter.

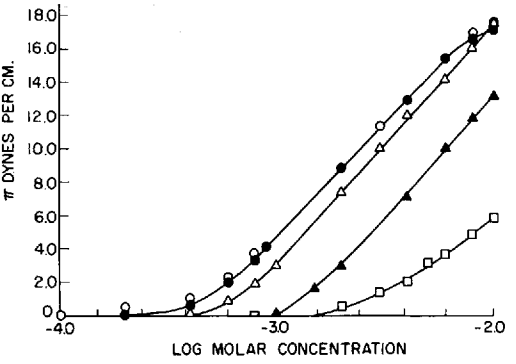

Fig. 3.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of 0.1 *M* NH_4Cl and various tetraalkylammonium salts. Key: O, 0.1 *M* NH_4Cl; ●, 0.1 *M* $(CH_3)_4N^+Cl^-$; △, 0.1 *M* $(C_2H_5)_4N^+Cl^-$; ▲, 0.1 *M* $(C_3H_7)_4N^+Cl^-$; □, 0.1 *M* $(C_4H_9)_4N^+Cl^-$.

RESULTS

In general, two criteria may be used to evaluate surface activity. The first, and perhaps the most important, is the concentration of drug required to achieve measurable surface pressures. The second involves estimating the surface excess or surface concentration at various bulk solution activities. This value requires measuring the change in surface pressure with changing solution activity and applying the appropriate form of the Gibbs adsorption equation (9). Unfortunately, in systems under study here bulk solution concentration cannot be easily equated or related quantitatively to solution activity. However, for the present, plots of surface pressure, π *versus* log *C*, will be given for the various systems studied, and it will be assumed that a greater slope at a given concentration *reflects* greater surface concentration.

Figure 1 indicates the relative tendency of chlorpromazine (CPZ) to develop surface pressure in the presence of buffer alone, and buffer plus 0.1 *M* and 0.5 *M* NaCl. The reduction in required concentration, the general increases in slope, and the appearance of an apparent critical micelle concentration (CMC) clearly indicate the strong tendency of chlorpromazine to exhibit marked surface activity once the apparent repulsive forces of the ionized polar group are reduced. The effect of different inorganic cations, as seen in Fig. 2, is relatively nonspecific except for small differences, particularly above the apparent CMC. At these higher drug concentrations, differences in hydration energies and ionic size may be accentuated.

Figures 3 and 4 demonstrate the inhibition of surface activity due to the presence of various tetraalkylammonium salts. It is interesting to note that 0.1 *M* tetramethylammonium ion acted exactly as an inorganic cation, while the 0.5 *M* solution offset the expected ionic strength effect by reducing surface activity. The surface pressure developed by these substances in the absence of drug was no greater than 1.0 dyne/cm. As can be seen, increasing the chain length of the four alkyl groups beyond one carbon greatly inhibits the surface activity, par-

ticularly 0.1 *M* tetrapropyl and tetrabutyl. The tetraethyl, tetrapropyl, tetrabutyl derivatives, at 0.1 *M* concentration without drug, exhibited surface pressures of 1.0, 5.0, and 10.0 dynes/cm., respectively. The 0.5 *M* solution of tetraethylammonium chloride exhibited a surface pressure of 2.0 dynes/cm. in the absence of drug.

A most interesting and revealing series of observations were made when the influence of various anions on surface activity was measured. Figure 5 illustrates the results with chlorpromazine hydrochloride plus 0.1 *M* NaCl, NaBr, and NaI. One can see that compared to NaCl a marked increase in surface activity occurs with a change in counter-ion. The iodide ion seems to exert the greatest effect and the bromide next. Note in particular the complete loss of curvature in the presence of iodide ion so that the plot of surface pressure *versus* log concentration is linear down to zero π. Under the conditions of this particular experiment, chlorpromazine hydrochloride was quite soluble (up to $10^{-2}M$) in the presence of excess bromide and chloride ion but separated as an oil at about $9 \times 10^{-3}M$ CPZ in the presence of iodide ion. The oil, upon standing, turned into a solid which, when collected and recrystallized from isopropyl alcohol, was identified as chlorpromazine hydroiodide by comparison with a sample on hand.

Since both the hydrobromide and the hydroiodide of CPZ were available, a solution of each salt was prepared in a 0.1 *M* solution of NaCl and in 0.1 *M* of its own sodium halide salt. These were compared with the previous experiments and the results are given in Fig. 6. It is interesting to note that each salt of CPZ in the presence of 0.1 *M* NaCl exerts a small but significant effect as compared to CPZ–HCl. The rapidly rising curve for CPZ–HI appears particularly significant and suggests that the iodide ion is strongly interacted with CPZ. A comparison of CPZ–HCl and CPZ–HBr in 0.1 *M* NaBr indicates no significant difference except as one approaches upper limits of surface pressure. This would indicate that bromide ion can essentially replace Cl^-, so that the system is behaving as CPZ–HBr. An exaggerated example of Cl^- displacement was apparently seen with CPZ–HCl and 0.1 *M* NaI (Fig. 5), since marked surface activity occurred at much lower concentrations of CPZ. The results of experiments with the hydroiodide salt of CPZ in 0.1 *M* NaI were most interesting since no significant concentration of CPZ could be dissolved. Reduction of the concentration of the common ion (iodide) resulted in higher solubilities, but no concentrations approaching that of the hydrochloride in 0.1 *M* NaI could be reached. Apparently in the former case the oil produced *in situ* is more soluble than the crystalline salt. Such oil formation was also observed in the presence of the phthalate buffer in a previous study (3).

Since the buffer effects noted in a previous study appeared in the presence of organic anions, the authors chose to study a series of sulfonates which are completely dissociated over a wide range of pH values. Figure 7 shows the effect of adding 0.1 *M* solutions of sodium methane-, propane-, and benzenesulfonate. Compared to the hydrochloride, note the apparent increase in surface activity due to the latter two, while a significant decrease results in the presence of methanesulfonate ion. It is interesting that these results appear to parallel those seen

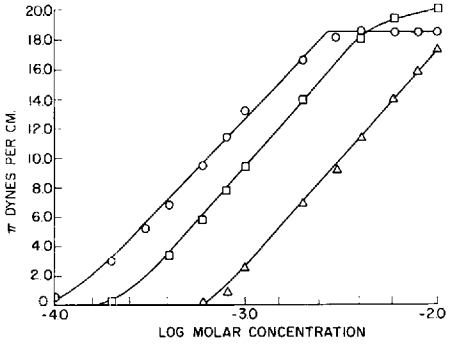

Fig. 4.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of 0.5 *M* NH_4Cl, tetramethylammonium chloride, and tetraethylammonium chloride. Key: ○, 0.5 *M* NH_4Cl; □, 0.5 *M* $(CH_3)_4 N^+ Cl$; △, 0.5 *M* $(C_2H_5)_4 N^+ Cl$.

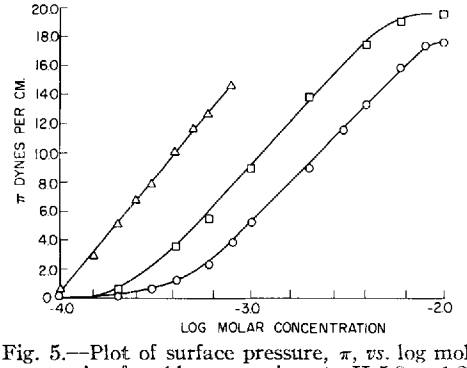

Fig. 5.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of 0.1 *M* NaCl, NaBr, and NaI. Key: ○, 0.1 *M* NaCl; □, 0.1 *M* NaBr; ▽, 0.1 *M* NaI.

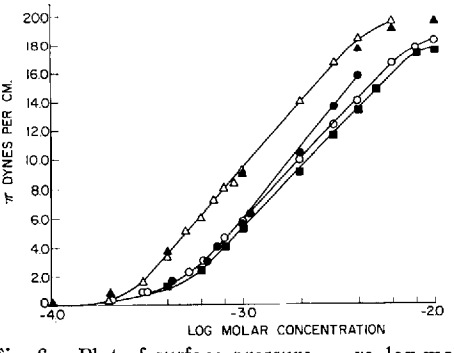

Fig. 6.—Plot of surface pressure, π, *vs.* log molar concentration for various chlorpromazine halides at pH 5.0 and 25° in the presence of various sodium halides. Key: ▲, CPZ–HCl in 0.1 *M* NaBr; △, CPZ–HBr in 0.1 *M* NaBr; ○, CPZ–HBr in 0.1 *M* NaCl; ●, CPZ–HI in 0.1 *M* NaCl; ■, CPZ–HCl in 0.1 *M* NaCl.

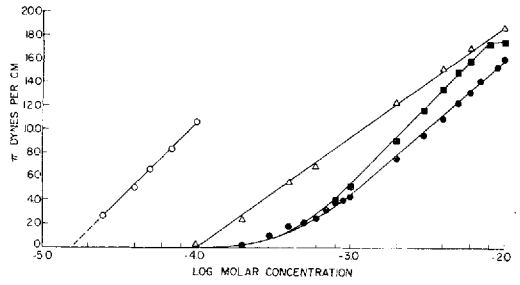

Fig. 7.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of various 0.01 *M* organic sulfonate solutions. Key: ○, 0.1 *M* $C_6H_5SO_3Na$; △, 0.1 *M* $C_3H_7SO_3Na$; ●, 0.1 *M* CH_3SO_3Na; ■, 0.1 *M* NaCl.

earlier with the buffers, in that the greatest increase in surface activity was seen with the aryl derivatives and an actual decrease was observed for the short chain species (3, 5). It may also be noted that, as in the case of iodide ion, there is complete loss of curvature for propane- and benzenesulfonate.

Figure 8 compares the same three sulfonates along with sodium-2-naphthalenesulfonate at a concentration of 0.01 plus 0.09 *M* NaCl. Here the marked surface activity due to the presence of the higher molecular weight anions may be noted. One can also observe that curvature is restored to the dilute propanesulfonate system and that there is no effect due to the methanesulfonate ion as compared to 0.1 *M* NaCl. As with the iodide system, oils were produced by the arylsulfonate system in the presence of CPZ but not by the aliphatics, but so far these oils have proved difficult to crystallize. Figure 9 indicates the effect of changing the benzenesulfonate concentration from 0.001 *M* to 0.1 *M* while maintaining an ionic strength of 0.1 with NaCl. Note the progressive reduction in concentration required for surface pressure development, the loss in curvature at higher concentrations of benzenesulfonate, and the fairly parallel slopes for all of the plots. Oil formation occurred at sulfonate concentrations of 0.01 *M* and higher.

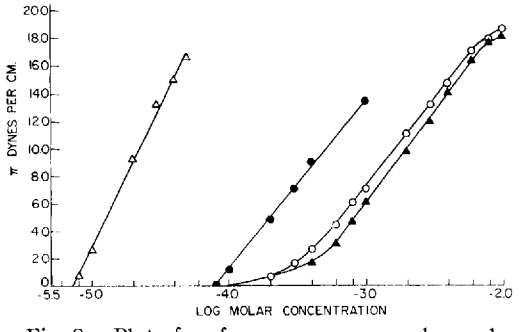

Fig. 8.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of various 0.01 *M* organic sulfonate solutions. Key: △, 0.01 *M* 2-naphthalenesulfonic acid (Na^+); ●, 0.01 *M* benzene sulfonic acid (Na^+); ○, 0.01 *M* propanesulfonic acid (Na^+); ▲, 0.01 *M* methanesulfonic acid (Na^+).

DISCUSSION

Factors Enhancing Surface Activity.—In general, it would appear that increases in surface activity in the presence of the various anions are due to some type of interaction with the CPZ cation. These interactions appear to be above and beyond the usual effects of electrolytes seen in Figs. 1 and 2. There, as seen with most ionic surfactants, the effect of increasing electrolyte concentration apparently produces penetration of the counterions between film molecules with a resulting increase in surface pressure (10). Additional electrolyte effects have been related to the hydration energies of the counterions (11) or, as the authors suspect with the anions considered here, to actual ion-pair formation of some type (12). Such interaction should produce a species which is more hydrophobic than the cation or anion alone. It is more than likely that the interactions involve secondary forces in addition to electrostatic forces since the increased hydrophobic nature and polarizability of the counterions appear to promote the effects we are seeing at the interface.

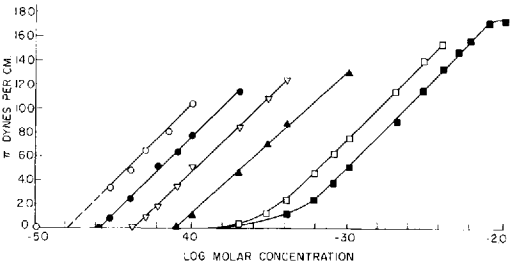

Fig. 9.—Plot of surface pressure, π, *vs.* log molar concentration for chlorpromazine at pH 5.0 and 25° in the presence of various concentrations of benzenesulfonate. Key: ○, 0.1 *M* $C_6H_5SO_3Na$; ●, 0.05 *M* $C_6H_5SO_3Na$; △, 0.025 *M* $C_6H_5SO_3Na$; ▲, 0.01 *M* $C_6H_5SO_3Na$; □, 0.001 *M* $C_6H_5SO_3Na$; ■, 0.1 *M* NaCl.

In all cases where marked increases in surface activity were noted the π *versus* log *C* curves appeared linear over the entire portion of the plot. Reduction of excess counterion concentration, while maintaining ionic strength, eventually restored curvature to the plots (Fig. 9, for instance) so that the degree of curvature seems related to the bulk solution state of the CPZ–anion pair. The linearity of such plots at concentrations just below the CMC has been reported for most common surfactants (13) but not over the entire plot. Such linearity implies a constant surface concentration, as seen when the Gibbs equation is applied, and it has been proposed that this is due to a hydration layer around the polar groups which prevents further increases in surface concentration (13). Elworthy and Mysels (14), however, have recently reported that constant surface concentration below the CMC, although thermodynamically possible, does not seem likely and does not occur as has been thought with sodium lauryl sulfate in water. Linear plots are believed to occur primarily because slight curvature is not easily detected and/or because corrections for activity coefficients and other bulk solution activity

effects are not included. In either case, therefore, in this region the linearity probably indicates that the change in surface concentration, if there is any, is very small and that the degree of surface coverage is very high. Fairly high surface coverage might be expected for the CPZ–anion systems since the anions probably produce fairly hydrophobic ion-pairs of rather large size; but, in order to prove this, radiotracer techniques (15) will be needed to directly measure surface concentration.

On the basis of the authors' experience with these systems, it is apparent that any situation producing a more hydrophobic form of chlorpromazine eventually produces an oil which appears to be more soluble than the crystalline form, at least in those cases where crystals have been isolated. This was seen also with the pure base as well as with the phthalate buffer in our earlier study (3). In addition, the solutions preceding the appearance of oil are quite surface active and yield the linear plots. In a sense, therefore, the solutions exhibiting linearity are in a supersaturated state which explains the apparent marked increase in thermodynamic activity. However, the exact state or degree of aggregation of the CPZ systems in this region remains unclear and is presently under study.

Factors Inhibiting Surface Activity.—The inhibition of surface activity by the quaternary ammonium salts and sodium methanesulfonate has raised many questions requiring more study. However, some clues related to the original observation of inhibition by the acetate buffer have been uncovered. If one only considers the effects of various quaternary ammonium ions, two major reasons for inhibition appear possible. Since they are cationic, as is CPZ, one might expect some competition at the air–solution interface. This could be a factor for the 0.1 *M* solutions of tetrapropyl and tetrabutyl derivatives since, in the absence of drug, they exhibited significant surface activity. However, the tetramethyl and tetraethyl derivatives are hardly surface active even at 0.5 *M* concentrations. Steigman *et al.* (16) have measured the effects of these quaternary ammonium ions on the CMC of hexadecyltrimethylammonium bromide with results similar to those reported here. Although some contribution was attributed to the surface activity of the short-chain compounds, the effects noted with the tetramethyl and tetraethyl derivatives caused them to consider an additional factor. This factor is the entropy change associated with the disorganization of water when molecules go to a surface or to a micelle. The authors feel that the presence of other alkyl groups tends to organize water structure in such a way as to reduce the tendency for nonpolar groups to leave an aqueous environment. Since alkyl anions such as methanesulfonate and acetate inhibit surface activity and yet cannot compete with CPZ, this mechanism seems quite plausible in the present case. Apparently beyond an anion molecular size involving one or two carbons the interaction of the anion with CPZ offsets this tendency to cause inhibition. A stronger insight into this picture is seen with some preliminary data which indicate that urea, methyl urea, and 1,3-dimethyl urea, in increasing order, inhibit surface activity and CMC (5). The influence of urea and its derivatives on CMC and protein denaturation has been discussed (17); and, although a number of theories are proposed, they all evolve around alteration of water structure.

SUMMARY AND CONCLUSION

The surface activity of chlorpromazine at pH 5.0 at the air–solution interface was studied in the presence of a variety of inorganic and organic ions.

In addition to expected increases in surface activity due to increases in ionic strength, marked effects, apparently due to some type of interaction, were noted with bromide, iodide, propanesulfonate, benzenesulfonate, and naphthalenesulfonate ions. These results appear related to buffer effects observed previously.

Significant decreases in surface activity were observed in the presence of short chain quaternary ammonium ions and methanesulfonate ion. This strongly suggests the marked dependence of CPZ surface activity on the structure of water and the ability of environmental factors to influence the thermodynamic activity of this drug without necessarily interacting with it. This and other possible mechanisms will be the subject of future work.

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Mono-*O*-isopropylidine Derivatives of Digitoxin, Digoxin, and Ouabain

By OLE GISVOLD

The mono-*O*-isopropylidene derivatives of digitoxin, digoxin, and ouabain have been prepared. In the case of digoxin, this derivative was more lipid soluble than the parent compound and more water soluble than digitoxin or acetyldigitoxin. All derivatives were less active than the parent glycosides when they were assayed intravenously in cats. Mono-*O*-isopropylidene digoxin was 50 per cent absorbed when administered orally.

IT IS WELL KNOWN that digitoxin taken orally is completely absorbed (1), whereas this is not the case with digoxin. Gitoxin and ouabain are very poorly absorbed if at all. Acetyldigoxin is better absorbed than is digoxin when orally administered to cats (2). Evidence in the literature to date strongly supports the concept that the lipophilic properties of the cardiac glycosides (3) are an important factor in the degree to which they are absorbed orally.

Although digitoxin is well absorbed orally, its prolonged duration of action and cumulative properties sometimes are not desirable. Digoxin's duration of action and cumulative properties are more favorable; however, its oral absorption is less dependable as measured by its oral dose *versus* its LD_{50} in intravenous assays in cats. MLD is 230 ± 10 mcg./Kg. for digoxin in cat assays and an average oral dose for humans is 0.5 mg. *versus* MLD 330 ± 8 mcg./Kg. for digitoxin in cat assays and an average oral dose for humans is 0.1 mg. Therefore, it seemed of interest to explore the possibilities of preparing a derivative of digoxin whose lipophilic properties would be enhanced with the retention of effective hydrophilic properties. Dioxolanes and dioxanes enhance the lipophilic properties of the parent compound together with the retention of some hydrophilic properties *via* hydrogen bonding to the two ether oxygens.

DISCUSSION

The cardiac glycosides digoxin and digitoxin have two free adjacent *cis* hydroxyl groups on the terminal digitoxose residue that should yield monodioxolanes upon proper treatment. The isopropylidene derivatives are the most readily prepared dioxolanes under mild conditions that should preclude any undesirable changes in the structures essential for cardiac activity. The sugar residue of ouabain also should lend itself to the preparation of a mono-*O*-isopropylidene derivative. The method that yielded the best results utilized anhydrous copper sulfate, anhydrous acetone, room temperature, and continuous shaking. The course of the reaction was followed by paper chromatography. In the case of digoxin, the mono-*O*-isopropylidene derivative was mobile on formamide-impregnated paper, whereas digoxin remained at the starting line when formamide-saturated benzene was used as the mobile phase (Fig. 1). The use of this simple system led to erroneous results because no amount of time and anhydrous copper sulfate would eliminate some material at the starting line. The use of solvent system II (4) demonstrated that after the most effective reaction conditions, the substance at the starting line (with benzene as the mobile phase) moved faster than digoxin (Fig. 2). This product was very minor in amount and as yet has not been characterized. All attempts at fractional crystallization failed to yield a homogeneous mono-*O*-isopropylidene product even though in the case of digoxin and digitoxin this derivative was quite soluble in anhydrous ether. Separation of the minor by-product was effected by the use of a formamide-impregnated microcrystalline cellulose[1] column and benzene saturated with formamide as the eluant or by Whatman No. 31 paper and preparative paper chromatography methods.

All the mono-*O*-isopropylidene derivatives showed greater lipid solubility properties when compared to the parent glycosides as measured by their pronounced solubility in ether and their movement on formamide-impregnated paper with solvent system II (Figs. 2 and 3). This increase in lipid solubility was greatest with digoxin and was about midway between digitoxin and acetyldigitoxin. When examined by a reverse phase paper chromatographic system (5), digoxin showed greater water solubility than mono-*O*-isopropylidene digitoxin or acetyldigitoxin (Fig. 4). Mono-*O*-isopropylidene ouabain still exhibited the greatest water solubility (Fig. 5). All these derivatives gave a positive Raymond reaction which suggests that the α,β-unsaturated lactone ring was still intact. These changes in water and liposolubilities would suggest that mono-*O*-isopropylidene digoxin probably has the more favorable lipophilic–hydrophilic balance for good oral absorption.

A preliminary screening for cardiac activity was carried out in pigeons and activity still was present although of a lower order. Intravenous assays

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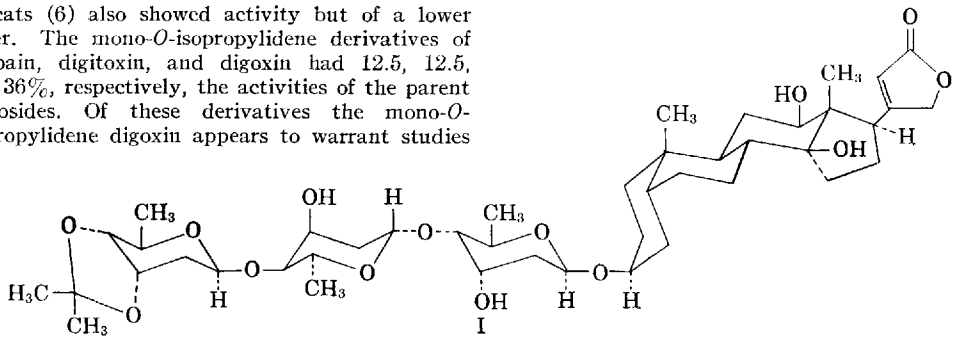
[1] Marketed as Avicel by American Viscose Co., Marcus Hook, Pa.

in cats (6) also showed activity but of a lower order. The mono-*O*-isopropylidene derivatives of ouabain, digitoxin, and digoxin had 12.5, 12.5, and 36%, respectively, the activities of the parent glycosides. Of these derivatives the mono-*O*-isopropylidene digoxin appears to warrant studies



3‴,4‴-*O*-Isopropylidene Digoxin

on its oral absorption because of its physical properties. Although it has 36% of the activity of digoxin, its degree and reliability of absorption might more than offset the reduction in activity. Its toxicity and duration of action also would have to be considered. These might be modified in view of the fact that some cyclic ketals are stable *in vivo* and also might be true of this compound. Thus, the rate of hydrolysis *in vivo* of the sugar residues and other *in vivo* transformations might be altered to provide favorable or unfavorable biological effects.

EXPERIMENTAL

The details of some of the paper chromatographic techniques used in these studies have been described previously (4). Solvent system II was used to very good advantage for the development of some of the paper chromatograms. The original glycosides as well as the reaction products separated effectively by this mobile system. Benzene saturated with formamide provided a simple and rapid mobile system to detect the extent of isopropylidene formation on Whatman No. 1 paper treated with 30% formamide in acetone. It also was used as a very fast moving system for preparative paper and powdered cellulose column studies. A descending reverse system described by Tschesche *et al.* (5) was used to compare the relative hydrophobic properties of the parent glycosides and their

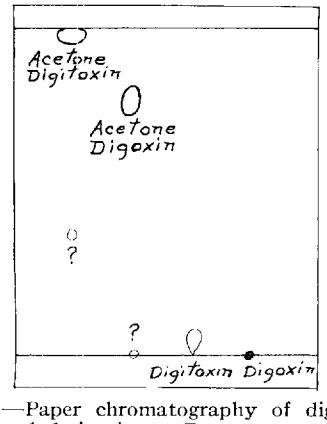


Fig. 1.—Paper chromatography of digoxin, digitoxin, and derivatives. Benzene as mobile phase.

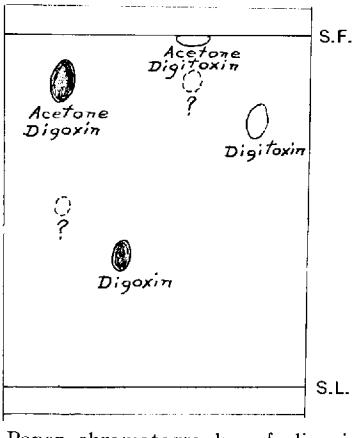


Fig. 2.—Paper chromatography of digoxin, digitoxin, and derivatives. System II as mobile phase.

isopropylidene derivatives. The Raymond reagent was used to detect the glycosides and their reaction products on paper.

Preparation of Isopropylidene Derivatives.—Reagent grade acetone was dried overnight with anhydrous calcium sulfate. It then was carefully decanted or filtered into a flask that was dried in the oven at 110°. (*Caution:* no powdered calcium sulfate should be in the distilling flask.) The acetone was distilled into a 250-ml. conical flask equipped with a drying tube. A forerun of 20% was discarded and subsequently 200 ml. of acetone was collected. The dry glycoside, 100 mg. of digoxin or ouabain or 400 mg. of digitoxin, was added to the flask and the flask carefully heated and shaken to dissolve as much as possible of the glycoside. After cooling to room temperature, 5 Gm. of anhydrous copper sulfate (Allied Chemical) was added. The flask was stoppered with a close fitting stopper and the mixture shaken continuously. The course of the reaction was followed by examining a sample by paper chromatography using formamide-impregnated paper and solvent system II. A period of 22 to 24 hr. of shaking was necessary to completely eliminate the presence of the parent glycoside, although in all cases a small amount of some other slower moving product also could be detected. This slower moving product gave an initial blue color with the Raymond reagent

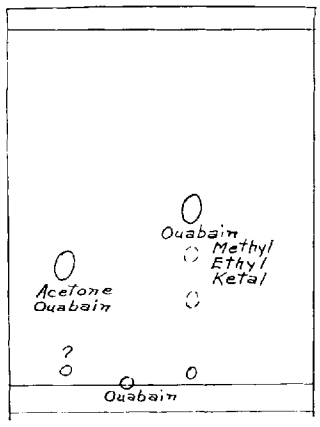


Fig. 3.—Paper chromatography of ouabain and derivatives. System II as mobile phase.

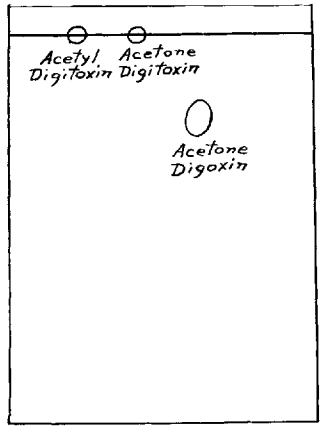


Fig. 4.—Reversed phase paper chromatography of derivatives of digoxin and digitoxin.

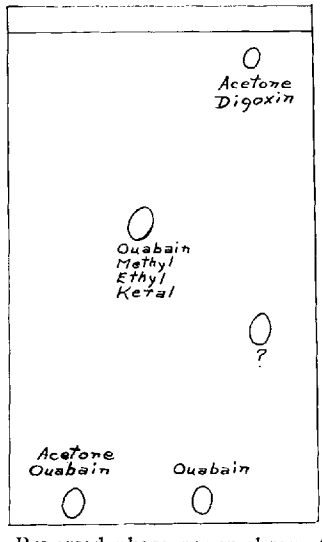


Fig. 5.—Reversed phase paper chromatography of ouabain and derivatives of ouabain and digoxin.

but very shortly turned a yellow green. This was not true with the faster moving isopropylidene derivative that retained its blue color as do the parent glycosides. The reaction mixture then was allowed to stand to permit most of the copper sulfate to settle out, after which time it was filtered through a dense filter paper to remove all the very finely divided suspended copper sulfate. The acetone was removed by distillation with the aid of a water pump and in the absence of air. The amorphous residue readily dissolved in 35 ml. of anhydrous ether in the case of digoxin and digitoxin; somewhat more ether was necessary in the case of ouabain. The ether was concentrated until turbidity appeared. The flask was stoppered, and upon standing a crystalline product was obtained. Upon further concentration, and in the case of digitoxin and digoxin the addition of a judicious amount of isopropyl ether, additional amounts of the crystalline product were obtained. In the case of ouabain the first and second crops of crystals exhibited homogeneity as manifested by their behavior on paper chromatograms using solvent system II and formamide-impregnated paper. Mono-*O*-isopropylidene ouabain melted at 174°.

Anal.—Calcd. for mono-*O*-isopropylidene ouabain, $C_{32}H_{48}O_{12}$: C, 61.54; H, 7.70. Found: C, 61.64; H, 7.42.

In the case of digitoxin and digoxin all attempts to fractionally crystallize their isopropylidene derivatives yielded products that still showed the presence of a small amount of a second product.

An attempt was made to improve upon the above described general method by adding drying agents anhydrous calcium sulfate or a molecular sieve[2] type 3A, to the reaction mixture. In the first case no advantage was gained in the yield of the acetonide. Furthermore, diacetone alcohol was obtained upon removal of the acetone. This substance moved with the solvent front on a paper chromatogram and gave a violet color. A combination of anhydrous calcium sulfate and a small crystal of *p*-toluenesulfonic acid was not satisfactory. In the case of the use of a molecular sieve, quite unsatisfactory results were obtained.

Prolonged shaking beyond 24 hr. did not lead to complete conversion of the glycosides to the acetonides. As the time of shaking was increased, the isopropylidene derivative slowly was converted to a secondary product. This was demonstrated by paper chromatography when the single spot of the original product appeared as two adjacent spots. About equal intensity of these two spots (Raymond reaction) was obtained after 120 hr. of shaking. No further investigations have been completed in this area at the time of this writing.

The general procedure used for the preparation of the isopropylidene derivatives was used with methyl ethyl ketone with analogous results. The derivatives were more lipid soluble and less water soluble than the acetonides. At the present time these derivatives have not been prepared in a homogeneous state. These studies have been suspended until more data have been obtained on the biological activities of the acetonides.

Purification of Mono-O-isopropylidene Digitoxin.—Microcrystalline cellulose,[1] 15 Gm., was

[2] Linde Molecular Sieves, Union Carbide Corp., Linde Division, New York, N. Y.

mixed with 25 ml. of 30% formamide and carefully dried on a large watch crystal with constant stirring. The microcrystalline cellulose then was intimately mixed with an equal volume of finely powdered quartz. A column 8 × 2.2 cm. was packed in a tube 10 × 2.2 cm. Mono-*O*-isopropylidene digitoxin, 100 mg., was powdered and intimately mixed with a small amount of the cellulose–quartz mixture and placed at the top of the above column. The column then was developed with benzene saturated with formamide. One-milliliter fractions were collected and those from 1 to 8 contained only mono-*O*-isopropylidene digitoxin (detected by paper chromatography). Fraction 16 contained only digitoxin and some of the intermediate fractions contained mixtures. This experiment was repeated three more times, and the rate of flow of the benzene held to 15 drops/min. Similar results were obtained as with the first trial. The mono-*O*-isopropylidene digitoxin fractions from all the runs were dissolved in a small amount of methylene chloride, isopropyl ether was added, and the methylene chloride was removed by distillation. The mono-*O*-isopropylidene digitoxin readily crystallized and appeared to be homogeneous by paper chromatographic analysis. It melted at 213–215°.

Anal.—Calcd. for mono-*O*-isopropylidene digitoxin, $C_{44}H_{68}O_{13}$: C, 65.60; H, 8.44. Found: C, 66.11; H, 8.95.

Purification of Mono-O-isopropylidene Digoxin.—When the same column technique as applied to mono-*O*-isopropylidene digitoxin was applied in an attempt to purify this derivative, very unsatisfactory results were obtained. This was not expected because of the similar results that were obtained on paper under the same conditions where only the desired derivative moved on paper impregnated with formamide and benzene saturated with formamide as the mobile phase. As an expediency measure, purification *via* Whatman No. 31 paper was carried out using the descending technique. The crude acetone digoxin product was heavily spotted all along the starting line which was 10 cm. from the end of the paper. This fast running paper provided complete elution of the mono-*O*-isopropylidene digoxin within 1 hr. with the retention of the unwanted products at the starting line. Two and even three starting lines, 2 cm. apart, equally impregnated with the same amounts of crude acetonated digoxin on the same length of paper gave results equally as good, with the retention of the unwanted products at each of the starting lines. Concentration of the benzene eluates left an amorphous residue that was stirred with water several times to remove the small amount of formamide that was present. This resulted in a solid product that gave a single spot on a paper chromatogram. Crystallization of this product was most difficult. A crystalline product readily can be obtained directly from the acetonation reaction; however, it will contain a small amount of the secondary reaction product. It melted at 155°.

Anal.—Calcd. for mono-*O*-isopropylidene digoxin, $C_{44}H_{68}O_{14}$: C, 64.31; H, 8.28. Found: C, 63.70; H, 8.14.

Establishment of Ketal Structure.—The periodate-benzidine test (7) on paper can be used very effectively to detect the presence of a *cis* glycol structure. The substance is spotted on paper and then sprayed with a saturated aqueous solution of potassium metaperiodate. After a suitable time it then is sprayed with a benzidine reagent (10 vol. of 0.1 *M* benzidine in 50% aqueous ethanol is mixed with 2 vol. of acetone and 1 vol. of 0.2 *N* HCl). The presence of a *cis* glycol structure is indicated by a colorless area surrounded by a blue background. Four milligrams each of digoxin, mono-*O*-isopropylidene digoxin, digitoxin, mono-*O*-isopropylidene digitoxin, ouabain, and mono-*O*-isopropylidene ouabain were dissolved in 1 ml. each of methanol. Equal quantities of each of these solutions were spotted on Whatman No. 1 paper and sprayed with the periodate solution. In the case of digoxin and digitoxin and their mono-*O*-isopropylidene derivatives a 5-min. waiting period was necessary. The parent glycosides all gave a positive test for the presence of a glycol structure, whereas the mono-*O*-isopropylidene derivatives gave a negative test.

It should be pointed out here that much of the benzidine in this reagent will crystallize out upon standing a few hours. If the mixture is warmed the benzidine will redissolve, and this reagent can be used directly with satisfactory results.

Bioassay of the Glycoside Acetonides.—These derivatives were assayed by Chen (6), who obtained the following mean $LD_{50} \pm$ S. E. mg./Kg. values. Ouabain acetonide, 0.8837 ± 0.0714 (10 cats); digitoxin acetonide, 2.520 ± 0.2147 (6 cats); and digoxin acetonide, 0.6392 ± 0.0356 (10 cats).

Subsequent to the review of this manuscript, oral studies (6) in cats indicated that mono-*O*-isopropylidene digoxin had 50% absorption from the jejunum which is the same as digoxin.

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Role of Ascorbic Acid Oxidase in the Hyoscyamine–Scopolamine Ratio in *Datura innoxia*

By HELEN LOUISE ROWLAND and MELVIN R. GIBSON

Isolated root cultures of *Datura innoxia* Miller to which benzoin anti-oxime (Cupron) was added showed a change in the habit of growth but no change in the weight of the roots nor in the hyoscyamine and scopolamine content, indicating that ascorbic acid oxidase activity was not involved in alkaloid biosynthesis. An improved method for the germination of *D. innoxia* seeds was developed.

ASCORBIC ACID OXIDASE, which is widespread in higher plants (1–3), has been implicated as a terminal oxidase in cellular respiration (4–7). Ascorbic acid itself is required for the rapid synthesis of large amounts of collagen and may be involved in the conversion of proline to hydroxyproline (8, 9). There is some question as to whether proline is hydroxylated before or after incorporation into collagen (9–11). The use of ^{18}O has shown that the hydroxyl oxygen of hydroxyproline is incorporated directly from the atmosphere (12, 13); ascorbic acid may act directly by generating free hydroxyl radicals (8); a direct effect of ascorbic acid on the collagen-synthesizing fibroblasts has also been suggested (14).

Proline is also involved in the biosynthesis of alkaloids in *Datura* species (15). Proline-^{14}C was incorporated into both hyoscyamine and scopolamine and other alkaloidal constituents of both *D. innoxia* Miller and *D. stramonium* L. variety *tatula* Torrey. However, hydroxyproline is apparently not a direct precursor of scopolamine or of the other alkaloids of *D. innoxia* (16). Scopolamine is apparently synthesized from hyoscyamine (17).

The present study was made to determine the possible relationship between ascorbic acid oxidase and tropane alkaloid biosynthesis in *D. innoxia*, since both the enzyme and the alkaloids appear to be related to proline–hydroxyproline metabolism. Ascorbic acid oxidase was inhibited by benzoin anti-oxime (C_6H_5-$CHOHC{:}NOHC_6H_5$)[1] since under physiological conditions it chelates only cupric ion (18).

EXPERIMENTAL

Growth and Processing of Root Cultures.—The methods used in this investigation for the growth and processing of root cultures were modified from those developed in this laboratory by French and Gibson (19) and Sullivan and Gibson (15).

Previous work in this laboratory was hindered by the low germination rate of *D. innoxia* as well as other species of *Datura*. When the seeds were incubated on damp filter paper, only 1 to 2% sprouted. Other workers have used various treatments to hasten the germination of *D. innoxia* (20, 21). Trials at the beginning of this investigation showed that when the seeds were presoaked in sterile White's nutrient solution (22), the germination rate increased to over 30%; also, the rate of injury to the radicles was less than when mechanical methods were used to weaken the seed coat. White's nutrient solution as used throughout this work was sterilized in an autoclave.

Seeds of *D. innoxia* were sterilized by shaking for 15 min. with 3% hydrogen peroxide. Then the seeds were placed on sterile filter papers in Petri dishes. The seeds were covered with 20 ml. of White's nutrient solution and placed in an incubator at 26–27°. After 3 days, the seeds were removed from the incubator; aseptically transferred one seed at a time, using forceps, to new sterile Petri dishes; and moistened with 5 ml. of sterile, demineralized, distilled water. The seeds usually started to germinate after the fourth day. The radicles were severed from the seed when they were 1 to 1.5 cm. long and placed in 50 ml. of nutrient solution. At the end of the first week the roots were transferred to fresh nutrient solution. After 2 weeks the roots were removed from the nutrient solution and dried at 55° to a constant weight.

Control roots were cultured in White's nutrient solution; the test roots were cultured in White's nutrient solution containing 10^{-3} *M* of benzoin anti-oxime.

Extraction and Separation of Alkaloids.—The procedure used for the extraction of the alkaloids from the dried root cultures was based on earlier work by French and Gibson (19), except that the root extract was allowed to drip directly onto a thin-layer chromatography plate rather than chromatographic paper strips.

Preliminary tests showed that it was necessary to combine roots in groups of four to seven, weighing about 25 mg., in order to obtain a concentration of hyoscyamine and scopolamine that could be measured spectrophotometrically. However, in computing the alkaloid contents, the weight of the crushed root samples was converted to a base of exactly 25 mg. These roots were weighed and then crushed together with a mortar and pestle. The crushed root powder was placed on top of a

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Abstracted in part from the thesis presented by Helen Louise Rowland to the Graduate School, Washington State University, in partial fulfillment of Master of Science degree requirements.

[1] Marketed as Cupron by Eastman Organic Chemicals, Rochester, N. Y.

TABLE I.—DRY WEIGHT OF CONTROL ROOTS OF *D. innoxia*

Root No.	Root Wt., mg.	Root No.	Root Wt., mg.	Root No.	Root Wt., mg.
1	4.3	45	5.3	89	3.9
2	6.2	46	2.7	90	7.6
3	3.4	47	2.8	91	7.9
4	4.7	48	6.5	92	5.6
5	5.9	49	7.8	93	4.8
6	5.1	50	2.2	94	5.1
7	6.0	51	6.1	95	4.7
8	4.7	52	6.5	96	3.7
9	5.5	53	3.7	97	4.2
10	7.5	54	5.6	98	4.3
11	7.7	55	4.4	99	6.3
12	4.6	56	7.4	100	7.5
13	6.0	57	2.8	101	1.5
14	7.7	58	3.6	102	7.2
15	5.0	59	7.3	103	7.0
16	8.4	60	8.7	104	6.1
17	3.4	61	2.9	105	5.6
18	3.9	62	7.5	106	4.3
19	9.8	63	2.5	107	4.6
20	8.2	64	6.3	108	8.8
21	4.3	65	3.8	109	1.6
22	4.1	66	1.8	110	5.7
23	2.9	67	2.1	111	4.7
24	6.1	68	2.9	112	4.8
25	5.9	69	2.4	113	8.5
26	5.9	70	11.0	114	7.8
27	7.6	71	7.8	115	6.8
28	5.4	72	5.8	116	4.6
29	11.0	73	6.3	117	6.2
30	4.4	74	5.5	118	4.1
31	10.1	75	5.7	119	7.4
32	8.5	76	5.9	120	4.5
33	3.8	77	8.2	121	9.5
34	10.7	78	6.0	122	8.1
35	7.4	79	5.8	123	7.0
36	3.2	80	9.1	124	5.1
37	2.4	81	5.2	125	5.0
38	6.6	82	4.0	126	2.7
39	5.8	83	3.0	127	9.4
40	1.5	84	5.7	128	3.7
41	3.2	85	5.0	129	3.1
42	5.9	86	4.9	130	11.5
43	2.1	87	6.8	131	5.2
44	5.9	88	5.5		
		Mean	5.6		
95% Confidence interval			5.4–5.8		

cotton pledget in an extractor cut from the lower end of a 50-ml. needle-valve buret, the extractor having a capacity of about 10 ml. The root powder was moistened with a 1:1 mixture of 10% ammonium hydroxide and 95% ethanol and allowed to stand for 1 hr. Then 2 ml. of chloroform was added to the extractor and 1 ml. of chloroform for each milligram of root powder was added to the reservoir above the extractor. The extract was allowed to drip directly onto a thin-layer chromatography plate, adjusting the stopcock of the extractor so that each drop dried completely before the next drop fell onto the plate. A jet of cool air was directed at the spot to facilitate drying.

The procedure used for the separation of alkaloids was developed by Sullivan and Gibson (15). The alkaloids of the root extract were separated on a 0.5-mm. thick matrix of Silica Gel G.[2] The root extract was placed 1.5 cm. from the bottom of the 8 by 8 in. plate and 3.0 cm. from the left edge of the plate. The chromatogram was developed in two directions, 15 cm. in each direction, using 10 ml. of diethylamine and 90 ml. of chloroform as the developing solvent for the first phase, and 15 ml. of diethylamine and 85 ml. of chloroform for the second phase. Both phases were developed in an incubator at 30°. Between phases and after the second phase the plates were dried at 55° for 15 min. in a Freas circulating dry air oven and then allowed to cool for 30 min. Preliminary chromatograms indicated the location on the plates of hyoscyamine and scopolamine. After cooling, the probable areas of location of hyoscyamine and scopolamine were covered for protection; then the rest of the plate was sprayed with iodoplatinic acid solution (23) to verify the position of the alkaloids. The hyoscamine and scopolamine spots were removed from the plate with a razor blade and a camel's hair brush.

The alkaloids were extracted from the Silica Gel G in an apparatus developed by Gibson in this laboratory (unpublished work). The extraction was set up in the following manner. Two circles of Whatman No. 2 filter paper, 6 mm. in diameter, were inserted into the bottom of the barrel of a 1-ml. hypodermic syringe. The alkaloid-containing silica gel was put on top of the filter paper. A small pledget of cotton was put on top of the silica gel, and the column was tamped down. A 2.5 in. long 19-gauge needle was attached to the syringe and the entire apparatus was inserted through the hole of a rubber stopper plugging

TABLE II.—DRY WEIGHT OF ROOTS OF *D. innoxia* GROWN IN 10^{-8} *M* BENZOIN ANTI-OXIME IN WHITE'S NUTRIENT SOLUTION

Root No.	Root Wt., mg.	Root No.	Root Wt., mg.	Root No.	Root Wt., mg.
1	1.8	29	9.6	57	10.4
2	2.8	30	8.7	58	7.3
3	4.0	31	12.0	59	12.0
4	2.8	32	7.8	60	8.2
5	4.6	33	9.0	61	7.7
6	4.2	34	6.2	62	25.5
7	2.8	35	4.9	63	4.0
8	1.7	36	7.4	64	4.4
9	4.8	37	4.2	65	8.2
10	1.4	38	4.7	66	5.9
11	5.6	39	5.3	67	6.3
12	4.0	40	10.8	68	7.5
13	5.4	41	9.2	69	3.3
14	2.5	42	9.3	70	4.3
15	3.5	43	3.4	71	7.1
16	5.2	44	5.2	72	5.4
17	1.3	45	9.8	73	6.6
18	9.3	46	8.3	74	3.9
19	4.8	47	7.4	75	7.4
20	8.0	48	8.8	76	5.3
21	8.4	49	7.4	77	3.3
22	7.5	50	7.4	78	5.5
23	7.0	51	8.5	79	3.1
24	11.7	52	5.0	80	9.4
25	7.3	53	8.3	81	6.0
26	6.3	54	9.4	82	6.7
27	6.3	55	9.7		
28	5.7	56	8.3		
		Mean	6.6		
95% Confidence interval			5.9–7.3		

[2] The Silica Gel G is a product of E. Merck AG., Darmstadt, Germany, and is distributed by Brinkmann Instruments, Inc., Great Neck, L. I., N. Y.

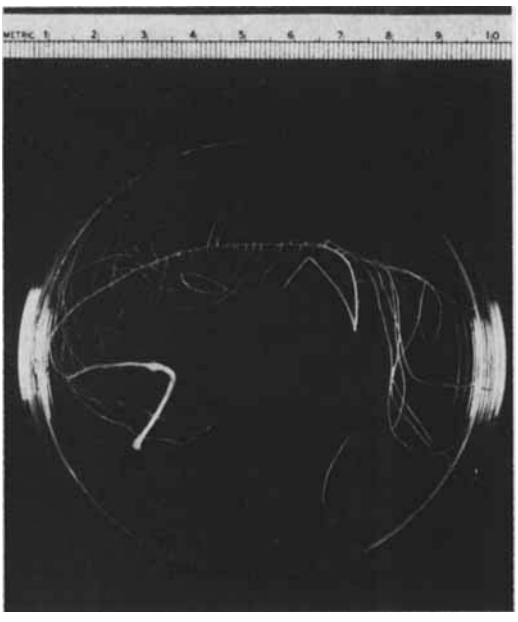

Fig. 1.—*D. innoxia* control root grown in White's nutrient solution.

the top of a small bell jar. One milliliter of absolute methanol was allowed to be in contact with the silica gel column for 30 min. Then 9 ml. of methanol was added to a reservoir above the extractor consisting of a 10-ml. hypodermic syringe attached to a 19-gauge needle inserted through a cork in the top of the 1-ml. syringe. By creating a vacuum in the bell jar, the extract was drawn into a 3-in. evaporating dish. The extract was evaporated to dryness over a water bath adjusted to approximately 65°.

Assay of Alkaloids.—The alkaloids hyoscyamine and scopolamine were quantitatively assayed spectrophotometrically using the Vitali-Morin reaction in a technique developed by French (24). Two-tenths of 1 ml. of fuming nitric acid was added to the dried methanol extract of silica gel containing hyoscyamine or scopolamine. The acid was then evaporated to dryness for 3 min. on a boiling water bath. The residue was redissolved in 4 ml. of acetone and made up to 25 ml. with successive 3-ml. washings of acetone. To determine the amount of alkaloid in each sample, 0.1 ml. of 3% w/v potassium hydroxide in absolute methanol was added to each 25-ml. flask of acetone solution of alkaloid. Each sample was measured against a blank prepared from 25 ml. of acetone and 0.1 ml. of 3% potassium hydroxide in methanol. The transmittance at 560 mμ with a 1-cm. light path was measured in a Beckman DU spectrophotometer, 3 min. after the addition of 3% potassium hydroxide in methanol for scopolamine, 7 min. for hyoscyamine.

RESULTS

The dry weights of both the control roots and the benzoin anti-oxime treated roots are summarized in Tables I and II. The confidence intervals indicate that there is no significant difference at the 5% level in dry weight between the control roots and the benzoin anti-oxime treated roots. However, benzoin anti-oxime does change the habit of growth of roots, as shown in Figs. 1 and 2.

To determine alkaloid content standard curves were prepared by plotting on semilog paper the transmittance values against known concentrations of pure hyoscyamine and scopolamine dissolved in 50% ethanol. The straight line curves then obtained showed adherence to Beer's law. The k values for hyoscyamine and scopolamine were computed from the standard curves and the Bouger-Beer relationship (25). The concentrations of alkaloid in milligrams were computed from these k values. The results of the alkaloidal assays of the control roots and benzoin anti-oxime treated roots are summarized in Tables III and IV. The overlapping confidence intervals indicate that there is no significant difference at the 5% level in alkaloid content between the control roots and the benzoin anti-oxime-treated roots.

DISCUSSION

The results obtained in this investigation seem to indicate that ascorbic acid oxidase is not related to hyoscyamine and scopolamine biosynthesis, nor is any other copper-containing enzyme.

At least five enzymes could serve as terminal oxidases in plants: polyphenol oxidase, lactase, ascorbic acid oxidase, cytochrome oxidase, and peroxidase (4). The first three, being copper-containing enzymes, are apparently eliminated from participation in alkaloid biosynthesis by the results of this study. The last two are iron-containing enzymes, which still may be considered, since they were not inactivated in this study. However,

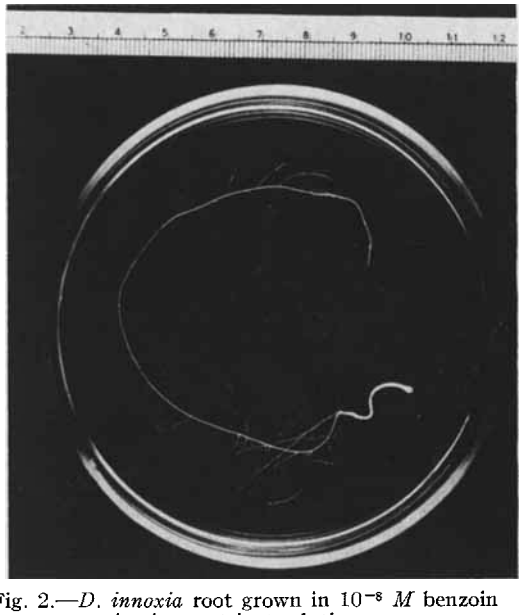

Fig. 2.—*D. innoxia* root grown in 10^{-8} *M* benzoin anti-oxime nutrient solution.

TABLE III.—ALKALOID CONTENT OF CONTROL ROOTS OF *D. innoxia*

Sample	Hyoscyamine[a]	Scopolamine[a]
1	0.00853	0.0220
2	0.00160	0.0188
3	0.00559	0.0195
4	0.00321	0.0141
5	0.00207	0.0127
6	0.00231	0.0145
7	0.00750	0.0102
8	0.0102	0.0182
9	0.00830	0.0210
10	0.00208	0.0153
11	0.00490	0.0141
12	0.00644	0.0225
13	0.0158	0.0238
14	0.0150	0.0274
15	0.0151	0.0155
16	0.0120	0.0183
17	0.0151	0.0226
18	0.00388	0.0191
19	0.00979	0.0213
Mean	0.00786	0.0185
95% Confidence interval	0.00577–0.00995	0.0164–0.0206

[a] Milligrams per 25 mg. crushed root sample.

TABLE IV.—ALKALOID CONTENT OF ROOTS OF *D. innoxia* GROWN IN 10^{-8} *M* BENZOIN ANTI-OXIME IN WHITE'S NUTRIENT SOLUTION

Sample	Hyoscyamine[a]	Scopolamine[a]
1	0.0125	0.0241
2	0.00964	0.0121
3	0.0113	0.0194
4	0.0154	0.0187
5	0.0101	0.0188
6	0.00402	0.0106
7	0.00660	0.0106
8	0.0114	0.0136
9	0.00846	0.00956
10	0.00358	0.0120
11	0.0133	0.0253
Mean	0.00966	0.0159
95% Confidence interval	0.00715–0.0122	0.0121–0.0196

[a] Milligrams per 25 mg. crushed root sample.

copper has also been suggested to be involved in cytochrome oxidase activity (26–28), but this has been disputed (29).

The effect on the habit of growth appears to be due to a growth inhibiting effect on the secondary roots compensated for, however, in total weight by greater primary root elongation. Another chelating agent, EDTA, has been shown to suppress the elongation of wheat roots by suppressing cell multiplication (30).

SUMMARY

The benzoin anti-oxime, at a concentration of 10^{-8} *M*, changes the habit of growth of isolated 2-week-old root cultures of *D. innoxia* but has no significant effect on the dry weight of the roots. At this concentration, the benzoin anti-oxime also has no significant effect on the amount of the hyoscyamine or scopolamine found in the roots, and these results indicate the lack of ascorbic acid oxidase involvement in the oxidation of hyoscyamine to scopolamine under the conditions of this study.

It was also concluded that the low germination rate of *D. innoxia* could be improved by presoaking the seeds in White's nutrient solution. The use of nutritive solutions to stimulate the germination of species of *Datura* is suggested.

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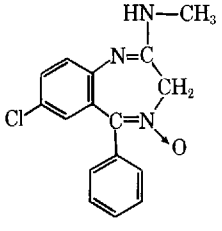
Metabolic *N*-Demethylation of Chlordiazepoxide

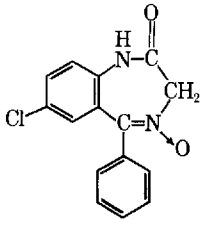
By MORTON A. SCHWARTZ and EDWARD POSTMA

Chlordiazepoxide was *N*-demethylated to 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepin-4-oxide (metabolite D-M) when incubated aerobically with either rat or dog liver preparations. Pretreatment of rats with phenobarbital resulted in an approximately threefold increase in the chlordiazepoxide metabolized to metabolite D-M after 2 hr. incubation with the liver microsome fraction. A fluorometric assay for metabolite D-M was developed which when used in conjunction with the Koechlin and D'Arconte fluorometric assay for chlordiazepoxide allowed for a specific determination of each compound in blood. The application of this assay to the study of chlordiazepoxide metabolism in man is illustrated.

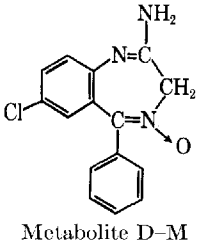
CHLORDIAZEPOXIDE,[1] 7-chloro-2-methylamino-5-phenyl-3H-1,4-benzodiazepin-4-oxide, is metabolized in the dog and man to 7-chloro-1,3-dihydro - 5 - phenyl - 2H - 1,4 - benzodiazepin-2-one-4-oxide ("lactam") (1) which is then metabolized to "opened lactam" metabolites (2). These metabolites have not as yet been detected in the rat which metabolized chlordiazepoxide to unidentified products (2). The present work, which was initiated to obtain information on the site and mechanism of the metabolic conversion of chlordiazepoxide to "lactam," has resulted in the discovery of a new chlordiazepoxide metabolite, 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepin-4-oxide (metabolite D-M), which is common to man, dog, and rat. The structures are given below.



Chlordiazepoxide



"Lactam"



Metabolite D-M

METHODS AND MATERIALS

In Vitro Studies.—Chlordiazepoxide hydrochloride (100 mcg. in 0.1 ml. of water) was incubated for 2 hr. at 37° with liver slices (450–490 mg.) or liver homogenate (0.3 ml. of a 1:4 homogenate in 1.15% KCl) prepared from 200–300 Gm. male rats. Also used as enzyme sources were 9000 × g supernatants (0.3 ml.) prepared as described by Fouts and Brodie (3) from 1:3 liver homogenates in 1.15% KCl. Liver supernatants prepared from four control rats were pooled as were those prepared from four rats given i.p. injections of 37.5 mg./Kg. of phenobarbital twice a day for 4 days to stimulate the drug-metabolizing enzymes of the liver microsomes. In addition, a liver supernatant from a dog who had been pretreated with phenobarbital (2 daily oral 20 mg./Kg. doses followed by 10 daily oral doses of 10 mg./Kg.) and which had been stored at −20° for 3 months was used. Besides substrate and enzyme source, the incubation mixture contained the cofactors described by Conney *et al.* (4): 0.2 ml. of 0.01 *M* ATP, 0.1 ml. of 0.003 *M* NADP, 0.1 ml. of 0.003 *M* NAD, 0.1 ml. of 0.6 *M* nicotinamide, 0.2 ml. of 0.03 *M* glucose-6-phosphate, 0.1 ml. of 0.1 *M* $MgCl_2$, 0.1 ml. of 2 *M* KCl, 1.0 ml. of 0.1 *M* potassium phosphate buffer, pH 7.4, and sufficient water to bring the total volume to 3.0 ml.

Following the 2-hr. incubation each sample was extracted twice with 6 ml. of ether. The ether extracts were evaporated to dryness, brought to 0.1 ml. with ethanol, and 0.025 ml. of the ethanol solutions were examined by thin-layer chromatography (TLC) using fluorescent silica gel (Camag Kieselgel DF-5) plates and a solvent system of ethyl acetate–ethanol (90:10) (system *1*). In this system the chlordiazepoxide R_f varied from 0.2–0.4 while the R_f values of "lactam" were 0.7–0.8. A second solvent system which proved useful for identifying metabolite D-M was heptane–chloroform–ethanol (1:1:1) (system *2*). Under shortwave U.V. light compounds on the plate appeared as dark areas against a fluorescent background. The plates were then sprayed with 10% sulfuric acid and viewed under long-wave U.V. light to detect compounds which were fluorescent against a dark background.

A quantitative determination of the amount of chlordiazepoxide metabolized to metabolite D-M was obtained by using as substrate chlordiazepoxide-2-^{14}C (specific activity of 1690 dpm/mcg.) which was purified by sublimation as previously described (2). The ^{14}C which was extracted from the incubation media by ether and which migrated on TLC as chlordiazepoxide and metabolite D-M was determined by the liquid scintillation techniques with the POPOP–PPO fluors previously described (5) and a Nuclear-Chicago Mark I liquid scintillation spectrometer equipped with a ^{133}Ba external standard. The instrument was programmed so that the ratio of the external standard counts in channel B/channel A was convertible from a standard curve into counting efficiency which when divided into the

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[1] Marketed as Librium by Hoffmann-LaRoche, Inc., Nutley, N. J.

channel C sample counts yielded the sample ^{14}C content in dpm.

Fluorometric Determination of Metabolite D-M, Chlordiazepoxide, and "Lactam." —The development of the fluorometric determination of metabolite D-M is given under *Results*. This assay has been incorporated into a slightly modified Koechlin and D'Arconte (1) assay for chlordiazepoxide and "lactam" so as to yield a fluorometric determination of each compound when all three are present in a single blood sample. All fluorometric determinations were made in a Farrand spectrofluorometer.

Reagents

A 1.0 *M* potassium phosphate buffer, pH 7.0–7.2, and 0.2 *M* potassium phosphate buffer, pH 6.7–6.8; 0.10 *N* H_2SO_4 and 7 *N* H_2SO_4, 5 *N* NaOH and 0.10 *N* NaOH, and 0.15 *M* diethanolamine aqueous solution.

Procedure

A 2-ml. control blood or plasma (blank) and 2 control bloods (or plasmas) containing internal standards are run with each set of unknowns. Five micrograms of chlordiazepoxide in 0.1 ml. of ethanol and 5 mcg. of "lactam" in 0.1 ml. of ethanol are added to one control blood, while 5 mcg. of metabolite D-M in 0.1 ml. of ethanol is added to the other; each control containing internal standard is taken through the entire procedure. Specimens of whole blood (heparinized or oxalated) and plasma (separated immediately after drawing the blood) must be kept frozen if the assay is not performed within 24 hr.

Two milliliters of blood is first diluted with 2.0 ml. of water and 4.0 ml. of 1.0 *M* phosphate buffer, pH 7.0–7.2, and is then extracted with 15 ml. of ether. A 12-ml. aliquot of the ether phase is extracted with 4 ml. of 0.1 *N* NaOH to remove "lactam" but not chlordiazepoxide or metabolite D-M from the ether. Two 5.0-ml. aliquots (to be used for the metabolite D-M and chlordiazepoxide assays) are then removed from the remaining ether phase.

Analysis A for "Lactam."—The 0.1 *N* NaOH extract is washed by extraction with 5 ml. of ether and is then placed 8 10 in. from a Pyro-Lux R-57 lamp (Luxor Lighting Products, Inc., New York, N. Y.) and light-exposed for 1 hr. This photochemical reaction may also be accomplished by exposure to bright sunlight for 1 hr. The fluorescence is then determined at 380 mμ excitation and 460 mμ emission.

Analysis B for Metabolite D-M.—One 5-ml. ether aliquot is extracted with 3.5 ml. of 7 *N* H_2SO_4 and the ether is removed by aspiration. After standing for 1 hr. (no light exposure required) the fluorescence of the acid extract is determined at 370 mμ excitation and 460 mμ emission.

Analysis C for Chlordiazepoxide.—The other 5-ml. ether aliquot is extracted with 1.5 ml of 0.1 *N* H_2SO_4. To the acid extract 0.5 ml. of 0.2 *M* phosphate buffer, pH 6.7–6.8, and 1 ml. of 0.15 *M* diethanolamine solution are added and the mixture is kept in a boiling water bath for 2 hr.[2] After cooling, 0.2 ml. of 5 *N* NaOH is added, and the sample is then exposed to light for 1 hr. and its fluorescence determined exactly as described above for the "lactam" determination.

Calculations

Analysis A ("Lactam").—The fluorescence reading (F) for the "lactam" internal standard (5 mcg.) corrected for the blood blank fluorescence (B) is divided by 5 to yield F-B per microgram of "lactam." This specific fluorescence value divided into the corrected fluorescence (F-B) obtained with each unknown yields microgram "lactam" per 2 ml. of blood and final division by 2 then gives the "lactam" concentration in mcg./ml.

Analysis B (Metabolite D-M).—The metabolite D-M internal standard fluorescence is used to get the specific fluorescence (F-B/mcg.) and the calculations proceed as described above.

Analysis C (Chlordiazepoxide.)—Since both chlordiazepoxide and metabolite D-M yield fluorescence in this part of the method, a correction for metabolite D-M fluorescence must be made before the fluorescence in an unknown due to chlordiazepoxide can be determined. The specific fluorescence for metabolite D-M in analysis *C* is determined. This value multiplied by the micrograms of metabolite D-M found in each unknown in analysis *B* yields the fluorescence in analysis *C* which is due to metabolite D-M. Subtraction from the total analysis *C* fluorescence (F-B of each unknown) yields the F-B value of each unknown which is due only to chlordiazepoxide. The remainder of the calculations are identical to those of the above analyses: the F-B per mcg. of chlordiazepoxide (obtained from the fluorescence of the chlordiazepoxide internal standard) is divided into the F-B of each unknown attributed to chlordiazepoxide to get the chlordiazepoxide content per 2 ml. of blood.

Comments

The sensitivity of this assay is 0.2–0.3 mcg. of each compound per ml. of blood or plasma.

The chlordiazepoxide assay is the least accurate of the three because it involves the subtraction of one fluorescence from another. An error in the determination of metabolite D-M will cause an error in the chlordiazepoxide determination.

RESULTS

In Vitro Metabolism of Chlordiazepoxide.—Aerobic incubation of chlordiazepoxide with rat liver slices, rat liver homogenate, and the 9000 × g supernatant of liver from a dog pretreated with phenobarbital all gave essentially the same results when the ether extracts were chromatographed in system *1*. A metabolite (metabolite D-M), which was not completely separated on the plate from nicotinamide (a constituent of the incubation medium which was also extracted by ether), was seen under shortwave U.V. light. The metabolite migrated 54–58% of the distance chlordiazepoxide migrated in this system. When the plates were sprayed with 10% sulfuric acid and examined under long-wave U.V. light this metabolite was seen more clearly because it fluoresced while nicotinamide did not. The metabolite was not detected in control samples incubated without chlordiazepoxide or in complete samples which were extracted immediately with

[2] This hydrolysis of chlordiazepoxide to "lactam" is a modification of the Koechlin and D'Arconte procedure suggested by M. Roth and J. Rieder, Hoffmann-LaRoche and Co., Basle, Switzerland.

ether without prior incubation. Furthermore, when chlordiazepoxide was incubated anaerobically (in evacuated Thunberg tubes) with rat liver homogenate the metabolite was not found, indicating that it was formed by an oxidative process. In none of these *in vitro* experiments, however, was any conversion of chlordiazepoxide to "lactam" detected.

Metabolite D-M was suspected of being *N*-desmethyl chlordiazepoxide because this compound would be a logical intermediate in the metabolism of chlordiazepoxide to "lactam" (see introduction for formulas). Authentic 7-chloro-2-amino-5-phenyl-3H-1,4-benzodiazepin-4-oxide (*N*-desmethyl chlordiazepoxide) was therefore spotted on TLC plates alongside an aliquot of the ether extract obtained after incubation of chlordiazepoxide with the 9000 × g supernatant of dog liver. *N*-Desmethyl chlordiazepoxide and metabolite D-M in the extract migrated identically in system *1* (R_f 0.25) and also in system *2* (R_f 0.53). The authentic *N*-desmethyl chlordiazepoxide also fluoresced under long-wave U.V. light after the plates were sprayed with 10% sulfuric acid. It was therefore concluded that metabolite D-M was *N*-desmethyl chlordiazepoxide.

The effect of phenobarbital pretreatment of rats on the metabolism of ^{14}C-labeled chlordiazepoxide by the liver microsome fraction (9000 × g supernatant) is shown in Table I. The first two flasks demonstrated the validity of the quantitation technique in that incubation of chlordiazepoxide-2-^{14}C in the absence of enzymes resulted in 95 and 94% of the ^{14}C being extracted by ether and 98% of the extracted ^{14}C being recovered on TLC as intact chlordiazepoxide. When labeled chlordiazepoxide was incubated with pooled liver supernatant from control rats (flasks 3 and 4) 90 and 92% of the ^{14}C was ether-extracted but only 74% of the extracted ^{14}C was recovered as unmetabolized chlordiazepoxide while 20% was found as metabolite D-M. Incubation with the pooled liver supernatant from phenobarbital-treated rats (flasks 5 and 6) resulted in a striking increase in chlordiazepoxide metabolism. Only 78% of the ^{14}C was extracted by ether indicating that significant quantities of polar metabolites were now formed. In addition the ether-soluble ^{14}C was mostly present in metabolite D-M (77 and 66%) while 22 and 23% was present as chlordiazepoxide and a significant amount of ^{14}C (5 and 6%) was now found at the origin. Calculation of the chlordiazepoxide-derived ^{14}C which was actually recovered as metabolite D-M yielded 18% for flask 3 and 56% as an average for flasks 5 and 6; this demonstrates that *N*-demethylation had been stimulated at least threefold (the metabolites not extracted by ether may also be *N*-demethylated) by pretreatment of the rats with phenobarbital. The stimulation of over-all chlordiazepoxide metabolism was similarly estimated to be approximately fourfold.

TABLE I.—EFFECT OF PHENOBARBITAL PRETREATMENT ON *In Vitro* METABOLISM OF CHLORDIAZEPOXIDE-2-^{14}C BY 9000 × g SUPERNATANT OF RAT LIVER[a]

Flask	Source of 9000 × g Supernatant	^{14}C Extracted by Ether After 2 hr. Incubation, %	^{14}C of Ether Extract in[b] Chlordiaz., %	Met. D-M, %	Origin, %
1	None, no enzyme	95	98	1	1
2	None, no enzyme	94			
3	Control rats	90	74	20	1
4	Control rats	92			
5	Phenobarb-treated rats	78	23	77	5
6	Phenobarb-treated rats	78	22	66	6

[a] Labeled chlordiazepoxide (100 mcg.) was incubated with the cofactors described under *Methods and Materials* and each designated enzyme source for 2 hr. at 37°. The 9000 × g supernatants of liver from four phenobarbital-treated rats were pooled as were the supernatants from four control rats. The pooled supernatant was run in duplicate as enzyme source. [b] Distribution of ether-extracted ^{14}C determined by TLC in system *1* and counting of the silica gel segments containing chlordiazepoxide, metabolite D-M, and origin. Chlordiazepoxide and metabolite D-M were spotted as internal standards with each extract.

Detection of Metabolite D-M in Blood.—The possibility that metabolite D-M was present in blood following the administration of chlordiazepoxide was investigated in three species. Blood (2 ml.) drawn from a squirrel monkey 4 hr. after an oral dose of 5 mg./Kg. of chlordiazepoxide was first mixed with equal volumes of water and 0.1 *M* potassium phosphate buffer, pH 7.4, and then extracted twice with 3 vol. of ether; the combined ether extracts were concentrated for TLC. In a similar manner ether extracts for TLC were prepared from rat blood (5 ml.) obtained 3.5 hr. after i.p. doses of 7.5 mg./Kg. and 15 mg./Kg. of chlordiazepoxide and from human blood (5 ml.) obtained from a woman who had taken an overdose of chlordiazepoxide. TLC in system *1* of each of the above concentrated ether extracts followed by inspection of the plates under U.V. light before and after acid treatment revealed the presence of both chlordiazepoxide and metabolite D-M. These findings established the need for an assay for metabolite D-M in blood.

Development of Metabolite D-M Assay.—Further studies revealed that the Koechlin and D'Arconte fluorometric determination of chlordiazepoxide (1) did not discriminate between chlordiazepoxide and metabolite D-M; 5 mcg. of metabolite D-M added to 1 ml. of blood and analyzed by this method resulted in approximately the same fluorescence as that obtained from 5 mcg. of chlordiazepoxide per ml. of blood. This made imperative the development of an assay specific for one or the other of these two compounds. Such an assay was developed for metabolite D-M using as the starting point the observed fluorescence of this compound on thin-layer plates which had been sprayed with 10% sulfuric acid. The compound was found to fluoresce in 10% sulfuric acid solution and an excitation wavelength of 370 mμ and emission wavelength of 460 mμ yielded the highest fluorescence reading. The fluorescence of 5 mcg./ml. of metabolite D-M increased sharply from 66 units after standing in the acid for 15 min. to 111 units after 1 hr. and then rose only to 119 units after 2 hr. It was further found that this was not a photochemical reaction; the same fluorescence was obtained whether metabolite D-M in 10% sulfuric acid was kept for 1 hr. in the dark or in daylight. Investigation of the effect of acid concentration on fluorescence revealed that metabolite D-M developed maximum fluorescence in 7 *N* H_2SO_4. Accordingly, 7 *N* H_2SO_4 was used to obtain data on the relationship of fluo-

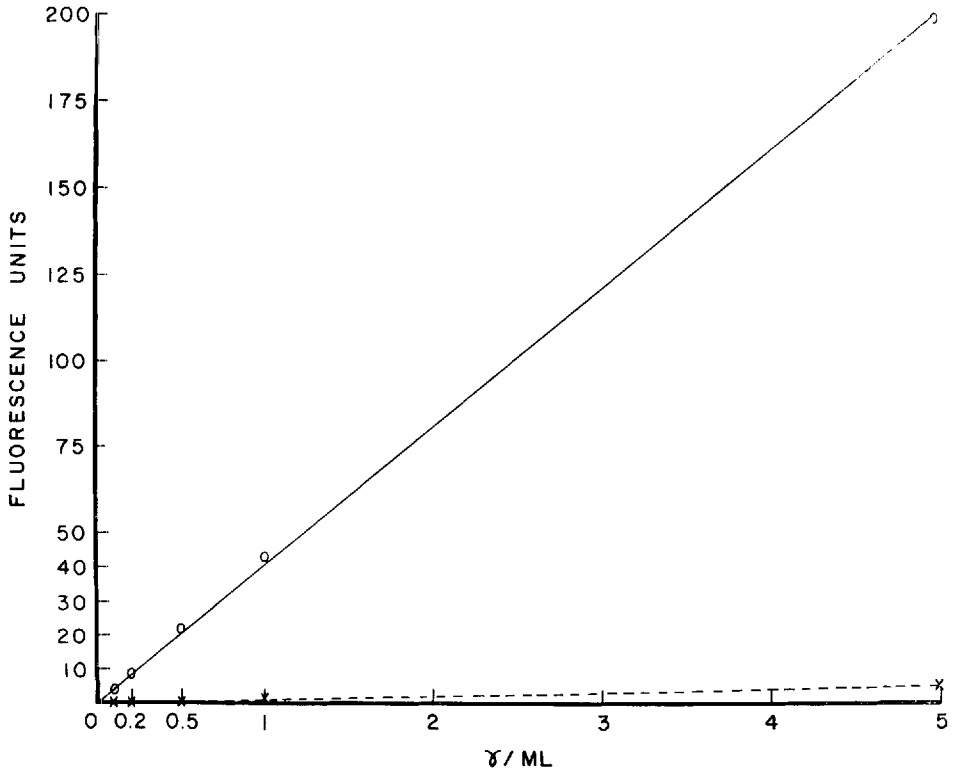

Fig. 1.—Relationship of fluorescence to concentration of metabolite D-M and chlordiazepoxide in 7 *N* sulfuric acid. After standing 1 hr. at room temperature, the solutions were read at 370 mμ excitation and 460 mμ emission. Key: O, metabolite D-M; X, chlordiazepoxide.

TABLE II.—RECOVERY OF CHLORDIAZEPOXIDE, "LACTAM," AND METABOLITE D-M IN BLOOD AND PLASMA CONTAINING ALL THREE COMPOUNDS[a]

		Recovery of Each Compd.								
		"Lactam"			Metabolite D-M			Chlordiazepoxide		
Expt.	Sample	Added, mcg.	Found, mcg.	Recovery, %	Added, mcg.	Found, mcg.	Recovery, %	Added, mcg.	Found, mcg.	Recovery, %
1	Human plasma	5.0	5.4	108	5.0	4.8	96	5.0	5.1	102
	Human plasma	3.0	2.7	90	2.0	2.0	100	4.0	3.5	88
2	Dog blood	4.0	4.1	103	6.0	6.1	102	2.0	1.9	95
	Dog blood	4.0	3.6	90	2.0	2.2	110	6.0	6.2	103
3	Dog plasma	5.0	4.8	96	5.0	5.4	108	5.0	4.5	90
	Dog plasma	3.0	3.1	103	2.0	2.3	115	4.0	3.9	98

[a] The method for metabolite D-M was incorporated into the Koechlin and D'Arconte assay to yield the combined method described under *Methods and Materials*.

rescence to metabolite D-M concentrations. As seen from Fig. 1 the fluorescence of metabolite D-M was a linear function of the concentration from 0.5 mcg./ml. The fluorescence obtained from 0.1–1 mcg./ml. of chlordiazepoxide under these conditions was barely measurable while the fluorescence from 5 mcg./ml. of chlordiazepoxide was about the same as that obtained from 0.1 mcg./ml. of metabolite D-M.

This metabolite D-M assay was therefore of sufficient specificity so that it could be used together with the Koechlin and D'Arconte assay to achieve specific determinations of chlordiazepoxide, "lactam," and metabolite D-M. This combined assay (described under *Methods and Materials*) was tested by determining the recoveries of each compound added to the same blood sample. It is seen from Table II that good recovery of each compound from blood and plasma was obtained in a series of recovery experiments in which the ratio of one compound to another in the added mixture was varied.

Applications of the Combined Assay for Chlordiazepoxide, Metabolite D-M, and "Lactam."—The plasma levels of each compound following 100 mg. of chlordiazepoxide hydrochloride given intravenously to each of two subjects are shown in Fig. 2. In both subjects the half-life of plasma chlordiazepoxide was roughly 16 hr. In subject No. 1 metabolite D-M appeared before "lactam," reached higher levels, and fell off faster; in subject No. 2

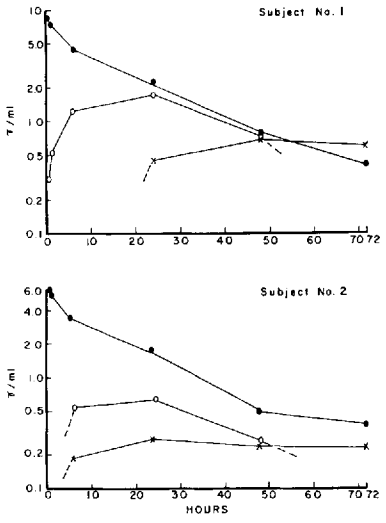

Fig. 2.—The plasma levels of chlordiazepoxide (●), metabolite D-M (O), and "lactam" (×) plotted logarithmically as a function of time. Each of the two subjects received a 100-mg. intravenous dose of chlordiazepoxide hydrochloride. The broken lines denote that the levels have either risen from or descended to levels which were below the sensitivity of the assay.

TABLE III.—PLASMA CHLORDIAZEPOXIDE, METABOLITE D-M, AND "LACTAM" IN A SUBJECT WHO RECEIVED 10 mg. OF CHLORDIAZEPOXIDE HYDROCHLORIDE q.i.d. FOR 14 DAYS

Time,[a] Days	Plasma Levels of— Chlordiazepoxide, mcg./ml.	Metabolite D-M, mcg./ml.	"Lactam," mcg./ml.
2	1.69	0.47	Nil
4	3.13	1.17	0.70
6	1.61	1.27	1.06
10	3.13	1.17	1.41
12	1.90	1.27	1.41
14	3.42	1.17	1.27
22	Nil	Nil	(0.24)[b]

[a] Blood was drawn on each day listed immediately before the first of the 4 doses was administered. Day 22 values represent residual plasma levels after 7 days of no drug.
[b] This value is at the limit of sensitivity of the assay.

both metabolites appeared at 6 hr., but the metabolite D-M levels were more than twice the "lactam" levels and metabolite D-M again fell off faster.

Table III presents the plasma levels of each compound in a subject who received chlordiazepoxide hydrochloride chronically. The levels of the metabolites are of particular interest because it is again seen that metabolite D-M is detectable in the blood before "lactam" and that after drug administration was stopped for 1 week both metabolite D-M and chlordiazepoxide were no longer detectable while a very slight amount of "lactam" appeared to be still present.

The limitation of the combined assay was illustrated when 30 mg. of chlordiazepoxide hydrochloride was administered intravenously to a subject. The chlordiazepoxide blood levels declined from 2.3 mcg./ml. 15 min. after the dose to approximately 0.4 mcg./ml. at 48 hr.; metabolite D-M was not seen until the eighth hour and from 8–48 hr. low levels at the sensitivity threshold of the assay appeared to be present; and "lactam" was not detected in any of the blood samples. In this subject the blood levels of metabolite D-M were too low for accurate measurement, and since the determination of blood chlordiazepoxide is dependent on an accurate assay of metabolite D-M, it was not possible to get an accurate measurement of chlordiazepoxide.

DISCUSSION

The *in vitro* studies demonstrate that rat and dog liver are capable of producing metabolite D-M by oxidative *N*-demethylation of chlordiazepoxide. This metabolite is also formed *in vivo* as evidenced by its detection in the blood of chlordiazepoxide-treated rats, squirrel monkeys, and humans. In previous studies with ^{14}C-chlordiazepoxide (2) most of the radioactivity excreted as neutral or basic urinary metabolites by two humans was identified as "lactam." It is possible that a small but significant amount of metabolite D-M was excreted but escaped detection. In the rat the basic metabolite fraction of the urine contained at least two labeled metabolites which were not identified. Studies on the characterization of chlordiazepoxide metabolites in rat urine have been reinstituted to determine if metabolite D-M is one of these excretion products.

Although the structure of metabolite D-M makes this compound a logical intermediate in the formation of "lactam" from chlordiazepoxide, only indirect evidence for this pathway is presently available. In all human blood level studies to date, including those reported above, the appearance of "lactam" in the blood of chlordiazepoxide-treated subjects has never preceded the appearance of metabolite D-M indicating that this latter compound may be the immediate precursor of "lactam" in man and dog. It must be noted, however, that *in vitro* experiments with dog liver aimed at demonstrating "lactam" formation from either chlordiazepoxide or metabolite D-M have been unsuccessful.

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Hydrolytic Behavior of Isoalloxazines Related to Riboflavin II

Kinetics of Degradation of 9-Methylisoalloxazine in Alkaline Media

By DEODATT A. WADKE and DAVID E. GUTTMAN

The kinetics of hydrolysis of 9-methylisoalloxazine under aerobic conditions was investigated at hydroxide-ion concentrations both below and above its pKa. The nature of the pH profile indicated that a specific base-catalyzed reaction was responsible for the degradation and that the unionized species was much more susceptible to hydrolysis than the ionized form. The hydrolysis in pH 9.0 borate buffer and in strongly alkaline medium was investigated rather extensively. Here quantitative separation of the reaction intermediates and products was achieved by the use of ion-exchange chromatography. Rate studies showed that under strongly basic conditions the parent isoalloxazine disappeared faster than the intermediate anil, while the reverse was true at pH 9.0. These studies also showed that the rate of disappearance of the anil paralleled the rate of appearance of its decomposition products. Evidence was obtained to indicate that the degradation of the isoalloxazine involved an initial hydroxide-ion attack at the C_α position to yield a carbinolamine which underwent further reaction to yield the anil or the ureide and then the final products.

A PREVIOUS COMMUNICATION (1) summarized the results of some preliminary studies on the hydrolytic degradation of 9-methylisoalloxazine. These studies showed that at least three different products could be formed from the parent compound and that these were 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid, 1,2,3,4 - tetrahydro - 1 - methyl - 2,3 - dioxoquinoxaline, and 1,2-dihydro-1-methyl-2-oxoquinoxaline. The relative concentrations of the products formed were seen to depend on the pH of the reaction medium. It was further shown that 5-(6-methylaminophenylimino)barbituric acid anil was formed from the parent isoalloxazine and subsequently disappeared from the reaction mixture. Evidence was presented to suggest that the anil existed in equilibrium with 1,2-dihydro-1-methyl-2-oxo-quinoxaline-3-carboxy ureide, and it was postulated that the ureide could further decompose by at least three mechanisms to yield three different products.

In the present investigation the kinetics of various reactions involved in the transformations of the isoalloxazine to products were investigated. Studies were restricted to reactions occurring in solutions which were exposed to air. Anaerobic systems which were shown to exhibit more complex behavior (1) were not investigated. The pH profile for the disappearance of the isoalloxazine in the alkaline range of pH was determined and extensive kinetic studies were conducted on reactions which occurred in strongly basic solution and in pH 9.0 buffer. These quantitative studies showed that in strongly alkaline medium 80% of the parent isoalloxazine was transformed into the keto acid, that the anil was formed and disappeared at the same rate as the formation of the keto acid, and that another transient intermediate, possibly a carbinolamine, was initially formed from the parent isoalloxazine. The data also revealed that the rate of disappearance of the suspected carbinolamine closely paralleled that of the anil and suggested the possibility that the anil and the suspected carbinolamine were in equilibrium in solution. Studies at pH 9.0, where the major product was the dioxo compound, showed that the rate of appearance of this compound paralleled the rate of disappearance of the parent compound and indicated that the rate-determining step in the formation of the dioxo compound was one involving the loss of 430 mμ absorbance of the isoalloxazine.

EXPERIMENTAL

Materials.—9 - Methylisoalloxazine (I), 5 - (6-methylaminophenylamino)barbituric acid anil (III), 1,2-dihydro-1-methyl-2-keto-3-quinoxaline carboxylic acid (V), and 1,2,3,4-tetrahydro-1-methyl-2,3-dioxo-quinoxaline (VI) were obtained as described previously (1, 2).

All other materials used in this study were obtained from commercial sources.

Procedure.—*Kinetic Studies.*—The experimental method used was the same as that described previously (3). The rate of disappearance of 9-methylisoalloxazine was determined by following the decrease in absorbance at 430 mμ. The rates of hy-

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Previous paper: Wadke, D. A., and Guttman, D. E., *J. Pharm. Sci.*, **55**, 1088(1966).

drolysis were investigated over the pH range 7.9 to 14.0 and at 35° ± 0.01°. In addition, systems in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide were also studied at 45° ± 0.01°.

The degradation of the anil (III) was investigated in pH 9.0 borate buffer and in 0.5 *N* sodium hydroxide. This study was conducted at 45°. Samples were withdrawn at various time intervals and complete spectra were determined using a Beckman DB spectrophotometer.

Ion-Exchange Chromatography.—One and one-half grams of a strongly basic, anion-exchange resin,[1] 100–200 mesh, was made into a slurry with distilled water and packed in a 25-ml. buret using a glass wool plug as a support. Prior to use, the column was washed with 100 ml. of 2 *M* sodium chloride followed by distilled water until the washings were free from chloride. Preliminary studies with the synthetic mixtures of the isoalloxazine (I), the anil (III), and the keto acid (V) showed that when such a mixture at about pH 5 was introduced on the column and eluted with water, the eluate (which will be subsequently referred to as the neutral fraction) contained the isoalloxazine. When the solvent was changed to 0.01 *M* acetic acid, the eluate (which will be referred to as the acidic fraction) contained the anil. Finally, elution with 2 *M* sodium chloride effected removal of the keto acid. These studies also showed that the recovery of the three compounds was quantitative within experimental limits.

This technique was utilized to effect quantitative separation of the residual isoalloxazine, the anil, and the keto acid at various stages of degradation of the isoalloxazine. Forty-five-milliliter samples were withdrawn from the reaction mixture at different times and mixed with 1 ml. of glacial acetic acid in the case of pH 9.0 samples and with 2 ml. in the case of high pH (0.5 *N* base) samples in a 50-ml. volumetric flask and the volume was adjusted to the mark with distilled water. The pH of the resulting solution was approximately 5. Forty milliliters of the acidified sample was introduced on the ion-exchange column and elution carried out in three stages to obtain the neutral fraction, the acidic fraction, and the salt fraction. Each fraction was collected in a 100-ml. volumetric flask. After adjusting to volume, the spectrum of each was recorded using a Beckman DB spectrophotometer.

RESULTS

pH Profile for the Hydrolysis of 9-Methylisoalloxazine.—Loss of absorbance at 430 mμ was used to indicate the disappearance of the isoalloxazine from solution. When the logarithm of the absorbance at 430 mμ was plotted as a function of time, a typical pseudo first-order plot resulted. Figure 1 illustrates such a plot for 9-methylisoalloxazine which was hydrolyzed in 0.5 *N* sodium hydroxide. As is seen, some deviation from linearity became apparent beyond approximately three half-lives. Similar behavior was also observed by Song *et al.* (4) in their study on photochemical degradation of flavins. As a result, rate constants were determined from initial straight line portions of the curves. The results of the studies conducted over a range of hydroxide-ion concentrations are summarized in Fig. 2, where logarithm of the observed rate constant was plotted as a function of the logarithm of the hydroxide-ion concentration. This pH profile shows that the reaction was approximately first order with respect to hydroxide ion at pH values below and above the pKa (9.82), but that in the region of the pKa, the order was changing.

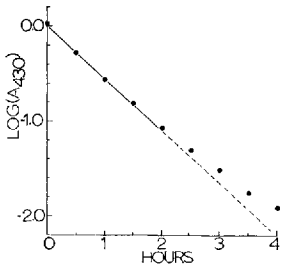


Fig. 1.—A plot illustrating the first-order disappearance of 9-methylisoalloxazine in 0.5 *N* sodium hydroxide and at 45°.

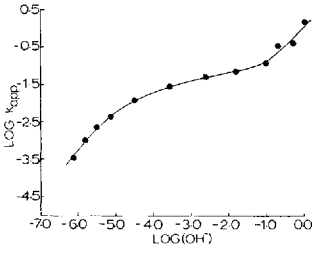


Fig. 2.—The pH profile for the hydrolysis of 9-methylisoalloxazine at 35°.

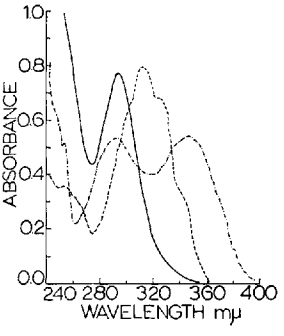


Fig. 3.—Spectra of the anil at a concentration of 1.8 × 10^{-4} *M* (———), the keto acid at a concentration of 8 × 10^{-5} *M* (– · – · –), and the dioxo compound at a concentration of 7.3 × 10^{-5} *M* (– – – –).

Kinetic Studies on the Hydrolysis of the Anil (III).—The kinetics of transformations involving the anil was also followed spectrophotometrically. Figure 3 shows the spectra of the anil (III), the keto acid (V), and the dioxo compound (VI). It is seen that the anil possesses negligible absorbance at 340 mμ, where both the keto acid and the dioxo compound exhibit significant absorbance. The increase in absorbance at this wavelength was thus used to follow the progress of the reactions. Although it was shown in a previous study (1) that the anil probably existed in solution in equilibrium with the ureide (IV), the predominant species, under the pH conditions of this study, was the anil. The concentration of the ureide was apparently so small that it did not contribute to the absorbance at this wavelength. Figure 4 illustrates that, in 0.5 *N* sodium hydroxide solution, the rate of appearance

[1] Marketed as Amberlite CG-400 by the Rohm & Haas Co., Philadelphia, Pa.

of the keto acid exhibited a first-order dependency on anil concentration. The rate constant was calculated to be 0.18 hr.$^{-1}$ at 45°. In a pH 9 system it was found that rate of appearance of the major product here, the dioxo compound, was also first order with respect to anil and that the rate constant for the reaction at 45° was 5.0×10^{-2} hr.$^{-1}$.

Quantitative Ion-Exchange Chromatography.—Figure 5 illustrates the results of kinetic studies which were conducted with 9-methylisoalloxazine in 0.5 *N* sodium hydroxide. It is seen that there occurred in this system a rapid disappearance of the parent isoalloxazine, appearance and disappearance of the anil, and appearance of the keto acid. From these data, rate constants were estimated for the various processes and are tabulated in Table I.

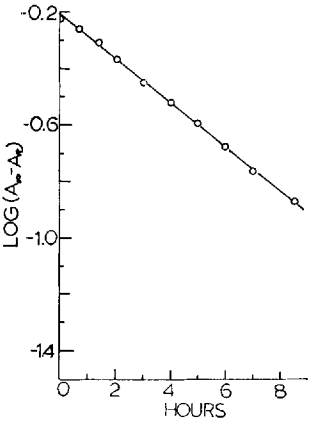


Fig. 4.—A plot illustrating the first-order dependency of keto acid formation on the concentration of the anil compound in 0.5 *N* sodium hydroxide and 45° ± 0.01°. A_∞ represents the maximum absorbance achieved at 340 mμ, while A_t represents the absorbance observed at this wavelength at time *t*.

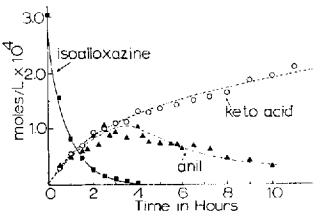


Fig. 5.—A plot of concentration *versus* time for the isoalloxazine, the anil, and the keto acid as observed in the reaction mixture that was degraded in 0.5 *N* sodium hydroxide and at 45°.

TABLE I.—RATE CONSTANTS FOR APPEARANCE AND DISAPPEARANCE OF VARIOUS COMPOUNDS IN 0.5 *N* NaOH AT 45° ± 0.01°

Reaction	Rate Constant, hr.$^{-1}$
Disappearance of isoalloxazine	1.3
Disappearance of the anil in the degrading soln. of isoalloxazine	0.19
Appearance of the keto acid from isoalloxazine	0.17
Disappearance of the suspected carbinolamine	0.22

It is pertinent to note that the rate constant characterizing the appearance of the keto acid is approximately the same as that found for the disappearance of the anil and that these constants are close to that obtained when pure anil was used as the starting material under similar conditions of temperature and hydroxide-ion concentration. The study also showed that approximately 80% of the parent isoalloxazine was eventually transformed to the keto acid. It is also apparent from the figure that the relatively rapid disappearance of 9-methylisoalloxazine from solution was not accompanied by as rapid an appearance of the anil. This was rather surprising in view of the evidence that the anil is an intermediate in the transformation of the parent compound to products. It was also interesting to observe that, in the initial stages of the reaction, the final product, the keto acid, was found in concentrations which were approximately the same as those formed for the anil. This behavior strongly suggested that another reactive intermediate was formed and that it was in equilibrium with the anil. The existence of such a species in the reaction mixture was also indicated by spectral examination of the neutral fractions obtained in the chromatographic procedure. The spectrum of such a fraction is shown in Fig. 6. It appears to be that of a mixture. The spectrum of 9-methylisoalloxazine alone is also shown for comparative purposes. It was assumed, based on the first-order decay of absorbance at 430 mμ, that absorbance at this wavelength was due solely to 9-methylisoalloxazine and its concentration in the fraction was calculated. Absorbance values at other wavelengths, resulting from this concentration, were then determined using appropriate molar absorptivities. When these values were subtracted from those obtained for the fraction, a difference spectrum was generated which is also shown in Fig. 6. The difference spectrum has a maximum at 350 mμ and is apparently due to a transient intermediate. Difference spectra were obtained for a number of fractions representing different reaction times and the logarithm of the absorbance at 350 mμ of the difference spectrum was plotted as a function of time. Figure 7 represents this plot and shows that this unknown intermediate initially formed rather rapidly and as time progressed disappeared from the medium at a rate which was slower than the rate of formation. The rate content for the disappearance was estimated from the later linear portion of the curve to be 0.22 hr.$^{-1}$ and is tabulated with other rate constants in Table I. This value is reasonably close to that obtained for the disappearance of anil and further supports the possibility of the existence of a rapidly achieved equilibrium between the unknown intermediate and the anil. The absolute concentration of the unknown compound at any time cannot be determined. However, if it was in equilibrium with the anil and if the equilibrium was established fairly rapidly then, at any time [unknown] = $K_{eq.}$[anil], where $K_{eq.}$ = equilibrium constant and the brackets represent concentrations. From the knowledge of the absorbance at 350 mμ due to the unknown compound and the concentration of the anil at the same time, one can calculate a term equivalent to the product of the molar absorptivity of the unknown at 350 mμ and the equilibrium constant.

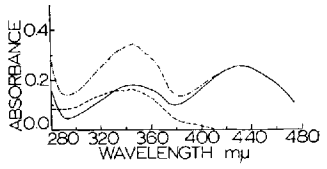


Fig. 6.—Spectrum of the aqueous fraction isolated from the reaction mixture that was partially degraded in 0.5 *N* sodium hydroxide (– · – · –) and that of the pure isoalloxazine (————). The dotted line represents the difference spectrum (see text).

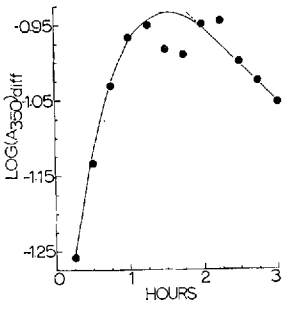


Fig. 7.—A curve showing the appearance and subsequent disappearance of the suspected carbinolamine intermediate.

Table II shows the values of this parameter calculated from data obtained at different times. The concentrations of the anil used in these calculations were obtained from the smooth curve represented in Fig. 5. It can be seen that the values agree fairly well among themselves and further support the hypothesis concerning the equilibrium. It is rather surprising that this supporting evidence was obtained since the analytical method employed does physically separate the two compounds which are thought to be in equilibrium, *i.e.*, the anil was held on the column while the unknown compound was passed through. Apparently, the forward and the reverse rates for the equilibrium are quite slow at the pH of the acid-quenched samples as compared to the rate of passage of solutions through the column.

Table II.—Values of the Product of the Molar Absorptivity of the Suspected Carbinolamine and the Equilibrium Constant, as Calculated from the Data Corresponding to Different Times[a]

Time, hr.	$K_{eq.}$ × molar Absorptivity × 10^{-3}
2.25	0.93
2.50	0.91
2.75	0.81
3.00	0.81
3.25	0.82
3.50	0.80
3.75	0.84
4.00	0.82
4.25	0.82

[a] See text.

Solutions of 9-methylisoalloxazine which were maintained at a pH of 9 did not contain measurable concentrations of the anil or the keto acid. Consequently, quantitative studies here were restricted to the determination, as a function of reaction time, of the concentrations of residual isoalloxazine and dioxo compounds. Both compounds appeared in the neutral fraction of the chromatographic elution scheme. Figure 8 shows the spectrum of such a fraction. Since the dioxo compound does not absorb in the visible region of the spectrum, a simultaneous spectrophotometric analysis of the two component mixture was readily made. Figure 9 shows the time course for the disappearance of isoalloxazine and appearance of the dioxo compound. It was determined that 70% of the parent compound was eventually converted to dioxo compound. The data shown in Fig. 9 were used to estimate rate constants for the processes and these are tabulated in Table III. The rate constant describing the disappearance of the isoalloxazine is sufficiently close to that found for the appearance of dioxo compound to suggest that the rate-determining step in the formation of the product from the parent compound was that involving the ion of the 430 mμ bond of the isoalloxazine. Intermediates such as the anil, the ureide, or the unknown species referred to earlier were apparently present in steady-state concentrations which were too low to detect. It is apparent from the data of Table III that the anil, under these conditions, degraded more rapidly than the parent isoalloxazine. This is in agreement with results obtained in the earlier study (1) where the anil could not be detected in degrading solutions of 9-methylisoalloxazine maintained at a pH of 9.

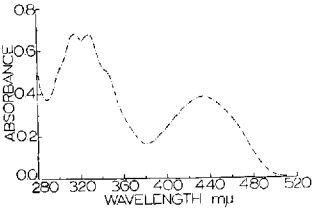


Fig. 8.—Spectrum of a neutral fraction obtained by ion - exchange chromatography of a reaction mixture which was partially degraded in pH 9.0 borate buffer at 45°.

DISCUSSION

As is seen in Fig. 2, the pH profile for 9-methylisoalloxazine exhibits curvature over the pH range bracketing the pKa of the compound (9.82). This was not unexpected and indicated that both ionized and unionized species underwent a base-catalyzed reaction, but at different rates. Thus, the apparent rate constant for the reaction can be defined in terms of ionization of the compound as:

$$k_{app.}(I_t) = k_1(OH^-)(I^-) + k_2(OH^-)(IH) \quad \text{(Eq. 1)}$$

where

$k_{app.}$ = the observed pseudo first-order rate constant,
I_t = the total concentration of the isoalloxazine,
IH = the stoichiometric concentration of the unionized isoalloxazine,
I^- = the stoichiometric concentration of the ionized isoalloxazine,
k_1 = the second-order rate constant for the disappearance of the ionized form,

k_2 = the second-order rate constant for the disappearance of the unionized form.

Equation 1 can be rearranged in the manner suggested by Schwartz *et al.* (5) to show:

$$k_{app.}/OH^- = k_1 + (k_2 - k_1)f_{1H} \quad \text{(Eq. 2)}$$

where, f_{1H} = the fraction unionized of the isoalloxazine. Equation 2 predicts a linear relationship between $k_{app.}/OH^-$ and f_{1H}. Such a plot for the system investigated is shown in Fig. 10. The excellent linearity observed over a wide range of hydroxide-ion concentrations supports the underlying hypothesis. The values of k_1 and k_2 can be calculated from the slope and the intercept of such a plot. These values as determined by the method of least squares were found to be 4.45 L. mole.$^{-1}$ hr.$^{-1}$ for k_1 and 5.01 $\times$ 10^2 L. mole^{-1} hr.$^{-1}$ for k_2. As would be expected from electrostatic considerations the rate constant characterizing the reaction of the negatively charged species is considerably smaller than that of the neutral species.

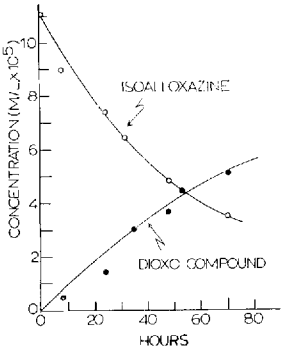


Fig. 9.—A concentration *vs.* time plot for the disappearance of 9 - methylisoalloxazine and the appearance of the dioxo compound in a pH 9 borate buffer at 45°.

TABLE III.—RATE CONSTANTS FOR APPEARANCE AND DISAPPEARANCE OF VARIOUS COMPOUNDS IN pH 9.0 BORATE BUFFER AT 45° ± 0.01°

Reaction	Rate Constant, hr.$^{-1}$ $\times$ 10^2
Disappearance of isoalloxazine	1.6
Appearance of the dioxo compd. from isoalloxazine	1.8
Disappearance of the anil	5.0

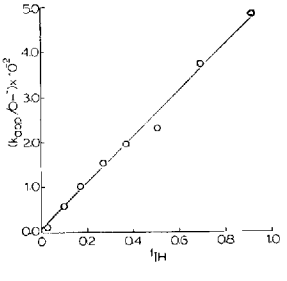
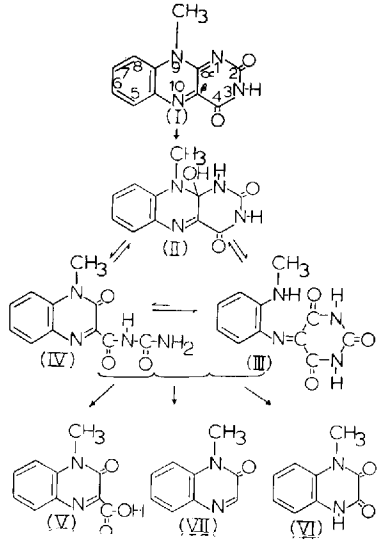


Fig. 10.—A plot showing the relationship expressed by Eq. 2 for 9-methylisoalloxazine at 35° ± 0.01°.

The reaction shown in Scheme I was presented and discussed earlier (1). It was suggested at that time that a carbinolamine intermediate could be initially formed from the isoalloxazine. It is indeed possible that the compound detected in the neutral fraction during the course of this study was this carbinolamine intermediate. It would result from the attack of hydroxide-ion at the Cα position of the isoalloxazine. Thus loss of absorbance at 430 mμ can be explained on this basis since such an attack would destroy the conjugated system $-N_{10} = C_\beta - C_\alpha = N_1-$, which is responsible for absorption of radiation in the visible region of the spectrum. The carbinolamine would be expected to possess a pKa similar to the parent compound and to appear, therefore, in the neutral fraction of the ion-exchange separation. In addition, the charge-density calculations for isoalloxazines reported by Pullman and Pullman (6) suggest that Cα is a good center for nucleophilic attack. Tetrahedral carbinolamine intermediates have been postulated by others in, for example, the hydrolysis of Schiff bases (7) and iminolactones (8). The work of Cunningham and Schmir (8) on the hydrolysis of iminolactones is of special interest here. According to their reasonings, a carbinolamine of the postulated structure (II) could undergo rupture of Cα—N$_9$ bond to yield the anil or breaking of Cα—N$_1$ bond to yield the ureide. It is conceivable then that both the anil and the ureide existed in equilibrium with the carbinolamine and the suspected ureide–anil equilibrium, reported earlier (1), occurred through the carbinolamine compound.

Results have been presented to show that the major products of alkaline hydrolysis of 9-methylisoalloxazine, in the presence of air, were the keto acid (V) and the dioxo compound (VI). In the earlier study (1) the presence of a third product—namely, 1,2 - dihydro - 1 - methyl - 2 - oxoquinoxaline (VII)—was reported. This compound could



Over-all Reaction Mechanism for the Degradation of 9-Methylisoalloxazine.

Scheme I

be detected only under anaerobic conditions at pH 9 where the competing reactions leading to the formation of the dioxo compound and the keto acid were suppressed. Quantitative studies of the kinetics of the anaerobic hydrolysis are presently being conducted and will be reported at a later time.

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Proteinaceous Antitumor Substances from Plants II

Mirabilis multiflora

By AYHAN ULUBELEN* and JACK R. COLE

Preliminary screening of the aqueous extract of *Mirabilis multiflora* (*Nyctaginaceae*) has shown antitumor activity against Sarcoma 180 in mice. The isolation, purification, and partial characterization of proteinaceous material is reported. This material has shown activity against Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), and lymphosarcoma.

As a result of a routine screen of southwestern plants for potential antitumor activity, the aqueous extract of *Mirabilis multiflora*[1] (*Nyctaginaceae*) Britton and Rusby demonstrated activity toward the Sarcoma 180 test system in mice.[2] This screening program was carried out by the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md. Further fractionation has resulted in activity in the Lewis lung carcinoma, Walker carcinosarcoma 256 (intramuscular), lymphosarcoma, and Sarcoma 180 test systems.

The plant is an herbaceous perennial with a large tuberous root. The above-ground portion of the plant dies during freezing weather or extended drought and regrowth occurs from subterraneous parts of the plant.

Part of the collection used in this study was obtained on rocky slopes about 5 miles south of St. David, Ariz., at an elevation of about 3,700 ft., May 31, 1962. Another part of the collection was obtained at the Boyce Thompson Southwestern Arboretum near Superior, Ariz., on July 1, 1964. It was in cultivation in a sandy loam soil within a grove of eucalyptus.

EXPERIMENTAL

Fresh roots of *M. multiflora* (3.5 Kg.) were extracted with approximately 15 L. of petroleum ether followed by 15 L. of water at room temperature. The water solution was then washed with benzene and chloroform and lyophilized in a Repp Industries model 15 sublimator. The yield was 320 Gm. One-hundred grams of this crude material was dissolved in 1000 ml. of water at room temperature. The insoluble part was separated by centrifuge and discarded (32 Gm.). The remaining aqueous portion was extracted with 5 × 200 ml. of ether to remove the remaining fatty materials. The aqueous solution was increased in volume threefold by the addition of 95% ethanol. A light-colored precipitate formed and was separated by centrifuge. It was dissolved in water and lyophilized. Twenty-one grams of the material was obtained. This crude material showed an activity against the Sarcoma 180 test system of the CCNSC. The requirement for activity in this system is two successive dose response tests showing a reduction of at least 56% in tumor size. The crude material showed a decrease in tumor size of 84% at a dose of 22 mg./Kg. The elementary analysis of this material showed 13.4% inorganic material which consists mostly of magnesium and phosphate ions and also the presence of sulfur and nitrogen. Upon hydrolysis of the material a number of amino acids were obtained. Two grams of the crude material was dissolved in water and dialyzed against distilled water, using a dialyzing tube of $^5/_8$ in. diameter (Arthur H. Thomas Co., No. 4465-A2, Philadelphia, Pa.). After a period of 7 days, the precipitate in the dialysis tube was separated from

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Previous paper: Ulubelen, A., Caldwell, M., and Cole, J. R., *J. Pharm. Sci.*, **54**, 1214(1965).
* Visiting Research Associate Professor. Present address: College of Pharmacy, University of Istanbul, Istanbul, Turkey.
[1] Identification confirmed by Robert Barr, Research Associate, College of Pharmacy, and Dr. Charles Mason, Curator of the Herbarium, Botany Department, University of Arizona, Tucson. A reference specimen was also deposited.
[2] Preparation of the preliminary extraction was carried out by Dr. M. E. Caldwell.

be detected only under anaerobic conditions at pH 9 where the competing reactions leading to the formation of the dioxo compound and the keto acid were suppressed. Quantitative studies of the kinetics of the anaerobic hydrolysis are presently being conducted and will be reported at a later time.

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Proteinaceous Antitumor Substances from Plants II

Mirabilis multiflora

By AYHAN ULUBELEN* and JACK R. COLE

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TABLE I.—*In Vivo* TUMOR INHIBITION[a]

	Dose, mg./Kg.	% T/C[b]
Lewis lung carcinoma	12	38
P-1798 Lymphosarcoma	12	22
	12	22
	8	55
	5.3	72
	3.5	53
	40	...
	27	...
	18	...
	12	33
Sarcoma 180	10	8
	10	11
	4.4	44
	2.9	63
Walker carcinosarcoma 256 (intramuscular)	45	39
	12	58
	8	79
	5.3	73
	3.5	69

[a] "Protocols for Screening Chemical Agents and Natural Products Against Animal Tumors and Other Biological Systems," Cancer Chemotherapy National Service Center, *Cancer Chromatography Rept.*, No. 25, December 1962. [b] The criteria for activity is defined as being a per cent T/C (test/control) value of less than 42 in a satisfactory dose response test.

TABLE II.—TUMOR INHIBITION OF SEPHADEX-TREATED FRACTIONS

Sephadex	Dose, mg./Kg.	% T/C
G-50	10	13
G-100	10	29
G-200a	10	15
G-200b	10	20

the solution. The solution was then lyophilized, with a yield of 957.7 mg. (47.8%) which contained only 2.1% inorganic compounds. The lyophilized material (compound *A*) was subjected to screening against several test systems. The results are indicated in Table I.

A series of column chromatographic purifications were attempted. They included column substrates of DEAE Sephadex A-50, CM Sephadex C-50; G-50, G-100, and G-200 Sephadex. The first two substrates were ineffective, whereas the latter three were successful. A series of fractions were collected and the presence of protein was indicated by means of absorption at 280 mμ in an ultraviolet spectrophotometer. One protein fraction was obtained from the G-50 and G-100 Sephadex columns and two different protein fractions were obtained from the G-200 Sephadex column. After subsequent dialysis and lyophilization, the materials obtained were submitted to tests on Sarcoma 180. The results are tabulated in Table II. Comparison of Tables I and II shows that essentially no increase in activity was obtained by purification techniques.

Physical and Chemical Characteristics of Compound A.—Paper electrophoresis of compound *A* was run utilizing a barbital buffer of pH 8.6, 0.05 ionic strength in a Spinco model instrument, and after 18 hr. of electrophoresis using a 5-ma. current, the papers were developed by two different procedures. Bromophenol blue indicated the presence of two proteins. One appeared at the point of application and the other had a mobility of 6.6×10^{-7} cm.²/sec. v. (Fig. 1). The second development procedure used was periodic acid and Schiff reagent which indicated a glycoprotein at the application point. The closed aldehyde groups in the polysaccharide conjugate are oxidized by periodic acid, then fuchsin sulfite stains the polysaccharides. Other substances such as glucose, glycogen, and the amino acids, serine and threonine, can be stained with fuchsin sulfite, but they are removed in the prerinse.

The above would indicate that compound *A* is a combination of a glycoprotein and protein. The two proteins were separated by a Sephadex G-200 column using a series of phosphate buffer systems pH 8.6, ionic strength 0.075. The compound was then hydrolyzed with 6 *N* hydrochloric acid in a sealed and evacuated glass tube at 105°. The resulting solution was applied on Whatman No. 1 paper using the same solvent system reported in a previous paper (1). Figure 2 shows the presence of 22 amino acids and perhaps some polypeptide residues.

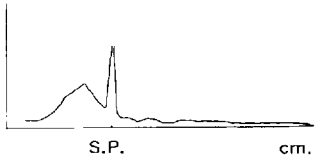

Fig. 1.—Absorbance relationship of the proteins. One unit of integration = 0.1 cm.². Key: SP, starting point.

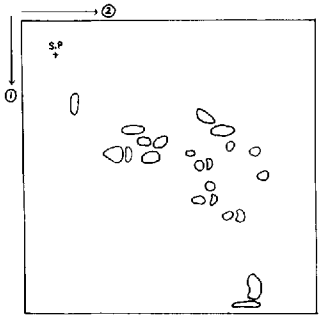

Fig. 2.—Paper chromatography of hydrolyzed compound *A*. Key: SP, starting point; 1, phenol–water (3:1); 2, butanol–formic acid–water (7:1:3).

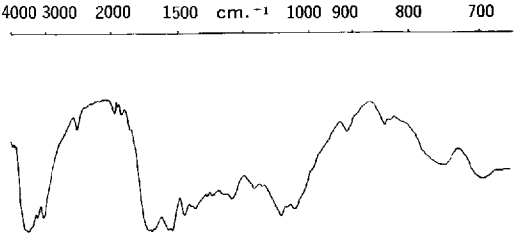

Fig. 3.—Infrared spectrum of compound *A*.

TABLE III.—PRELIMINARY AMINO ACID ANALYSIS OF *Mirabilis* PROTEIN[a]

Lysine	5.74%
Histidine	1.09
Arginine	4.32
Aspartic acid	12.49
Threonine	6.44
Serine	4.95
Glutamic acid	8.00
Proline	3.45
Glycine	6.10
Alanine	2.95
Half-cystine	6.54
Valine	5.24
Methionine	0.89
Isoleucine	6.91
Leucine	4.89
Tyrosine	6.54
Phenylalanine	4.62

[a] Based on three determinations of the dried sample.

One-hundred milligrams of compound *A* was hydrolyzed with 3 *N* hydrochloric acid in a boiling water bath for 24 hr. in order to determine the sugars. Using Whatman No. 1 paper and the solvent system *n*-butanol–pyridine–water (6:4:3) and comparing with standard sugar samples the following were detected: galactose, mannose, galactosamine, and fucose. The latter two were present in very small amounts. Two additional solvent systems were employed to verify the presence of the above sugars. They were (*a*) ethyl acetate–acetic acid–ethanol–benzene–water (325:93:236:200:146) and (*b*) *n*-butanol–acetic acid–water (4:1:2).

Ultraviolet absorption of compound *A* showed maxima at 280, 243, and 210 mμ. Infrared curve shows characteristic peaks at 3350, 3150, 3030, 2900, 1600, and 1535 cm.$^{-1}$. (See Fig. 3.) Nitrogen determination of compound *A* using microkjeldahl method yielded 14.06% nitrogen. Results of a preliminary amino acid analysis utilizing a Beckman model 120B instrument are shown in Table III.

DISCUSSION

In the work being carried out in this laboratory, glycoproteins have been found on several occasions to be antitumor agents.

A review of the literature has shown a number of publications concerning the relationship of malignant tumors and the increase of glycoprotein concentration in the blood of test animals and humans. There are, however, several varied opinions as to the cause of this increase. Seibert and co-workers (2) reported that the source of glycoproteins was the breakdown of the products of tissue necrosis. Several other workers (3–5) suggested complex mechanisms involving increased hepatic synthesis and release of glycoproteins into the circulation in response to an appropriate stimulus which has a tumor or inflammation. Patterson *et al.* (6) suggest the increase in glycoprotein is a function of bacterial contamination. Other groups of workers (3, 7, 8) reported that this increase is due to a liberation of products of the metabolic activity of rapidly reproducing cells. Catchpole (9) has found a high concentration of glycoprotein in the neighboring tissues of a malignant tumor. Therefore, this could be the depolymerization of the ground substance of this adjacent connective tissue with subsequent release into the circulation. Engel (10), Gilmore and Schwarz (11) are in accord with this theory. The carbohydrate portion of these serum glycoproteins has been shown to contain mannose, galactose, glucosamine, fucose, and sialic acid (12, 13). Macbeth *et al.* (14–18), in a private communication (19), indicated that the liver is the most active agent in the synthesis of these glycoproteins. By using isotopic techniques they also have shown that the tumor system is capable of synthesizing and subsequently liberating glycoproteins into the blood stream. This work was done on intact and hepatectomized rats.

The authors believe that further investigation should be carried on to determine whether the introduction of a certain type of a plant glycoprotein into tumor-implanted animals may result in an inhibition of the synthesis and a reduction of the serum glycoprotein followed by a reduction in overall tumor size. In addition to the two plants mentioned above, there are three other plants under investigation in our laboratories which appear to have glycoproteins as the active antitumor agents.

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Rheological Evaluation of Lipophilic Suspending Agents I

Dimethyl Dialkyl Ammonium Hectorite

By CHARLES T. LESSHAFFT, JR.

A modified Brookfield viscometer was used to evaluate dispersions containing various concentrations of dimethyl dialkyl ammonium hectorite (DDAH) and ethanol in light mineral oil. The optimum amount of ethanol needed to disperse the DDAH in the oil was found to be an amount equal to the weight of the DDAH. A new method of determining the static yield value was used as one of the rheological parameters in the evaluation. The static yield value (Y_s) in dynes/cm.^{2} was found to be related to the percentage concentration (C) of the DDAH as follows: log $Y_s = mC + \log b$ in which the constants m and b equal 1 and 2, respectively, for dispersions containing an ethanol content equal to the DDAH; and equal to 0.6 and 3.5, respectively, for dispersions containing an ethanol content twice that of the DDAH. A 1.25 per cent DDAH–1.25 per cent ethanol dispersion was evaluated for its ability to suspend several drugs. It was postulated from the sedimentation data that the small amount of supernatant liquid which occurred in these suspensions was due to syneresis.

THE EVALUATION of hydrophilic suspending agents as stabilizers for aqueous suspensions has been the subject of numerous investigations. However, there are no such lipophilic suspending agents in use for preparing pharmaceutical oil suspensions with the exception of aluminum monostearate. Aluminum monostearate is used to gel the oil in the preparation of oily suspensions of procaine penicillin G, not for the purpose of stabilizing the suspension (even though it does this), but for the purpose of prolonging the absorption of the penicillin.

Oily mediums still serve a need as vehicles for dermatological drugs as attested to by the number of U.S.P. XVII (1) and N.F. XII (2) ointments that use the petrolatums as the base. Rheologically, petrolatum has a high yield value at room temperature. The yield value becomes greater as solid substances are incorporated. This makes the resulting ointment difficult to spread and the excessive pressure needed for its application may be painful. It would seem that a liquid vehicle, such as mineral oil, might serve the same purpose as an ointment prepared with petrolatum and be easier to apply.

Mineral oil is a viscous liquid but is not a good suspending medium because of its Newtonian character. A good suspending medium should have a yield value, yet it should be capable of being poured from a bottle (3–6). The evaluation of a substance that would alter the flow properties of mineral oil to produce such an effect is of interest here. Previous reports indicate that dimethyl dialkyl ammonium hectorite[1] (DDAH) might be a likely candidate to produce this effect. For example, it has been used to prepare ointments with heavy mineral oil (7) and with hexamethyltetracosane (8). Viscous dispersions of DDAH in volatile organic solvents have been tested as granulators and binders in the production of tablets (9).

DDAH is a finely divided light cream-colored powder. Unlike hectorite, which is highly hydrophilic, this organic derivative of hectorite is hydrophobic and organophilic. As a result, it produces gels with organic liquids. The mechanism of the gelling process has been investigated by Jordan and co-workers (10–12). Although they worked with organic derivatives of bentonite, their results and interpretations can be applied to DDAH because both bentonite and hectorite have the same montmorillonite lattice structure (13). In the gelling process it is necessary for the solvating liquid to first penetrate between the small primary particles and effect a separation of them before solvation can occur. The liquid must possess some degree of polarity which is needed to overcome the attractive forces that hold these particles together and allow its penetration. Liquids consisting primarily of hydrocarbons, such as mineral oil, do not possess sufficient polarity to accomplish this. The use of polar additives and mechanical shear can be used to overcome these forces and allow the penetration of the oil. The low molecular weight alcohols, such as methanol and ethanol, are recommended as polar additives for this purpose.

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[1] Made by the National Lead Co. under the name of Bentone 38. The alkyl groups are a mixture of C_{18} and C_{16} in a ratio of approximately 70 to 30, respectively.

TABLE I.—DIMENSIONS OF CYLINDRICAL SPINDLES

Spindle No.	Radius (R_b), cm.	Ht., cm.	Effective Ht. (h),[a] cm.
1	0.942	6.510	7.493
2	0.513	5.395	6.121
3	0.294	4.288	4.846
4	0.159	3.101	3.396

[a] The effective height is the actual height plus the correction for the end effect.

It is also suggested that the alcohols contain 5% water (14).

The amount of ethanol recommended as a polar additive for organic derivatives of montmorillonite clays is about 45% based on the weight of the clay derivative (14). It is likely that the amount of ethanol would vary, depending on the particular derivative and the polarity of the vehicle. The purpose of this investigation was to determine the optimum concentration of DDAH and ethanol and the most efficient procedure for the preparation of an oily suspending medium. Rheological evaluations of the dispersions were used to select the combination of DDAH and ethanol in light mineral oil that could be used as a suspending medium. Various rheological parameters were used in the evaluation for the purpose of correlating concentrations with a rheological parameter.

EXPERIMENTAL

Instrumentation.—A Brookfield viscometer (LVT model)[2] was modified so that it would function as an absolute rheometer. The No. 2 and No. 3 disk shaped spindles were replaced with cylindrical spindles. The dimensions of the spindles are given in Table I. The guard that is supplied with the viscometer was removed to prevent the guard from interfering with shearing and also to facilitate the use of a cup having a smaller diameter and volume than the cup recommended for use with the guard attached to the instrument. These modifications were similar to those of Wood *et al.* (15) with the exception of the cup. The cup used in this investigation was a 100-ml. graduated cylinder having a diameter of 2.6 cm. and cut at the 70-ml. mark to reduce its height.

The shearing stress (F) was calculated utilizing the following equation

$$F = \frac{M}{2\pi R_b^2 h} \qquad \text{(Eq. 1)}$$

in which M is the torque, R_b is the radius of the spindle, and h is the effective height of the spindle (16). The effective height of the spindle is the actual height plus a correction factor for the end effect of the spindle. The torque is obtained by multiplying the dial reading of the instrument by 6.737. Each unit division of the dial is equal to 6.737 dynes-cm. for the Brookfield LVT model. The equation for calculating the shearing stress for this instrument is then

$$F = \frac{6.737D}{2\pi R_b^2 h} \qquad \text{(Eq. 2)}$$

in which D is the dial reading.

The rate of shear (G) was calculated at the wall of the spindle by the following equation

$$G = \frac{2\omega R_c^2}{R_c^2 - R_b^2} \qquad \text{(Eq. 3)}$$

in which ω is the angular velocity, R_c is the radius of the cup, and R_b is the radius of the spindle (16).

Rheograms could now be prepared by plotting rate of shear in sec.$^{-1}$ *versus* shearing stress in dynes/cm.^{2}. These flow curves would visually describe the flow character and relative viscousness of the liquid. However, a mathematical description of the curve would be more useful.

Plastic Viscosity and Bingham Yield Value.—The rheogram of a Newtonian liquid is a straight line which passes through the origin. The reciprocal of the slope of this line is the coefficient of viscosity in poises. Theoretically, the rheogram for a plastic liquid is a straight line which does not pass through the origin but intercepts the shearing stress axis at zero rate of shear. The reciprocal of this line is the plastic viscosity in poises and the intercept is known as the Bingham yield value in dynes/cm.^{2}. Both parameters, the plastic viscosity and the Bingham yield value, are needed to describe a plastic liquid. In an actual experimental determination the rheogram of a plastic liquid will not be a straight line throughout the whole range of shear rates but will curve toward the origin at the lower rates of shear. This curve is due to an instrumental characteristic. The low angular velocity of the spindle is not great enough to cause the liquid to flow throughout the annular space between the walls of the spindle and cup. The straight line portion of the curve can be projected to the shearing stress axis for the determination of a Bingham yield value, the reciprocal of the straight line being the plastic viscosity. In this study these parameters were calculated from the straight line connecting the two highest shear rates of the rheogram.

Power Law Equations.—Two power law equations have been used to describe the flow curve for non-Newtonian liquids. Equation 4 has been used by a number of investigators (17–19).

$$F^N = \eta' G \qquad \text{(Eq. 4)}$$

Written in logarithmic form the equation is

$$\log G = N \log F - \log \eta' \qquad \text{(Eq. 5)}$$

Equation 6 has also been frequently used (20–22).

$$F = K G^{1/N} \qquad \text{(Eq. 6)}$$

The logarithmic expression for this equation is

$$\log F = 1/N \log G + \log K \qquad \text{(Eq. 7)}$$

While both equations have shearing stress (F) and rate of shear (G) in common, Eq. 4 utilizes the slope (N) of the curve and Eq. 6 uses the reciprocal of the slope ($1/N$). The slope or its reciprocal indicates deviation from Newtonian behavior. The

[2] Brookfield Engineering Corp., Stoughton, Mass.

slope and the reciprocal of the slope are equal to one for Newtonian liquids; $N > 1$ and $1/N < 1$ for pseudoplastic (shear thinning) liquids; $N < 1$ and $1/N > 1$ for dilatant (shear thickening) liquids. The η' in Eq. 4 is the reciprocal of the intercept on the rate of shear axis, while the K in Eq. 6 is the intercept on the shearing stress axis at a rate of shear of 1 sec.$^{-1}$. Therefore, K is the apparent viscosity in poises at a shear rate of 1 sec.$^{-1}$. The relationship between K and η' is given in Eq. 8.

$$\log K = 1/N \log \eta' \qquad \text{(Eq. 8)}$$

Static Yield Value.—Newtonian liquids flow when acted on by any force, however small. Some liquids, other than Newtonian, do not flow until the applied shearing stress exceeds a certain minimum value. This minimum stress necessary to initiate flow is the yield value of the liquid. The present methods of determining yield value either measure the stress at a very low rate of shear or project a stress at zero rate of shear. These methods are referred to as dynamic since the yield value is determined as a result of shearing.

A method of determining static yield value was reported by Levy (23). The advantage of his method is that a different and previously undisturbed region of the liquid is continuously sheared as the Helipath spindle descends into the liquid. The method has the disadvantage in that the instrumental design does not permit the yield value to be expressed in terms of dynes/cm.^{2}. It is also questioned whether the method is static since the spindle was rotated at 6 r.p.m.

It was noticed in the preliminary rheological evaluations of the dispersions made in this study that the pointer of the Brookfield viscometer did not move immediately after the instrument was turned on, even though the dial moved at the designated r.p.m. The pointer started to move only after some stress value was reached as indicated by the dial reading. This condition did not occur when known Newtonian liquids were tested. It was determined that the spindle did not move until the pointer moved, which meant that the liquid was not being sheared. It was reasoned that the liquid prevented the spindle from moving until the strain on the torsion spring of the instrument overcame the yield value of the liquid. It was also determined that the yield value taken at zero rate of shear was independent of the spindle size used.

It was known prior to this investigation that a yield value could be determined directly with a Brookfield viscometer using cylindrical spindles (24). If the liquid has a yield value the pointer will not drift to zero when the motor of the instrument is stopped. Instead, the pointer will drift and come to rest at some position on the dial before reaching zero. The stress equivalent to the dial reading would be the yield value. This yield value, however, would depend on the shear history of the sample even though the yield value was determined at zero rate of shear.

Methodology.—The method used in this study for taking the static yield value consisted of a repeated on-off technique of operating the instrument. The viscometer was turned on at a speed of 0.3 r.p.m. and then off almost immediately. The pointer would remain stationary after the instrument was turned off until the yield value was exceeded, then it would drift toward zero, stopping at some distance from zero. The last stationary reading taken before the pointer started to drift toward zero was taken as the static yield value.

It was necessary to control the shear history of the samples since the dispersions were time-dependent; that is, at a constant rate of shear the shearing stress decreased with time. All of the rheological evaluations were made 2 days after the preparation of the dispersion unless otherwise specified. The sample was transferred to the cup. The cup was immersed in a water bath and held in place by a clamp. The temperature of the bath was regulated at 25° ± 0.1° by a constant-temperature circulator.[3] After the spindle was immersed in the sample, a period of 30 min. was allowed to elapse before taking the static yield value. Immediately after taking the static yield value, the sample was sheared for exactly 10 min. at each r.p.m. of the instrument starting at the highest r.p.m. The stress was recorded at the end of each 10-min. shearing period. Preliminary investigations of the methodology and instrumentation of the rheological evaluation indicated that results could be satisfactorily duplicated.

Preparation of the Dispersions.—The dispersions of DDAH were prepared in quantities of 900 Gm. The percentages used to express the concentration of the ingredients are by weight. Light mineral oil N.F. which had a specific gravity of 0.8379 and a viscosity of 25 cps. was used as the vehicle. Alcohol U.S.P. which is referred to in this paper as ethanol was used as the polar additive.

Various orders of mixing were investigated to determine the most efficient procedure for dispersing DDAH in light mineral oil. Dispersions containing 1% DDAH and 0.5% ethanol were prepared by each of the following methods.

Method A.—Add the ethanol to a suspension of DDAH in the oil while mixing with the Dispersator.[4]

Method B.—Add the ethanol to the DDAH contained in a beaker and lightly mix with a stirring rod. Add this damp powder to the oil while mixing with the Dispersator.

Method C.—Add the ethanol to the DDAH contained in a beaker and lightly mix with a stirring rod. Add 100 ml. of the oil to the damp powder. Transfer this thick slurry to the remaining oil and mix with the Dispersator.

Method D.—Mix the ethanol intimately with the DDAH in a mortar. Incorporate 100 ml. of the oil with this mass. Transfer this mixture to the remaining oil and mix with the Dispersator.

The mechanical mixing was uniform for all of the above methods. The high viscosity head of the Dispersator was used for 5 min. with a Powerstat setting of 45 which was equivalent to approximately 4500 r.p.m. The use of this head was necessary in order to disperse the large clumps of DDAH resulting from the preliminary mixing in methods *C* and *D*. The Simplex head was then used to shear the dispersion for 10 min. at a full Powerstat setting 120 which was equivalent to approximately 13,000 r.p.m. The sample prepared by method *D* was sheared for an additional 10 min. with the

[3] Bronwill's Constant Temperature Circulator, Bronwill Scientific, Rochester, N. Y.

[4] Series 2000 Standard Premier Dispersator, 1/3 HP, single phase, universal type motor. Made by Premier Mill Corp., Temple, Pa.

TABLE II.—RHEOLOGICAL PARAMETERS OF DISPERSIONS PREPARED BY VARIOUS METHODS[a]

Method	Static Yield Value	Bingham Yield Value	Plastic Viscosity	K	$1/N$
A	None	0.77	0.36	0.57	0.89
B	0.3	3.1	0.39	1.5	0.67
C	2.	6.2	0.79	4.9	0.47
D	3.	12.	1.18	10.	0.39

[a] Spindle No. 2 used to collect data.

Simplex head at the full Powerstat setting. No change in the rheological properties of the sample was detected. No attempt was made to find the minimum time or shearing rate necessary to prepare an equivalent sample. Since method D produced the most viscous sample, this method was used throughout the rest of this investigation.

In order to remove the air entrapped by the mixing, the dispersions were subjected to reduced pressure for 1 hr. at approximately 35 mm. of mercury immediately after their preparation. It was reasoned that some ethanol would be removed in the process of removing the air and it was of interest to determine what effect this alcohol would have on the dispersion if allowed to remain for a longer period of time before evacuating. A 1% DDAH dispersion was prepared using 2% ethanol. Half of this dispersion was subjected to reduced pressure immediately after preparation and a rheological evaluation was made 2, 6, and 8 days after preparation. The other half was subjected to reduced pressure 4 days after preparation and a rheological evaluation made 6 and 8 days after preparation.

Various concentrations of ethanol were used to prepare 1% dispersions of DDAH. The concentrations of ethanol used were 0.25, 0.5, 0.75, 1, 2, and 3%. A sample was also prepared without ethanol.

Dispersions of various concentrations of DDAH were then prepared with ethanol concentrations equal to that of the DDAH and with ethanol concentrations twice that of the DDAH.

RESULTS

Methods of Preparation.—The results of the rheological evaluation of the dispersions which were prepared by the various methods and which contained 1% DDAH and 0.5% ethanol are recorded in Table II. All of the rheological parameters indicate that maximum gellation occurred in the sample prepared by method D.

Although the various methods of preparing the dispersions appear to be similar, it should be emphasized that the principal difference is in the manner of incorporating the ethanol. In method D the ethanol was intimately mixed with the DDAH resulting in a mass that was visibly wet and of a pilular consistency while in methods B and C the mixture was a flowable powder.

In method B the DDAH–ethanol powder was added to all of the oil, while in method C the powder was initially mixed with only a portion of the oil. The greater concentration of ethanol in this preliminary mix of method C is probably the reason for the greater degree of gellation in the sample prepared by method C as compared to method B.

The least contact of DDAH with ethanol occurred in method A in which the ethanol was added to the mixture of DDAH and the oil. The sample prepared by this method also exhibited the least gellation.

These results seem to substantiate the mechanism of the gelling process proposed by Jordan and co-workers (10–12) in which the polar additive must first effect a separation of the primary particles in order for the oil to penetrate and solvate these particles. The preliminary investigation of these methods of preparing the dispersions indicated that reproducibility could be obtained within an acceptable range of ±10%.

There was no change in any of the five samples of the 1% DDAH dispersion prepared with 2% ethanol during 8 days of storage, regardless of whether the entrapped air was removed immediately after preparation or 4 days after preparation. It was thought that a longer storage period before the air was removed might affect the viscosity of the dispersion. It was possible that some ethanol would be removed in the process of removing the air. A longer storage period before evacuation would mean that the ethanol would be in contact with the DDAH for a longer period of time. Since there were no rheological differences between these samples, it would seem to indicate that the small amount of ethanol lost during the evacuation of the air was too minimal to affect their rheological properties. It is possible that the attraction of DDAH for the ethanol minimized or prevented its removal during the evacuation process.

Rheological Evaluations.—The results of the rheological evaluations of the 1% DDAH dispersions prepared with various concentrations of ethanol are recorded in Table III. All parameters indicate that the dispersion prepared with 1% ethanol was the most viscous. The sample prepared without ethanol does not appear in the table since it had a Newtonian viscosity of 27 cps. which was practically the same as the light mineral oil. Table IV lists the results of the rheological evaluations of the dispersions containing various concentrations of DDAH with ethanol concentrations equal to that of the DDAH and with ethanol concentrations twice that of the DDAH.

Figures 1 and 2 illustrate the flow curves of the dispersions containing various concentrations of DDAH with ethanol concentrations equal to that of the DDAH and with ethanol concentrations

Table III.—RHEOLOGICAL PARAMETERS OF DISPERSIONS[a] PREPARED WITH VARIOUS CONCENTRATIONS OF ETHANOL[b]

% Ethanol	Static Yield Value	Bingham Yield Value	Plastic Viscosity	K	$1/N$
0.25	2.	7.2	1.05	6.8	0.45
0.50	3.	12.	1.18	10.	0.39
0.75	10.	29.	1.84	21.	0.37
1.00	21.	38.	2.10	29.	0.31
2.00	15.	32.	1.84	26	0.30
3.00	9.	23.	1.71	21.	0.28

[a] One per cent DDAH. [b] Spindle No. 2 used to collect data.

TABLE IV.—RHEOLOGICAL PARAMETERS OF DISPERSIONS PREPARED WITH VARIOUS CONCENTRATIONS OF DIMETHYL DIALKYL AMMONIUM HECTORITE AND ETHANOL[a]

% DDAH	% Ethanol	Static Yield Value	Bingham Yield Value	Plastic Viscosity	K	$1/N$
1.00	1.00	21.	31.	3.77	25.	0.43
1.00	2.00	15.	30.	2.87	22.	0.42
1.25	1.25	36.	66.	4.83	46.	0.42
1.25	2.50	21.	52.	3.77	41.	0.33
1.50	1.50	61.	84.	10.4	69.	0.42
1.50	3.00	27.	58.	4.83	49.	0.32
1.75	1.75	120.	134.[b]	18.6[b]	102.	0.49
1.75	3.50	38.	60.	6.04	59.	0.28
2.00	4.00	64.	116.	8.45	101.	0.28

[a] Spindle No. 3 used to collect data. [b] Projected value.

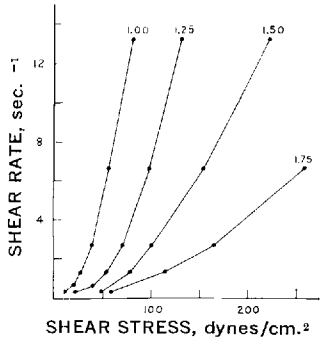

Fig. 1.—Rheograms of dispersions prepared with various concentrations of DDAH using an ethanol concentration equal to that of the DDAH.

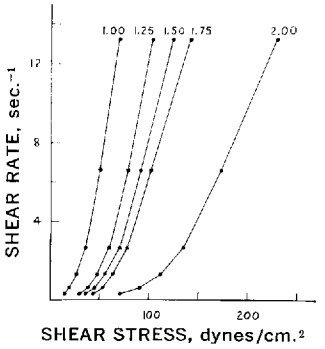

Fig. 2.—Rheograms of dispersions prepared with various concentrations of DDAH using an ethanol concentration twice that of the DDAH.

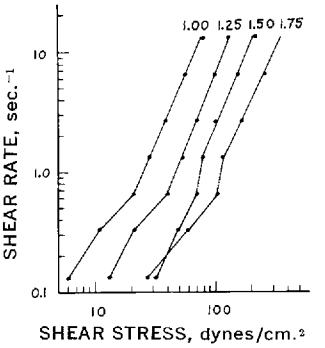

Fig. 3.—Log-log rheograms of dispersions prepared with various concentrations of DDAH using an ethanol concentration equal to that of the DDAH.

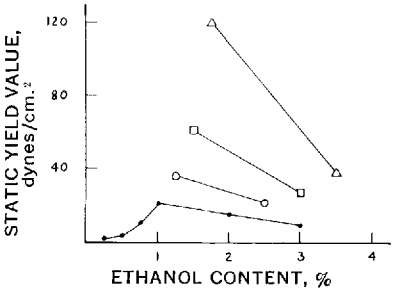

Fig. 4.—Plot of static yield value *vs.* the ethanol content present in dispersions containing various concentrations of DDAH. Key: ●, 1%; ○, 1.25%; □, 1.5%; △, 1.75%.

twice that of the DDAH, respectively. The upper portion of the flow curve for the 1.75% DDAH dispersion in Fig. 1 could not be determined because the stress exceeded the upper limits of the instrument for the No. 3 spindle at this high r.p.m. Even though a reading could not be made, the spindle was allowed to revolve at the designated velocity (60 r.p.m.) for the specified time (10 min.) so that the shear history would be the same as the other samples. The upper portion of this curve could be projected on a log-log plot of shear rate *versus* stress (see Fig. 3) to obtain a projected stress at the rate of shear equivalent to 60 r.p.m. For this reason the plastic viscosity and Bingham yield value given in Table IV for this sample are recorded as projected values.

A 2% DDAH dispersion using 2% ethanol was not prepared since it would have been too viscous to evaluate with the No. 3 spindle. Because of the time-dependency of these systems a comparison could not be made between data collected from two different spindles.

A log-log plot of shear rate *versus* stress for the DDAH dispersions prepared with ethanol concen-

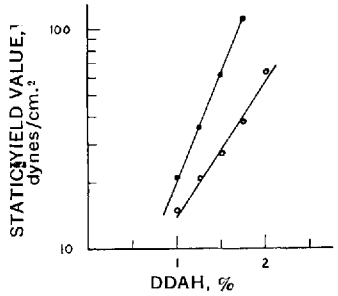

Fig. 5.—Plot of the log static yields value *vs.* the DDAH concentration. Key: ●, dispersions containing an ethanol concentration equal to that of the DDAH; ○, dispersions containing an ethanol concentration twice that of the DDAH.

trations equal to the DDAH is presented in Fig. 3. These log-log rheograms are representative of the others encountered in this study and are presented here for the purpose of showing the apparent effect of the static yield value upon them. A straight line relationship was found to exist between log rate of shear and log stress at the higher shear rates but not for the very lowest ones. It is interesting to note that the break in the straight line always occurred near a stress corresponding to the static yield for that particular dispersion. From this observation it is assumed that the straight line relationship between log shear rate and log stress does not exist throughout the whole shear rate range due to interference of the yield value of the dispersion at the low shear rates.

Effect of DDAH and Ethanol Concentrations on the Static Yield Value.—The static yield value of the dispersions containing 1% DDAH and various concentrations of ethanol were plotted against the ethanol concentrations. The results of this plot are presented in the lower portion of Fig. 4. The static yield value increases with an increase in ethanol concentration until a concentration of 1% ethanol is reached. As the concentration of ethanol is further increased a decrease in the static yield value occurs. From these results it is evident that the optimum ethanol concentration for a 1% DDAH dispersion is 1% ethanol which is equal to the concentration of the DDAH.

The static yield value of the dispersions containing various concentrations of DDAH with ethanol concentrations equal to that of the DDAH and with ethanol concentrations twice that of the DDAH are also illustrated in Fig. 4. As can be seen in this figure, the slopes of the lines become greater as the DDAH concentration increases. These slopes represent a decrease in static yield value as the ethanol concentration increases. The slope of each line was determined and the log of the slopes plotted against the DDAH concentrations. This semilog plot produced a straight line represented by the following equation:

$$\log \frac{\Delta Y_s}{E_2 - E_1} = -1.204\ C + \log 0.375 \qquad \text{(Eq. 9)}$$

The $\Delta Y_s/E_2 - E_1$ in Eq. 9 is the slope of the lines in Fig. 4 having a negative value; ΔY_s is the decrease in static yield value due to an increase in the percentage concentration of ethanol from E_2 to E_1; C is the percentage concentration of DDAH.

The log of the static yield value of the dispersions containing various concentrations of DDAH with ethanol concentrations equal to that of the DDAH and with ethanol concentrations twice that of the DDAH were plotted against the percentage concentrations of DDAH. These results, as illustrated in Fig. 5, are represented by the following equation

$$\log Y_s = mC + \log b \qquad \text{(Eq. 10)}$$

in which Y_s is the static yield value, m is the slope, b is the intercept on the y axis, and C is the concentration of DDAH. For the dispersions containing an ethanol concentration equal to the DDAH concentration, $m = 1$ and $b = 2$. For the dispersions containing an ethanol concentration twice that of the DDAH concentration, $m = 0.6$ and $b = 3.5$.

The results obtained from the plots in Figs. 4 and 5 indicate the validity of the static yield value as a rheological parameter. Although plots of the other rheological parameters were similar to those in Figs. 4 and 5, they were not so symmetrical and equations were not derived from them. These results also indicate that the ethanol concentration is an important factor in the formulation of a DDAH dispersion and that the optimum amount of ethanol needed to disperse DDAH in light mineral oil is an amount equivalent to the weight of the DDAH.

The dispersion having the highest static yield value would likely be the most effective in suspending insoluble material. However, it would also be impractical as a suspending medium because it would be too viscous to be pourable. Of the dispersions containing an ethanol concentration equivalent to the DDAH concentration, the 1% and 1.25% dispersions could be easily poured from a 2-oz. narrow-mouth bottle having an orifice of 14 mm.

Sedimentation Study.—The effectiveness of the

TABLE V.—SEDIMENTATION RATIO WITH TIME OF VARIOUS DRUGS SUSPENDED IN LIGHT MINERAL OIL

min.	Calcium Carbonate	Zinc Oxide	Ppt. Sulfur	Boric Acid
0	1.00	1.00	1.00	1.00
5	0.96	0.95	0.98	0.35
10	0.94	0.93	0.98	0.27
15	0.92	0.86	0.90	0.23
20	0.90	0.80	0.87	0.23
25	0.88	0.76	0.80	0.22
30	0.86	0.71	0.74	0.22
35	0.86	0.70	0.60	0.22
40	0.84	0.67	0.50	0.22
45	0.83	0.63	0.40	0.21
50	0.82	0.61	0.25	0.21
55	0.81	0.59	0.24	0.21
60	0.80	0.57	0.24	0.21
75	0.78	0.55	0.23	0.21
105	0.76	0.50	0.23	0.21
135	0.74	0.47	0.23	0.21
180	0.73	0.45	0.23	0.21
240	0.71	0.42	0.23	0.21
300	0.71	0.41	0.23	0.21
360	0.71	0.41	0.23	0.21
420	0.71	0.41	0.23	0.21

TABLE VI.—SEDIMENTATION RATIO WITH TIME OF VARIOUS DRUGS SUSPENDED IN LIGHT MINERAL OIL CONTAINING 1.25% DDAH AND 1.25% ETHANOL

Days	Control	Calcium Carbonate	Boric Acid	Pptd. Sulfur	Zinc Oxide
0	1.00	1.00	1.00	1.00	1.00
1	1.00	1.00	1.00	1.00	1.00
2	1.00	1.00	1.00	1.00	1.00
3	1.00	1.00	1.00	1.00	1.00[a]
6	1.00	1.00	1.00	1.00	0.992
13	1.00[a]	1.00[a]	1.00[a]	1.00[a]	0.984
21	1.00[a]	1.00[a]	1.00[a]	0.992	0.969
24	1.00[a]	1.00[a]	1.00[a]	0.992	0.962
30	1.00[a]	1.00[a]	1.00[a]	0.984	0.954
84	1.00[a]	1.00[a]	1.00[a]	0.977	0.931

[a] A film of light mineral oil, too small to measure, was observed on the surface.

1.25% DDAH–1.25% ethanol dispersion as a suspending medium was determined by comparing the sedimentation rate of various drugs suspended in this medium with the sedimentation rate of drugs suspended in light mineral oil. The drugs used were boric acid, precipitated calcium carbonate, precipitated sulfur, and zinc oxide in 5% w/v concentrations. All were of U.S.P. quality. The suspensions were prepared in a mortar and transferred to 50-ml. glass-stoppered cylinders having an internal diameter of 22 mm. A sample of the DDAH dispersion was also transferred to a cylinder and observed along with the suspensions. The height of the settled phase (H_s) was determined at various intervals of time and compared with the height of the original suspension (H_0). The sedimentation ratio (H_s/H_0) at the various time intervals are given in Table V for the light mineral oil suspensions and Table VI for the suspensions prepared with the DDAH dispersion.

A film of light mineral oil, too small to measure, was observed on the surface of the DDAH suspensions of calcium carbonate, boric acid, and sulfur on the 13th day of aging. At the same time a similar film was noticed on the surface of the DDAH dispersion which served as the control. It is likely that this separation of oil in the DDAH dispersion was due to syneresis. This phenomenon can also be observed in bentonite magma on standing. The fact that this separation occurred in the suspensions of calcium carbonate, boric acid, and sulfur at the same time as the control seems to indicate that syneresis also occurred in these suspensions.

The supernatant liquids of the DDAH suspensions of zinc oxide and sulfur could be measured after the 6th and 21st day, respectively. The supernatant liquids were perfectly clear throughout the 12-week aging period and were obviously light mineral oil. For practical purposes these drugs were considered as settling. However, syneresis cannot be ignored in these suspensions for it is possible that interaction between the DDAH particles and the drugs could occur to increase this effect.

A comparison of the sedimentation rates of boric acid and zinc oxide lends support to the theory that zinc oxide increased the syneresis of the DDAH dispersion. Boric acid settled at a much greater rate than zinc oxide in light mineral oil. Yet when suspended in the DDAH dispersion zinc oxide had a greater apparent rate of sedimentation than boric acid. A similar comparison can be made with boric acid and sulfur. Boric acid settled faster than sulfur in light mineral oil. However, the apparent sedimentation rate of sulfur was greater than boric acid in the DDAH suspending medium. From these observations it appears that the development of the supernatant liquid in the DDAH suspensions was due, not to the settling of the drugs as normally envisioned, *i.e.*, the insoluble particle falling in the suspending medium due to gravity, but rather the squeezing-out of the light mineral oil as the DDAH gel shrinks.

SUMMARY

The ability of dimethyl dialkyl ammonium hectorite (DDAH) to alter the flow properties of light mineral oil was evaluated for the purpose of preparing an oily suspending medium.

Dispersions of various concentrations of DDAH and ethanol in light mineral oil were prepared and rheologically evaluated by the use of a modified Brookfield viscometer. The optimum amount of ethanol needed to disperse DDAH in the oil was found to be an amount of ethanol equal to the weight of DDAH.

A new method of determining static yield value was found to be reliable. The static yield value (Y_s) in dynes/cm.^{2} was found to be related to the concentration (C) of DDAH as follows:

$$\log Y_s = mC + \log b$$

in which the constants m and b equal 1 and 2, respectively, for dispersions containing an ethanol content equal to the DDAH; and equal to 0.6 and 3.5, respectively, for the dispersions containing an ethanol content twice that of the DDAH.

A dispersion containing 1.25% DDAH and 1.25% ethanol in light mineral oil was used to prepare suspensions with several drugs. It was postulated that the formation of the small amount of supernatant liquid which occurred in these suspensions was due to syneresis rather than sedimentation of the insoluble particles.

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Phytochemical Investigation of *Arctostaphylos columbiana* Piper and *Arctostaphylos patula* Greene (*Ericaceae*)

By GEORGE H. CONSTANTINE, JR., PHILIP CATALFOMO, KIRTI SHETH, and LEO A. SCIUCHETTI

Improved methods of extraction and new techniques of isolation and identification of 11 components from the title plants were conducted. A solvent mixture of diverse polarity extracted four polycyclic compounds, a hydrocarbon, two flavonoids, and four phenols. Another phenolic, assumed to be widespread in the family, could not be shown to be present. Antibacterial and antifungal activity was found in the extracts obtained from both plants.

ETHNOBOTANICAL and early medical literature have often cited the usefulness of various *Arctostaphylos* species for a variety of medicinal uses. Members of the genus have been used by the Pacific Northwest Indians. These include their uses as healing poultices and as a curative for severe colds (1), as an eyewash and styptic (2), as a food (3–5), and as smoking tobacco (6). In fact, Reagan (7) reported drunken and erratic behavior of Indians who smoked the leaves of *A. uva-ursi* and stated that it was smoked as a medicine and in religious ceremonies.

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Phytochemical Investigation of *Arctostaphylos columbiana* Piper and *Arctostaphylos patula* Greene (*Ericaceae*)

By GEORGE H. CONSTANTINE, JR., PHILIP CATALFOMO, KIRTI SHETH, and LEO A. SCIUCHETTI

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extracts were reported to inhibit cultures of *Mycobacterium tuberculosis* (19).

EXPERIMENTAL

Plant Collection and Treatment

A. columbiana and *A. patula* were gathered in Marion County, Oreg., during the fall of 1965.[1] The plants were allowed to air-dry on greenhouse benches for not less than 2 weeks. They were then divided into their morphological parts, *i.e.*, leaves, roots, and stems, and ground to a course powder in an Abbé mill. The powdered material was then stored in air-tight plastic bags in the dark until time for analysis.

Preliminary Investigation.—Five-gram samples of each plant part were subjected to selective solvent extraction using petroleum ether U.S.P., chloroform U.S.P., alcohol U.S.P., and distilled water, successively. The extracts were then chromatographed (ascending technique) on Whatman No. 1 paper with the solvent systems *n*-butanol–acetic acid–water (4:1:5, organic phase) and *n*-butanol–pyridine–water (2:1:1). Antimony trichloride (10% w/w in chloroform) detected positive triterpene/sterol compounds (pink to red) in all except the aqueous fractions of both plants. Dragendorff's spray reagent demonstrated negative results in all of the extracts. Ferric chloride (1% in ethanol) and phosphomolybdic acid (5% in methanol) detected the presence of phenolic compounds. The alcoholic and aqueous extracts of *A. patula* contained 11 phenolic compounds, whereas, seven phenolic compounds were found in *A. columbiana*.

Additional testing was conducted on all of the selective solvent fractions from above. Anisaldehyde and Liebermann-Burchard spot tests also indicated the presence of triterpene/sterols in all but the aqueous fractions. Dragendorff's, Mayer's, and Wagner's reagents indicated the absence of alkaloid substances. Ferric ammonium sulfate (3% in water) and vanillin (1% in HCl) reagents also indicated the presence of phenolic compounds in the alcoholic and aqueous fractions.

Extraction and Fractionation.—One kilogram of the leaves from each plant was extracted individually for 72 hr. in a Soxhlet apparatus with 6 L. of methanol–skelly B (1:1). The dark green residue obtained after concentration in a flash evaporator was dissolved in skelly B–water (1:1) and placed in a large separator. On standing, a copious green interphase developed which was removed along with the organic phase and washed with water (3 × 125 ml.). The aqueous phase and washings (fraction II) were set aside for later examination of phenolic constituents. The organic phase (fraction III) was examined for triterpenes and sterols.

Triterpenes and Sterols.—Samples of fraction III were saponified according to the method of Huneck and Snatzke (20). Refrigeration of the acidified mixture yielded a copious greenish-yellow precipitate (fraction IV). The mother liquor was subjected to liquid–liquid extraction for 48 hr. using hexane (analytical reagent) as the lighter solvent. The soluble components were designated fraction V. Fractionation steps are summarized in Scheme I.

[1] Identified by Dr. K. L. Chambers, Professor and Curator of the Oregon State University Herbarium. Voucher specimens of both plants are located at same.

Isolation of Ursolic Acid.—Fraction IV gave a positive Liebermann–Burchard test (red to immediate purple) and anisaldehyde test (red). Thin-layer chromatography (TLC)[2] of fraction IV employing chloroform–acetone (9:1) as a solvent system and antimony trichloride as a spray reagent indicated only one spot (R_f 0.14) identical to ursolic acid which was also chromatographed on the same plate. The crude material (fraction IV) after repeated washings with cold chloroform was crystallized from ethanol. Fine needles of ursolic acid were obtained, m.p. 277–280°.[3] Literature values for ursolic acid range from 224° (21) to 291° (22). Superimposable infrared spectra were obtained in comparing the isolate with ursolic acid.

Column Chromatography of Fraction V.—Fraction V, when chromatographically screened on a thin-layer plate (chloroform–acetone, 9:1), revealed the presence of three compounds when sprayed with antimony trichloride spray reagent (R_f 0.39, 0.49, and 0.58). An 8-Gm. sample of fraction V was mixed with 10 Gm. of activated alumina[4] and placed atop a 300-Gm. alumina column (50 × 240 mm.). The column was eluted with successive solvents of increasing polarity. The results are summarized in Table I.

Fraction A upon drying gave a white lustrous product (1.1 Gm.) which was recrystallized from acetone, melted sharply at 64°, and was tentatively identified as nonacosane, m.p. 64–65°. Fractions B and C were combined, treated as above, and yielded additional nonacosane (0.7 Gm.).

Thin-layer chromatography of fraction D revealed the presence of only one compound which had an R_f value comparable to that of β-amyrin. Evaporation *in vacuo* yielded a semisolid yellow residue (0.8 Gm.) which was recrystallized from petroleum ether and yielded β-amyrin, m.p. 194–196°. A mixed melting point with an authentic sample of β-amyrin,[5] m.p. 192–196°, showed no depression. The infrared spectra of the compound and authentic β-amyrin were identical.

Fraction E (0.7 Gm.) contained a mixture of three compounds which had R_f values comparable to those of β-amyrin, β-sitosterol, and uvaol. The dried fraction was deposited on top of a small column of alumina (100 Gm., 30 × 240 mm.) and carefully eluted. Fraction E′ [chloroform–ether (3:1)] eluted only β-sitosterol (as revealed by TLC) which was crystallized from chloroform–methanol and gave a m.p. of 135°. Mixed melting points with an authentic sample of β-sitosterol,[5] m.p. 133–135°, did not cause any depression. Superimposable infrared spectra were obtained in comparing the isolate with the authentic sample.

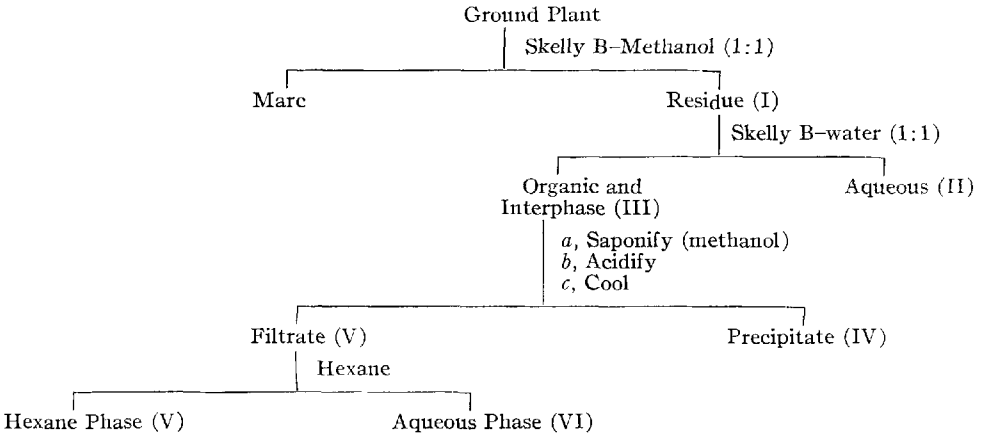
Fractions F and G contained only one component which had an R_f value comparable to that of uvaol. The dried fraction (0.8 Gm.) was recrystallized from ethanol and yielded fine white needles, m.p. 222–224°. Reference uvaol, m.p. 223–224°, was prepared by lithium aluminum hydride reduction of ursolic acid utilizing a modified procedure of Nystrom and Brown (23). Mixed melting points of

[2] TLC plates (20 × 20 cm.) prepared using Silica Gel G, according to Stahl. Apparatus: C. DeSaga, Heidelberg, Germany. U.S. Representative: Brinkman Instruments Co., Westbury, N. Y.

[3] Thomas-Hoover melting point apparatus, uncorrected.

[4] Alumina activated; Matheson, Coleman and Bell, East Rutherford, N. J.

[5] Nutritional Biochemicals, Cleveland, Ohio.



Extraction Scheme for Sterols and/or Triterpenes
Scheme I

TABLE I.—COLUMN CHROMATOGRAPHY OF FRACTION V FROM *A. patula*

Fraction	Eluant	Vol., ml.	*Rf* of Steroidal Component[a]
A	Hexane	1000	...
B	Hexane–benzene (1:1)	1000	...
C	Benzene	1000	...
D	Benzene–chloroform (7:3)	3000	0.58
E	Benzene–chloroform (1:1)	4000	0.58 0.49 0.39
F	Chloroform	2500	0.39
G	Chloroform–methanol (1:1)	2000	0.39

[a] Detected by TLC in chloroform–acetone (9:1); 10% w/w $SbCl_3$ in $CHCl_3$ as spray reagent and heating in an oven at 110° for 5 min.

isolated and reference uvaol showed no depression and the infrared spectra of the two were identical.

Co-Chromatography.—In these experiments the identity of β-amyrin, β-sitosterol, uvaol, and ursolic acid isolated from the extract was also verified by means of TLC. The isolated compound and the corresponding authentic sample were co-spotted. Controls of the isolated compound and the reference compound were spotted singly. These plates were then run in three different solvent systems and when developed showed that the co-spot which did not separate had the same R_f value as that of the two control spots. The results of co-spotting experiments are summarized in Table II.

Rather than repeating the entire procedure of isolation of compounds from *A. columbiana* a sample of fraction III of this plant was co-spotted along with standard β-amyrin, β-sitosterol, ursolic acid, and uvaol in three different solvent systems. The results are shown in Table III and indicate the presence of the same compounds in *A. columbiana*.

Phenolic and Related Compounds.—Portions of fraction II (Scheme I) of both plants were subjected to liquid–liquid extraction using ethyl acetate and ether, successively. Portions of fraction II were also subjected to acidic or alkaline hydrolysis and the hydrolysates were then extracted with ether. The extraction scheme is summarized in Scheme II. Samples were spotted in several channels on chromatoplates. Spots corresponding to reference compounds were removed from the plates with a vacuum-zone extractor (24) and the compounds were eluted from the extractor with hot methanol. Ultraviolet spectra of the methanolic solutions were run and the solutions were then co-spotted in three different solvent systems. Arbutin (hydroquinone-β-D-glucoside) was identified in fraction A by co-spotting and absorption maximum of 283 mμ. Ellagic acid was identified by co-spotting and absorption maximum of 253 mμ. Gallic acid was obtained from fraction D and identified by co-spotting and absorption maximum of 273 mμ. Hydroquinone was found both in the free state (fraction A) and in the acid hydrolyzed fraction (fraction D). Co-spotting identified its presence as did the absorption maxima, 289–290 mμ. Hyperin (quercetin-3-galac-

TABLE II.—CO-SPOTTING OF REFERENCE AND ISOLATED COMPOUNDS FROM *A. patula*[a]

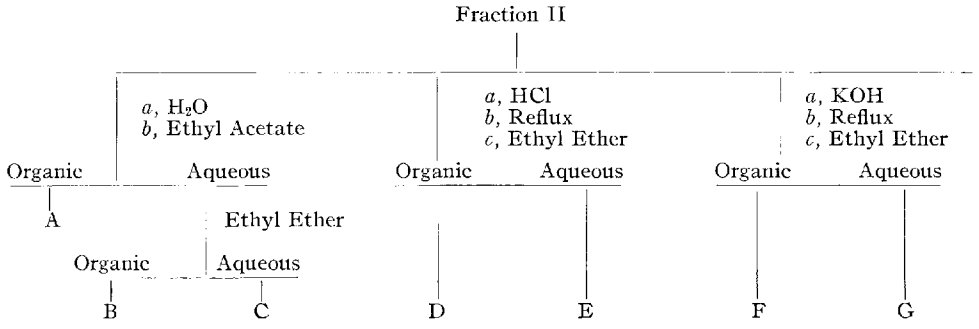
Compd.	*Rf* Values BE[b]	CA[b]	HBE[b]
1, Ursolic acid	0.14	0.13	0.04
2, Fraction IV	0.13	0.12	0.04
3, Co-spot (1 and 2)	0.13	0.12	0.04
4, β-Amyrin	0.50	0.58	0.30
5, Fraction D	0.51	0.58	0.30
6, Co-spot (4 and 5)	0.51	0.58	0.30
7, β-Sitosterol	0.41	0.49	0.22
8, Fraction E′	0.42	0.49	0.23
9, Co-spot (7 and 8)	0.42	0.49	0.23
10, Uvaol	0.32	0.38	0.07
11, Fractions F and G	0.33	0.39	0.08
12, Co-spot (10 and 11)	0.33	0.39	0.08

[a] Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110° for 5 min. [b] BE, benzene–ethyl acetate (4:1); CA, chloroform–acetone (9:1); HBE, heptane–benzene–ethanol (50:50:5).

TABLE III.—CO-SPOTTING OF REFERENCE COMPOUNDS AND *A. columbiana* FRACTION III

Solvent Systems	R_f Values[a] Fraction III[a]	β-Amyrin	Co-Spot, β-Amyrin and III	β-Sitosterol	Co-Spot, β-Sitosterol and III	Ursolic Acid	Co-Spot, Ursolic Acid and III	Uvaol	Co-Spot, Uvaol and III
BE[b]	0.14		0.14		0.14	0.14	0.14		0.14
	0.32		0.32		0.32		0.32	0.32	0.32
	0.38		0.38	0.38	0.38		0.38		0.38
	0.48	0.48	0.48		0.48		0.48		0.48
	0.08		0.08		0.08	0.08	0.08		0.08
CA[b]	0.33		0.33		0.33		0.33	0.33	0.33
	0.42		0.42	0.42	0.42		0.42		0.42
	0.54	0.54	0.54		0.54		0.54		0.54
	0.10		0.10		0.10	0.10	0.10		0.10
HBE[b]	0.18		0.18		0.18		0.18	0.18	0.18
	0.29		0.29	0.29	0.29		0.29		0.29
	0.43	0.43	0.43		0.43		0.43		0.43

[a] Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110° for 5 min. [b] BE, benzene–ethyl acetate (4:1); CA, chloroform–acetone (9:1); HBE, heptane–benzene–ethanol (50:50:5).



Fractionation Scheme of Phenolic Compounds
Scheme II

toside) was also found in fraction A and identified by co-spotting and absorption maxima of 360 and 255 mμ. Free quercetin was also identified in fraction A and by its absorption maxima of 370 and 252 mμ. The results of the co-spotting experiments are found in Table IV.

Ibrahim (15) recently isolated *o*-pyrocatechuic acid from various members of the *Ericaceae*, including *A. uva-ursi*. A standard sample[6] was chromatographed in six different solvent systems along with all of the plant phenolic fractions (A–G), and no evidence could be obtained for its presence in *A. columbiana* or *A. patula*. The six solvent systems used were BAW, BMA, CEF, TEF (*cf.* Table IV), BPA (benzene–pyridine–acetic acid, 36:9:5) and BDA (benzene–dioxane–acetic acid, 90:25:4).

Antibacterial–Antifungal Screen.—The small tube method of Catalfomo and Schultz (25) was used for screening the crude plant extracts and the compounds found to exist in the plants. Crude extracts consisted of skelly B–methanol (1:1) and 70% ethanol fractions (5 Gm./150 ml.) from each plant. These fractions were then dried *in vacuo* and redissolved in 70% ethanol shortly before use. The known compounds were also dispersed in 70% ethanol. Controls, tubes innoculated with the organism only, and blanks, tubes with the solvent and the organism, showed adequate growth. The results are shown in Table V.

[6] Obtained through the courtesy of Dr. R. V. Ibrahim, Alexandria, Egypt, U.A.R.

RESULTS AND DISCUSSION

A new procedure was devised in order to remove as many components of interest in one extraction procedure. Miscible solvents of diverse polarity had to be selected and experimentation demonstrated that a skelly B–methanol (1:1) mixture was best suited for this need.

Five compounds were isolated from the sterol/triterpene fraction of *A. patula*. Ursolic acid and uvaol had been previously identified in the genus but β-amyrin, β-sitosterol, and nonacosane had not. All of the compounds were identified in *A. columbiana* by means of co-chromatography except for nonacosane which was also isolated.

The new extraction solvent also removed phenolic components which were fractionated from other components by physical and chemical methods and were separated from one another by TLC. Ultraviolet spectra and co-spotting verified the presence of two flavonoids, quercetin and hyperin; two quinonoids, arbutin and hydroquinone; and two phenolic acids, gallic and ellagic acid. *o*-Pyrocatechuic acid, previously assumed to be widespread in the family, could not be shown to be present in the two species investigated.

Antibacterial–antifungal screening was conducted on the crude plant extracts and the compounds found to be present in the plants. All of the crude extracts demonstrated fungicidal activity against *Trichophyton mentagrophytes* which could not be attributed to the known compounds. Further frac-

TABLE IV.—CO-SPOTTING OF PHENOLIC COMPONENTS AND REFERENCE COMPOUNDS[a,b]

	BMA[c]	CAW[c]	CEF[c]	TEF[c]	BAW[c]	BAW'[c]
Arbutin	0.11	0.68	...	0.04	...	...
Ellagic acid	0.04	...	...	...	0.03	0.02
Gallic acid	0.19	...	0.29	0.28	...	...
Hydroquinone	0.38	...	0.56	0.44	...	...
Hyperin	0.10	0.64	...	0.03	...	...
Quercetin	0.36	0.74	...	0.31	...	...

[a] R_f values consist of standards and eluted components (co-spot) from both species. [b] Detection: arbutin, Millon's spray reagent; ellagic acid, 1% ethanolic ferric chloride; gallic acid, hydroquinone, hyperin, and quercetin, 5% methanolic phosphomolybdic acid (PMA). [c] BMA, benzene–methanol–acetic acid (10:2:1); CAW, chloroform–acetic acid–water (35:50 17.5); CEF, chloroform–ethyl acetate–formic acid (5:4:1); TEF, toluene–ethyl formate–formic acid (5:4:1); BAW, butanol–acetic acid–water (4:1:1); BAW', butanol–acetic acid–water (4:1:5).

tionation of these extracts is in progress to determine wherein this activity may reside. The skelly B–methanol extract from *A. columbiana* also demonstrated fungicidal activity against *Candida albicans*, whereas neither of the *A. patula* extracts did. In contrast, the skelly B–methanol extract of *A. patula* was antibacterial against *Escherichia coli* but neither *A. columbiana* extract was so effective.

Hydroquinone was effective against both bacteria, *E. coli* and *Staphylococcus aureus*, whereas the glycoside arbutin was effective only in higher concentrations against *S. aureus*.

Quercetin demonstrated equivocal results against all organisms except *S. aureus*, whereas no such activity was noted with the galactoside, hyperin.

The triterpene, β-amyrin, demonstrated the widest spectrum of activity among the triterpene/sterols and the triterpene alcohol, uvaol, contained no activity.

Newer applications of techniques for the extraction, fractionation, and identification of compounds from two diverse chemical classes have been demonstrated. Eleven compounds from two previously uninvestigated plants have been identified and the microbiological activity of the crude plant extracts and the compounds have been recorded.

TABLE V.—ANTIBACTERIAL–ANTIFUNGAL STUDIES

Compd. and Concn.	*E. coli*	*S. aureus*	*C. albicans*	*T. mentagrophytes*
A. columbiana[a]				
1:100	±[c]	±	±	−
1:500	±	+	±	−
A. columbiana[b]				
1:100	+	+	−	−
1:500	+	+	−	−
A. patula[a]				
1:100	+	+	±	−
1:500	+	+	±	−
A. patula[b]				
1:100	−	+	±	−
1:500	−	+	±	−
Arbutin				
1:100	+	−	+	+
1:500	+	+	+	+
Hydroquinone				
1:100	±	−	+	+
1:500	±	−	+	+
Quercetin				
1:100	±	+	±	±
1:500	±	+	±	±
Hyperin				
1:100	+	+	+	+
1:500	+	+	+	+
Gallic acid				
1:100	±	−	+	±
1:500	+	±	+	±
Ellagic acid				
1:100	±	+	+	+
1:500	±	+	+	+
β-Amyrin				
1:100	±	±	±	+
1:500	±	±	±	+
β-Sitosterol				
1:100	+	±	+	±
1:500	+	±	+	±
Ursolic acid				
1:100	+	±	+	+
1:500	+	±	+	+
Uvaol				
1:100	+	+	+	+
1:500	+	+	+	+

[a] Dried 70% ethanol extract. [b] Dried skelly B–MeOH (1:1) extract. [c] +, growth; −, no growth; ±, equivocal growth.

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Catecholamine-Induced Release of 5-Hydroxytryptamine (5-HT) from Perfused Vasculature of Isolated Dog Intestine

By T. F. BURKS* and J. P. LONG

The ability of exogenously administered epinephrine, norepinephrine, and tyramine and stimulation of sympathetic nerves to release 5-HT from the vasculature of isolated dog intestinal segments has been investigated. These stimuli were all found to produce significant release of 5-HT. Administration of an α-receptor blocking agent (tolazoline) significantly reduced the 5-HT release by norepinephrine but not by epinephrine.

IT HAS BEEN demonstrated recently (1) that isolated segments of dog small intestine release 5-hydroxytryptamine (5-HT) into physiological solution perfusing the vasculature. Stimuli which produced smooth muscle contraction or enhanced motility of the perfused segment increased its release of 5-HT. Such stimuli as acetylcholine, angiotensin, $BaCl_2$, increased intraluminal pressure, and scratching the serosal surface of the gut section all significantly increased 5-HT release. It was felt that such increased 5-HT release could simply be due to mechanical distortion of the tissues produced by the various stimuli.

More recent work indicates that the above explanation may be oversimplified since some stimuli that relax the intestinal musculature also release 5-HT.

METHODS

Adult mongrel dogs of either sex weighing 8 to 15 Kg. were anesthetized with 15 mg./Kg. of sodium thiopental and 250 mg./Kg. of sodium barbital administered intravenously. The small intestine was exposed and a small branch of the superior mesenteric artery with its juxtaintestinal arterial fan was cannulated with polyethylene tubing. The artery was perfused with warmed Krebs bicarbonate solution which was aerated by bubbling with a mixture of 95% oxygen and 5% carbon dioxide. Perfusion pressure, provided by use of a Sigmamotor model T-8 constant-flow peristaltic infusion pump, was maintained at 80–100 mm. Hg and was measured from a T-tube between the pump and the artery by a Statham pressure transducer and recorded on an Offner Dynograph (type RS). Since flow into the artery was held constant, changes of perfusion pressure were then proportional to changes in arterial resistance.

After flow was established through the artery, the associated vein was cannulated so that the venous effluent could be collected. Ligatures were tied around the intestinal segment supplied by the cannulated arterial fan and the segment surgically removed, placed on a cotton pad, and covered with a warm, saline-soaked gauze sponge. The section was then kept warm by use of an incandescent lamp.

In some of the experiments a balloon, attached to a Statham pressure transducer, was tied into the lumen of the intestinal section and intraluminal pressure recorded on either an Offner Dynograph or a Gilson (GME) polygraph.

In one preparation the venous effluent from the dog intestinal segment was superfused over an isolated uterus horn from an oestrus rat.

A constant recording of 5-HT concentration in the venous effluent was obtained by use of a flow-through cell in an Aminco-Bowman spectrophotofluorometer. The excitation monochromator of the spectrophotofluorometer was set at 295 mμ and the fluorescence monochromator was set at 330 mμ where the native fluorescence of 5-HT is maximal at neutral to slightly alkaline pH. Calibration of the instrument was performed with appropriate concentrations of 5-HT dissolved in Krebs solution placed in the cell. An illustration of the preparation employed is provided in Fig. 1.

The 5-HT concentration in the venous effluent was recorded in mcg./ml. of the effluent solution, then this figure was divided by the weight (to the nearest 0.1 Gm.) of the gut segment to give a 5-HT estimation of ng./ml./Gm. of wet tissue. The data are all reported in ng./ml./Gm.

In preparations from 15 dogs, the periarterial sympathetic nerves were isolated and fixed on a stimulating electrode. Nerve stimulation was performed with a Grass Instrument Co. model S4 stimulator. Parameters of stimulation were within the following ranges: frequency 20–30 c.p.s., duration 10–20 msec., and at 6–15 v. for 5 30 sec.

Test drugs were injected in volumes of 0.002–0.1 ml. intra-arterially *via* the arterial cannula. Agents employed were l-epinephrine hydrochloride, l-norepinephrine bitartrate (calculated as the base), tyramine hydrochloride, and tolazoline hydrochloride.

Experiments were so designed that each preparation served as its own control, and statistical comparisons were performed by the Student's t test (2). A P value equal to or less than 0.05 was considered significant.

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* U. S. Public Health Health Service Fellow.

RESULTS

The initial observation responsible for this series of experiments is illustrated in Fig. 2. It was noted that stimulation of the periarterial sympathetic nerve of the isolated intestinal section resulted in enhanced contraction of the rat uterus being superfused with the venous effluent from the gut segment.

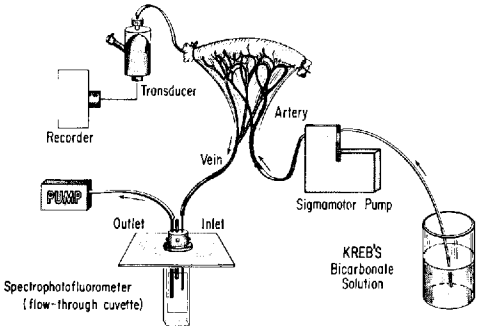

Fig. 1.—A drawing of the isolated intestinal preparation employed. A T-tube was inserted between the pump and the artery to monitor perfusion pressure. Intraluminal pressure was measured from a baloon in the lumen of the isolated intestinal segment. The effluent solution from the cannulated vein could be allowed to superfuse a rat uterus or to flow through the cell for analysis. A quartz baffle plate divides the quartz cell into two compartments, and the solution flows past and around this divider.

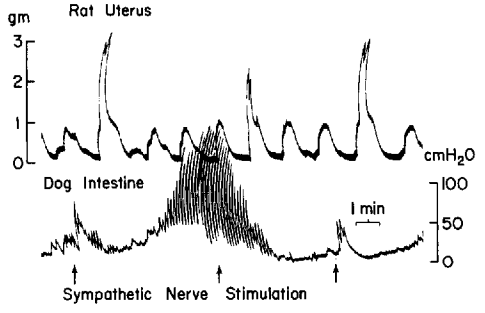

Fig. 2.—Key: top, tracing produced by an isolated rat uterus superfused with venous effluent from the dog intestinal segment; bottom, intraluminal pressure of intestinal segment. Stimulation of the periarterial sympathetic nerves of the intestinal segment produced contractions of the rat uterus which were not blocked by atropine.

Since norepinephrine usually produces only relaxation of the rat uterus, it was felt that the stimulation of the sympathetic nerve and catecholamine administration might release 5-HT.

In preparations from 16 dogs, epinephrine (4 mcg./dose) was found to significantly release 5-HT from the intestinal segment (see Table I). The musculature of the segment was relaxed by the epinephrine, but often enhanced activity of the section was observed after the period of relaxation. This increased activity occurred after the release of 5-HT and may have been produced by the released 5-HT (Figs. 3 and 4). It was noted that repeated injections of epinephrine into the vasculature of the section released progressively diminishing amounts of 5-HT. On some occasions when there was no longer any release of 5-HT by the epinephrine, 2-mcg. doses of acetylcholine were injected, and these treatments invariably released additional 5-HT.

Injected norepinephrine (4 mcg./dose) behaved qualitatively in the same manner as epinephrine (Figs. 3 and 4). In preparations from 10 dogs, the norepinephrine significantly ($p < 0.01$) increased 5-HT release (see Table I).

Since the above catecholamines were found to be active as releasers of 5-HT from the perfused intestinal vasculature, tyramine (200 mcg./dose) was injected to determine if endogenous catecholamines would similarly produce 5-HT release. In preparations from 10 dogs, the administered tyramine resulted in 5-HT release ($p < 0.01$) which is illustrated in Fig. 3 and Table I.

In perfused intestinal segments from 15 dogs, stimulation of the periarterial sympathetic nerves resulted in significant ($p < 0.01$) release of 5-HT (see Table I). The effects of nerve stimulation on intraluminal pressure were no different from that observed following the above agents (Fig. 4). Similarly, after repeated nerve stimulation there was loss of ability to release additional 5-HT, in which case administration of epinephrine, norepinephrine, or tyramine would not usually produce any 5-HT release. On some occasions administration of 2 mcg. of acetylcholine under these conditions would produce some 5-HT release.

A separate study was undertaken to determine if 5-HT release by the catecholamines was dose related. Five isolated intestinal segments were prepared from each of 13 dogs. One segment received first a low (0.2 mcg.) dose of epinephrine followed by a high (0.4 mcg.) dose of epinephrine; a second preparation received the same treatments but in reverse order; a third section received a low (0.3 mcg.) dose of norepinephrine followed by a high (0.6 mcg.) dose of norepinephrine; a fourth seg-

Table I.—Release of 5-HT by Catecholamines and Sympathetic Nerve Stimulation

Stimulus	ng. 5-HT Base/ml./Gm. Before[a]	ng. 5-HT Base/ml./Gm. After[a]	P^b	N^c
Epinephrine, 4 mcg.	5.7 ± 0.8	13.5 ± 1.9	<.01	16
Norepinephrine, 4 mcg.	6.7 ± 0.8	16.7 ± 3.4	<.01	10
Tyramine, 200 mcg.	6.0 ± 1.5	8.9 ± 1.8	<.01	10
Sympathetic nerve stimulation	12.7 ± 1.8	20.8 ± 2.7	<.01	15

[a] Mean ± standard error. [b] Level of significance, Student t test, paired comparison. [c] Number of animals employed in the experiment.

ment received the norepinephrine doses in the reverse order; a fifth segment first received tolazoline hydrochloride (250 mcg./dose) then 0.6 mcg. norepinephrine and 0.4 mcg. epinephrine, the order of the latter two agents being alternated from dog to dog. The treatments were randomly applied to each of the five loops from each dog. The results of these experiments may be seen in Tables II and III.

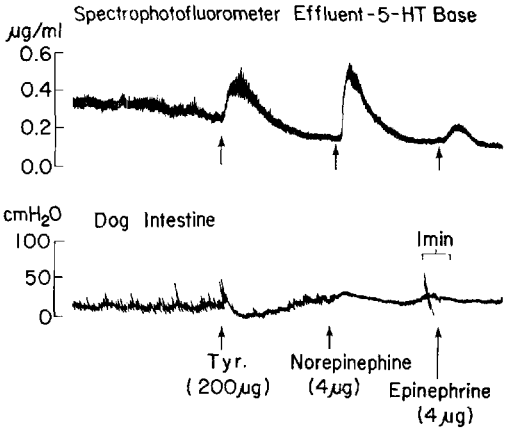

Fig. 3.—Demonstration of release of 5-HT from dog intestinal segment by tyramine (Tyr.), norepinephrine, and epinephrine. Key: top, continuous recording of 5-HT concentration in venous effluent; bottom, intraluminal pressure in dog intestinal section. The relatively small response to epinephrine was due to its being third in this particular series.

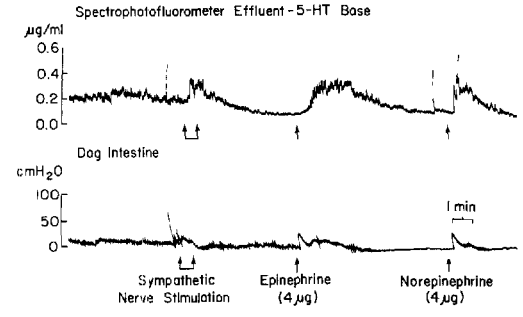

Fig. 4.—Release of 5-HT by 30-sec. stimulation of periarterial sympathetic nerves, epinephrine, and norepinephrine. Key: top, 5-HT content of venous effluent; bottom, intraluminal pressure of intestinal segment.

A dose-response curve was obtained for 5-HT release by the catecholamines and this release appeared to be related to pressor responses observed. It was noted that tolazoline alone was capable of producing pressor responses and releasing 5-HT and is therefore included in Table II.

Comparison of the responses to epinephrine and norepinephrine after tolazoline to the mean responses produced by the same doses of these agents in the absence of tolazoline revealed that while the pressor response to 0.4 mcg. epinephrine was significantly antagonized ($p < 0.05$) there was no statistically significant difference in 5-HT release by this agent whether or not tolazoline was present. On the other hand, the release of 5-HT by 0.6 mcg. norepinephrine was significantly antagonized by tolazoline ($p < 0.05$) but there was no significant antagonism of the norepinephrine pressor response. These data are summarized in Table III.

DISCUSSION

The ability of catecholamines, whether endogenous or added exogenously to release 5-HT from perfused dog intestinal segments does not constitute evidence for the hypothesis that intestinal 5-HT is released only by mechanical distortion of that organ. Since the catecholamines relax the musculature of the dog intestine and also release 5-HT, the above hypothesis must be considerably modified. It is possible that there is more than one method (perhaps there are many) by which the intestine can be stimulated to release 5-HT. Some agents, such as acetylcholine, $BaCl_2$, angiotensin (1), increased intraluminal pressure (1, 3), hydrochloric acid (4), and intraluminal hypertonic sucrose solutions (5) could produce intestinal contractions with incidental, secondary release of 5-HT. Catecholamines, however, may release 5-HT in some other manner, perhaps by a more direct mechanism. The 5-HT released by these latter agents may represent a second source or "pool" of 5-HT, perhaps another binding site in the intestinal mucosa, which is separate or distinct from that released by mechanical deformation. Evidence for this may be represented by the fact that acetylcholine can still release 5-HT after catecholamines have lost the ability to do so; the converse situation has also been observed.

The means by which the catecholamines release 5-HT in the dog intestine is open to speculation.

TABLE II.—EFFECTS OF CATECHOLAMINES AND TOLAZOLINE ON RELEASE AND INCREASE IN PERFUSION PRESSURE, $N = 13$[a]

Stimulus, mcg.	ng. 5-HT Base/ml./Gm. Before[b]	ng. 5-HT Base/ml./Gm. After[c]	P[d]	Rise in Perfusion Pressure,[e] mm. Hg
Epinephrine, 0.2	5.0 ± 0.5	7.2 ± 0.9	<.01	8 ± 4.8
Epinephrine, 0.4	5.1 ± 0.5	8.4 ± 1.3	<.01	14 ± 5.3
Norepinephrine, 0.3	5.1 ± 0.5	7.9 ± 1.1	<.01	17 ± 4.7
Norepinephrine, 0.6	5.2 ± 0.6	8.9 ± 1.2	<.01	23 ± 5.6
Tolazoline, 250[f]	6.7 ± 0.6	9.4 ± 1.0	<.01	12 ± 2.2

[a] Number of animals employed in each experiment. [b] Mean ± standard error before stimulus. [c] Mean ± standard error after stimulus. [d] Level of significance, Student t test, paired comparisons. [e] Mean response ± standard error. [f] Tolazoline included in this table only to show that it is capable of producing effects alone.

TABLE III.—EFFECTS OF 250 mcg. OF TOLAZOLINE ON 5-HT RELEASE AND INCREASE IN PERFUSION PRESSURE, $N^a = 13$

Stimulus, mcg.	Before Tolazoline: Increase 5-HT Base,[b] ng./ml./Gm.	Before Tolazoline: Increase in Perfusion Pressure,[b] mm. Hg	After Tolazoline: Increase 5-HT Base,[b] ng./ml./Gm.	After Tolazoline: Increase in Perfusion Pressure,[b] mm. Hg
Epinephrine, 0.4	3.3 ± 0.9	14 ± 4.6	2.1 ± 2.4	3 ± 1.1[c]
Norepinephrine, 0.6	3.7 ± 1.1	23 ± 5.8	1.7 ± 0.8[c]	13 ± 7.4

[a] Number of animals employed in each experiment. [b] Mean ± standard error. [c] Significantly ($p < 0.05$) decreased after tolazoline.

Perhaps the most plausible explanation would be that of displacement by the catecholamine of 5-HT at its mucosal binding sites. Stacey has stated (6) that tyramine, for example, can inhibit the uptake of 5-HT by blood platelets. Bertler *et al.* (7) have found that injected norepinephrine can replace 5-HT in its pineal gland sympathetic nerve storage sites. Similarly, administered metaraminol causes a pronounced depletion of pineal 5-HT. Born *et al.* (8) have suggested as an intracellular storage site for 5-HT, a 5-HT–ATP complex similar to that proposed for catecholamines in the adrenal medulla. In the fraction of the dog small intestine rich in 5-HT (mucous membrane) there is a molar ratio of approximately 3 to 1 between 5-HT and ATP (9). So it is quite possible that either exogenously administered catecholamines or those released by sympathetic nerve stimulation or tyramine could simply displace 5-HT from its storage granules in the intestinal mucosa.

More evidence will be required to demonstrate a positive role for 5-HT as a neurotransmitter in autonomic innervation of the small intestine. There have been some data presented, however, that may help define any such function. Van Harn found that, in the cat, stimulation of the thoracic sympathetic chain is inhibitory when the jejunum is spontaneously active but may cause an increase in activity in a previously inactive gut (10). It seems possible from the data reported in this communication, that the intestinal stimulation reported by Van Harn could have been due to 5-HT release. Klingman (11) has reported that immunosympathectomized rats have decreased levels of norepinephrine in their intestines, but that there is no alteration in that tissue in DOPA-decarboxylase activity. Since DOPA-decarboxylase and 5-hydroxytryptophan decarboxylase may be the same enzyme (12), it would be interesting to know whether there is a concomitant alteration of 5-HT levels in immunosympathectomized animals. A decrease in gastrointestinal 5-HT following immunosympathectomy could suggest that an intact sympathetic system is required for 5-HT elaboration, storage, or participation in transmission. An increase in 5-HT levels might indicate that there is competition between 5-HT and catecholamines for common storage sites in the intestine.

Ahlquist (13) states that the canine intestine has both α and β adrenergic receptors which produce intestinal inhibition. There may be "receptor sites" in the dog intestine with which catecholamines interact to produce 5-HT release which are different from the usual α and β receptors. The present data indicate that by use of an α-receptor blocking agent, tolazoline, some separation of pressor activity in the intestinal vasculature and 5-HT-releasing activity can be achieved with epinephrine and norepinephrine.

There may be some evidence for a homeostatic role of 5-HT in the intestine, as has been proposed for this substance in the cardiovascular system (14). It has previously been reported (1) that 5-HT release from the dog intestine can occur after either an increase or a decrease in intraluminal pressure. Similarly, agents that produce intestinal constriction (acetylcholine) or relaxation (catecholamines) similarly produce 5-HT release. Van Harn's experiments (10) contain related suggestions. It could be proposed that 5-HT somehow serves a buffer capacity, or as one component of a buffer system for intestinal motility. Any such function, of course, as yet eludes elucidation.

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Effect of pH on the *In Vitro* Absorption of Flufenamic Acid

By ARMANDO J. AGUIAR and RICHARD J. FIFELSKI

The effect of pH on the *in vitro* absorption of flufenamic acid is studied using the "everted sac" technique. The study reveals that the passage of this drug through the gut membrane is by passive diffusion. The amount diffusing through is dependent on the pH. Using Fick's diffusion law, a method is presented to calculate the apparent permeability constant at each pH value, and to determine the permeability constant. The relative surface area of the membrane through which diffusion of the drug takes place is estimated from the amount of "bound" drug.

AFTER ORAL administration, a drug to act systemically must be absorbed from the gastrointestinal tract. The rate of absorption is dependent on two independent processes: the rate of solution of the drug in the media and its rate of permeation through the gastric wall or intestinal lumen. For a relatively insoluble drug (less than 0.01 mg./ml.), the rate of solution becomes a fundamental factor affecting the rate of absorption. The reason is that unless the drug dissolves at a sufficiently rapid rate the necessary build-up of an effective concentration at the site of absorption will never occur. On the other hand, a relatively soluble drug will immediately saturate the system. In this case, the permeation rate becomes the important factor.

There have been many *in vitro* and *in vivo* studies (1, 2) dealing with the passage of drugs through the gut. However, as far as the authors have been able to determine, there has been no attempt to treat the data quantitatively as a diffusion phenomenon.

This study is concerned with the effect of pH on the *in vitro* absorption of flufenamic acid,[1] [*N*-(α,α,α-trifluoro-*m*-tolyl) anthranilic acid], with a pKa of 3.9 and solubility of 1 mg./ml. at pH 7.0. Based on Fick's law of diffusion, the apparent permeability constant at each pH value is calculated and the permeability constant is determined. A method is presented to estimate the relative surface area of the gut membrane through which diffusion of the drug takes place.

THEORETICAL CONSIDERATIONS

It has been known for many years (1) that cellular permeability to weak electrolytes may be dramatically affected by relatively small changes in the pH of the suspending medium. Although this phenomenon is of general significance in all cells, only recently were good examples described in the case of the intestinal absorption (3–8).

Absorption involves the transfer or permeability of materials from the intestinal lumen into the mucosal blood and lymph vessels. In the classification of permeability, a primary division is made between passive diffusion and special mechanisms such as active transport, facilitated diffusion, and pinocytosis. It has been shown that most drugs are absorbed by passive diffusion (3–5, 9).

Passive or simple diffusion describes the passage of a molecule across a barrier from a region of high to a region of low concentration. This phenomenon is quantitatively described by Fick's law (10), which states that the driving force which causes the transfer of a substance from regions of high to low concentrations is proportional to the concentration gradient or,

$$\frac{ds}{dt} = \frac{K\,(A)\,(C_0 - C_1)}{h} \qquad \text{(Eq. 1)}$$

where

$\frac{ds}{dt}$ = rate of movement of solute, s in mcg./min.,
A = area of the membrane in cm.^{2},
K = constant,
C_0 = amount of solute on the outside in mcg.,
C_1 = amount of solute on the inside in mcg.,
h = thickness of the membrane in cm.

When dealing with membranes, such as biological specimens, the thickness, h, of the membrane is not known. This factor is then commonly combined with the constant K to give a new constant P_1, the apparent permeability constant. Equation 1 then becomes

$$\frac{ds}{dt} = P_1(A)(C_0 - C_1) \qquad \text{(Eq. 2)}$$

If the permeability rate follows Eq. 2, a plot of amount *versus* time should be linear initially. The slope (l_1) of this line is equal to the change in amount with time, *i.e.*, to $\frac{ds}{dt}$.

Differentiating Eq. 2 with respect to area (A), we get

$$\frac{d^2s}{dt} = dA\,P_1(C_0 - C_1) \qquad \text{(Eq. 3)}$$

or

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[1] To be marketed as Arlef, Parke, Davis & Co., Detroit, Mich.

$$P_1 = \frac{d^2s}{dtdA(C_0 - C_1)} \qquad \text{(Eq. 4)}$$

The term $d^2s/dtdA$ can be evaluated by plotting the slope l_1 *versus* the area A and determining the slope l_2 of the resulting line. l_2 is then numerically equal to $d^2s/dtdA$. Knowing $C_0 - C_1$, P_1 can be calculated from Eq. 4.

If the permeation rate is measured at different pH values, P_1 can be calculated for each pH. Furthermore, the fraction of unionized moiety of the drug at different pH values can be determined theoretically, and assuming that it is only the unionized species that traverse the barrier, the permeation constant P can then be calculated, from the relationship

$$P = \frac{P_1}{U} \qquad \text{(Eq. 5)}$$

where U is the fraction of the unionized drug. P should then be a constant and independent of the pH.

The determination of the surface area of the membrane is difficult, particularly due to the presence of the villi on the surface of the intestinal wall. In this study, the concept of relative surface area is used. The apparent permeability constants derived are, therefore, relative; nevertheless, they are useful for comparing the transfer of the drug under different conditions.

Flufenamic acid is "bound"[2] to the intestine. At a particular concentration, the quantity "bound" is directly proportional to the surface area exposed or one can write

$$Q = kA \qquad \text{(Eq. 6)}$$

where Q = quantity of drug "bound" at a given concentration, k = constant, A = surface area.

If, then, for a series of experiments, at a particular concentration of the drug, one determines an average Q and assumes that A is 1, for the series, k can be calculated. This k can then be used to calculate other relative surface areas if the concentration of "bound" drug is known.

METHODOLOGY

To study adsorption of drugs both *in vivo* and *in vitro* procedures have been used. The selection of the method depends on the type of information desired. In this connection, Laster (11) points out that *in vivo* methods, *e.g.*, measuring the disappearance of a test substance from the gut or its appearance in the body fluids, tend to yield over-all results determined by a number of processes of which absorption is only one. On the other hand, he states that if excised intestinal tissue is studied *in vitro*, discrete absorption patterns may be established. However, it must be remembered that excised segments are deprived of their blood and lymph flows and depend on their oxygen supply on the diffusion of the gas across the epithelium.

From a number of *in vitro* methods available to study absorption, the authors used in these studies the everted-sac technique as modified by Crane and Wilson (12).

[2] The use of the term "bound" does not imply adsorption. The exact nature of physical and/or chemical interaction of flufenamic acid with the gut membrane is being investigated and will be the subject of a future presentation.

An excised segment (about 6 cm. in length) of the small intestine of a golden hamster is everted so that the mucosa faces outward. The segment is suspended in a glass tube, with a side arm, by tying to a cannula which in turn is supported by a rubber stopper. Forty-five milliliters of a buffered solution at the desired pH containing a known amount of the drug is added to the tube and bathes the mucosal surface of the intestine. A mixture of 95% oxygen and 5% carbon dioxide is bubbled through the solution through a longer cannula. Two milliliters of the buffer (without the drug) is placed inside the sac. The whole assembly is placed in a constant-temperature bath set at 37° ± 0.5°.

At known time intervals, the solution inside the intestine is removed for assay of flufenamic acid. Two milliliters of buffer is added to the inside of the intestine to rinse out any adhering drug and the solution is assayed. At the same time period, 1 ml. of the outside solution is removed for assay.

At the end of a run, the intestinal segment is taken out from the assembly, both surfaces washed 3 times with water, dried at 65°, powdered and extracted with 10 ml. of 0.1 N sodium hydroxide solu-

Table I.—Permeation of Flufenamic Acid at pH 7.2 (Concentration of Outside Solution, 20 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4
15	13.6	14.6	7.5	—
20	—	—	—	8.68
30	25.9	17.3	8.2	—
40	—	—	—	19.9
45	36.35	25.1	15.9	—
60	43.35	29.35	15.4	30.6

Table II.—"Bound" Drug, Relative Surface Area, and Rate at pH 7.2

Run	"Bound" Drug, mcg.	Relative Surface Area	Rate of Permeation, mcg./min.
1	152	2.47	0.725
2	61	1.0	0.4
3	29	0.48	0.25
4	87	1.42	0.55

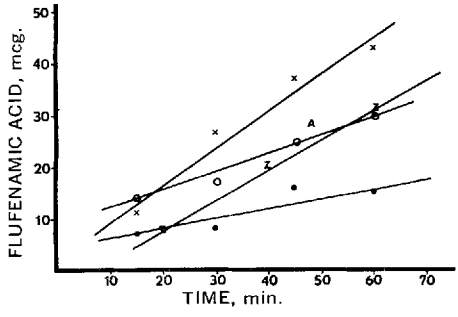

Fig. 1.—Plot of rate of permeation at pH 7.2 showing dependency on relative surface area. Key: Z, 1.42; ●, 0.48; O, 1.0; ×, 2.47.

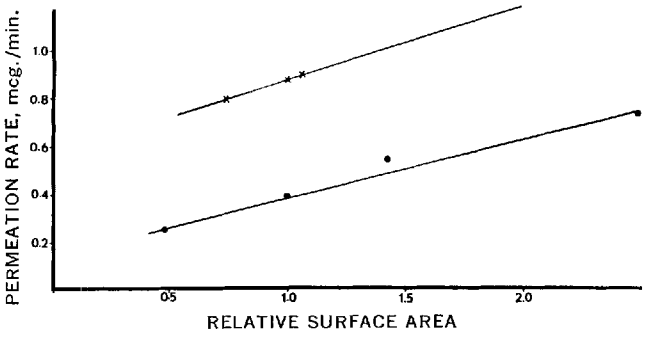

Fig. 2.—Plot of permeation rate *versus* relative surface area at pH 5.0 (×) and pH 7.0 (●).

TABLE III.—PERMEATION OF FLUFENAMIC ACID AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION 20 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3
15	10.88	15.5	—
20	—	—	13.95
30	27.0	—	—
50	—	49.05	—
60	52.0	—	31.0
75	—	—	56.0

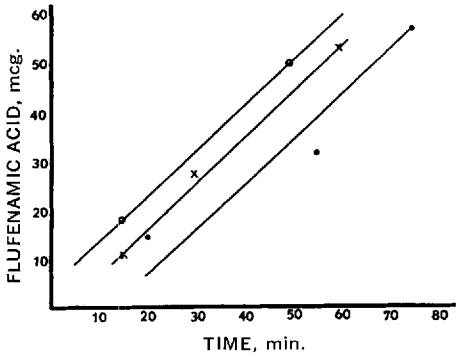

Fig. 3.—Plot of rate of absorption at pH 5.0 (three separate determinations). Initial concentration of outside solution 20 mcg./ml.

tion to remove any "bound" drug. It was necessary to follow this procedure to remove the "bound" drug since it adhered quite strongly to the gut membrane and could not be removed by simple washing with water or buffer solutions.

All samples are assayed using an Aminco Bowman spectrophotofluorometer, employing the assay procedure of Glazko and Dill (13).

The composition of the buffer used is:

	mmoles/L.
Sodium chloride	145
Potassium chloride	4.56
Calcium chloride	1.25
Sodium phosphate (dibasic)	1.33
Sodium phosphate (monobasic)	0.33
Distilled water	*q.s.*
pH of buffer 7.2	

The buffer is adjusted to pH 5.0, 3.9, and 2.5 by adding 20 mmoles of aspartic acid to the above solution and adjusting the pH with sodium hydroxide or hydrochloric acid solution.

RESULTS AND DISCUSSION

The quantity of drug which permeates through the intestine at different time intervals at pH 7.2 is given in Table I. This quantity represents the sum of the assays of the sample withdrawn from the inside of the intestinal segment, and the 2-ml. sample used to wash the inside. The initial concentration of the drug in the outside solution is 20 mcg. of flufenamic acid per ml. The results of four separate determinations are shown.

The quantity of "bound" drug for each of the runs and the relative surface area calculated from this are given in Table II. It is apparent from Table II that th rcelative surface area of the gut membranes, as gauged from the "bound" drug, varies considerably. This was due to the difference in thickness of the gut segments of the different animals used in this study.

The quantity of drug which permeates at pH 7.2 is plotted against time in Fig. 1. The slope of the

TABLE IV.—"BOUND" DRUG, RELATIVE SURFACE AREA, AND RATE AT pH 5.0

Run	"Bound" Drug, mcg.	Surface Area	Rate of Permeation, mcg./min.
1	168	1	0.875
2	179	1.06	0.9
3	130	0.774	0.8

TABLE V.—PERMEATION AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION, 15 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3
15	18.3	—	8.55
20	—	9.4	—
30	17.35	—	16.4
40	—	32.7	—
45	21.2	—	26.2
60	40.0	28.7	—
"Bound" drug, mcg.	91.3	105.6	142.8
Rate of permeation, mcg./min.	0.65	0.65	0.59

lines gives the rate of permeation in accordance with Eq. 2. These values are also included in Table II.

In Fig. 2, the rate of permeation is plotted against relative surface area. By determining the slope and using Eq. 4, the apparent permeability constant P_1 is calculated.

TABLE VI.—PERMEATION AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION, 5 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4
15	2.55	—	1.9	—
30	7.1	4.9	4.9	8.6
45	7.8	—	5.4	—
60	20.0	14.0	7.55	12.95
"Bound" drug, mcg.	38	24	36.7	47
Rate of permeation, mcg./min.	0.36	0.24	0.13	0.23

TABLE VII.—PERMEATION AT pH 5.0 (CONCENTRATION OF OUTSIDE SOLUTION, 2 mcg./ml.)

Time, min.	Run 1	Run 2
15	0.66	—
20	—	1.08
40	2.09	1.19
60	2.68	3.21
"Bound" drug, mcg.	6.93	11.6
Rate of permeation, mcg./min.	0.051	0.051

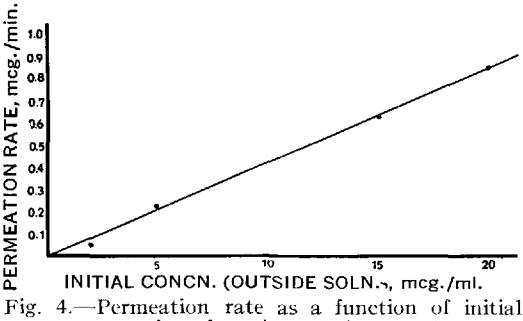

Fig. 4.—Permeation rate as a function of initial concentration of outside solution, pH 5.0.

Similarly, the quantity of drug which permeates at pH 5.0, when a concentration of 20 mcg./ml. is maintained on the outside, is given in Table III and shown in Fig. 3.

The quantity of "bound" drug, relative surface area, and rate of permeation at pH 5.0 are given in Table IV.

If Fick's law is applicable, the permeation rate should vary with the concentration of the drug in the outside solution, since the gradient across the membrane is proportional to the concentration. To test this hypothesis, permeation rates were determined at pH 5, using solutions containing 2, 5, and 15 mcg. of the acid per ml. The results are given in Tables V, VI, and VII. For convenience, the rate of permeation and concentration of "bound" drug are also included in these tables.

A plot of permeation rate *versus* concentration at pH 5.0 is shown in Fig. 4. It is apparent that a linear relationship exists between the rate and concentration of the outside solution. This is in agreement with Fick's law.

The quantity of drug "bound" in the membrane is also proportional to the concentration outside. Thus a plot of "bound" drug *versus* concentration should be linear, which is shown in Fig. 5. This is not surprising if one considers the nature of the membrane barrier. If one assumes that it is at least two to three cells thick, at a given time there is a certain amount of drug in the membrane. The quantity is proportional to the concentration gradient which in turn is determined by the concentration of the solution.

At low pH values, the solubility of flufenamic acid is limited; therefore, it is not possible to carry out studies using solutions containing 20 mcg./ml., as is done at pH 7.2 and 5.0. The studies at pH 2.5 and 3.9 are, therefore, carried out using solutions containing 2 mcg. of the drug per ml. In order to have a basis for comparison, the permeation rates for 20 mcg./ml. concentration are then computed from the value of 2 mcg./ml. It is felt that this procedure is valid since the concentration dependency should hold at pH 2.5 and 3.9 as was shown at pH 5.0.

The data are given in Tables VIII, IX, and X, and a plot of permeation rate *versus* relative surface area at these pH values is shown in Fig. 6.

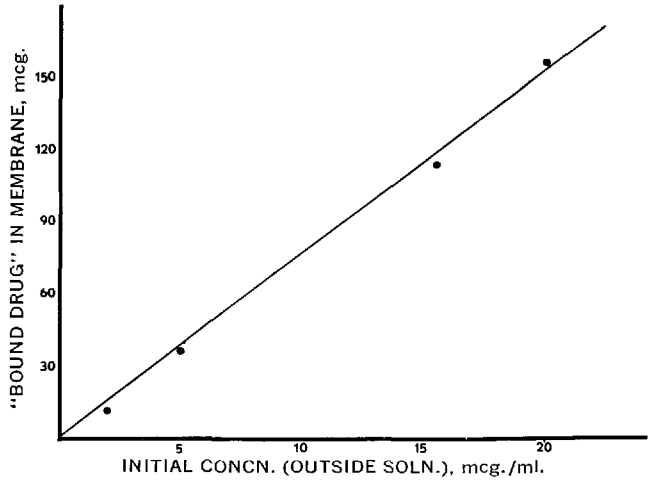

Fig. 5.—Plot of "bound drug" *versus* initial concentration of outside solution, pH 5.0.

TABLE VIII.—PERMEATION AT pH 3.9 (CONCENTRATION OF OUTSIDE SOLUTION, 2 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4	Run 5
15	4.22	1.82	1.90	1.7	1.20
30	—	2.35	1.40	5.70	—
45	4.82	2.67	2.20	6.45	3.32
60	8.1	6.3	3.75	9.8	—
"Bound" drug, mcg.	15.4	13	8.87	14.2	12.2
Relative surface area	1.19	1	0.68	1.09	0.94
Rate of permeation, mcg./min. computed to 20 mcg./ml. concn.	2.3	1.68	0.924	2.6	1.58

TABLE IX.—PERMEATION AT pH 2.5 (CONCENTRATION OF OUTSIDE SOLUTION, 2 mcg./ml.)

Time, min.	Amt. Found Inside, mcg. Run 1	Run 2	Run 3	Run 4
15	1.8	1.3	2.0	—
20	—	—	—	1.65
30	6.35	—	2.35	—
40	—	—	—	8.85
45	3.05	5.35	6.05	—
60	5.80	4.85	9.0	10.12
"Bound" drug, mcg.	29.25	27	33.75	36.98
Relative surface area	1	0.923	1.15	1.26
Rate of permeation, mcg./min. computed to 20 mcg./ml. concn.	1.56	1.56	2.32	2.85

TABLE X.—APPARENT PERMEABILITY CONSTANTS P_1 AND P

pH	Unionized Drug, %	$P_1 \times 10^{-4}$	$P \times 10^{-4}$
7.2	0.05	2.6	52
5.0	7.36	3.7	50
3.9	50.0	29.1	58
2.5	96.19	52.0	54

The apparent permeability constants P_1 at each pH value is calculated using Eq. 4. This is shown in Table X, together with the values for the permeability constant P, calculated from Eq. 5, and the per cent of unionized drug.

From Table X it is evident that the permeability constant P derived for measurements at each pH value is in excellent agreement. Furthermore, it is apparent that at pH 2.5 the permeation of flufenamic acid is 20 times faster than at pH 7.2. The study also shows that flufenamic acid follows the postulation (2–4, 8) that it is only the unionized moiety of the drug that passes through a cell, due to its lipoid solubility hypothesis. Since at pH 2.5 the drug is approximately 96% unionized, it is to be expected that the rate would be much faster than at pH 7.2, at which only 0.05% of the drug is in the unionized form.

In this study a procedure is described defining the permeation of flufenamic acid in terms of Fick's diffusion law. Perhaps this approach could be used in describing the passage of other drugs through the gut membrane, particularly those drugs which have an affinity for binding with the membrane. It is also suggested that in this type of measurement, the quantity of drug in the membrane should be determined. In some instances, as was found in this study, this can be considerable.

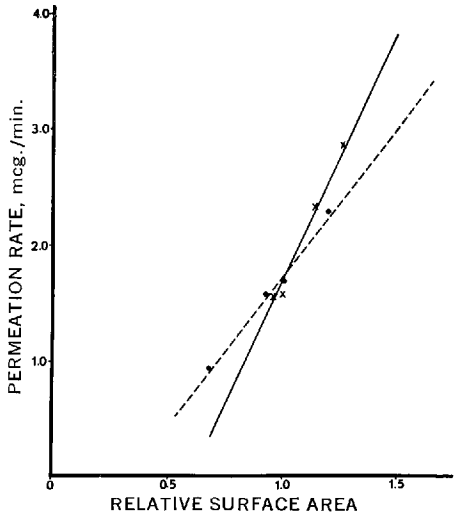

Fig. 6.—Plot of permeation rate *versus* relative surface area at pH 2.5 (×) and pH 3.9 (●).

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Bioassay of Hypocholesterolemic Steroids

By R. M. TOMARELLI, T. M. DOUGHERTY, and F. W. BERNHART

The hypocholesterolemic activity of a number of natural and synthetic steroids was determined in a 3-day oral assay using male rats with hypercholesterolemia of dietary origin. The reduction in serum cholesterol was linearly related to the log dose of the administered natural or modified estrogen; the effect was evident 6 hr. after oral administration and had reached a maximum in 24 to 30 hr. Active steroids lowered the elevated serum phospholipids, cholesterol/phospholipid ratios, triglycerides, α and β lipoproteins, and β/α lipoprotein ratios, but not the elevated serum globulins. The effect of the steroids on body weight permitted a semiquantitative estimation of feminizing activity.

THE LOWER incidence of atherosclerotic heart disease in premenopausal women than in men and the demonstration that estrogen administration will lower serum lipids in man and animals have prompted a search for antilipemic steroids that have little or no feminizing activity (1,2). The present report describes the development of a 3-day bioassay for the screening of orally active hypocholesterolemic compounds and presents the results obtained with a number of natural and synthetic steroids.

EXPERIMENTAL

Analytical Procedures.—The following methods were used for determining serum constituents: cholesterol, Zlatkis *et al.* (3) and Technicon Auto-Analyzer procedure (4); triglyceride, van Handel and Zilversmit (5); phospholipid, Whitley and Alburn procedure (6); protein, Lowry *et al.* (7).

Serum lipoproteins were estimated from the cholesterol content of serum protein fractions separated by paper electrophoresis. The relative proportions and locations of the protein fractions were determined on strips stained with azocarmine G; cholesterol associated with these areas was eluted from duplicate unstained strips and determined colorimetrically. The designation of protein fractions as α or β lipoproteins was according to Searcy *et al.* (8).

Assay Procedure.—The assay animals were male rats with serum cholesterol levels elevated 7 to 10 times normal by the feeding of a hypercholesterolemic diet. The diet used was essentially that of Nath *et al.* (9); it consisted of 18% casein,[1] 25% hydrogenated coconut oil,[2] 4% salts, (U.S.P. XIV) 1% cholesterol, 0.5% cholic acid, 0.2% choline chloride, an adequate amount of all required vitamins (10), and sucrose to make 100%. Groups of 70 to 80 young male Sprague-Dawley rats, 100–120 Gm. body weight, were prepared for assay by feeding this diet *ad libitum* for 3 weeks. Average weight gain during this period was 70–100 Gm. Average serum cholesterol levels determined on 0.01 ml. of serum from tail blood varied from 600–1000 mg.%.

The rats were divided into groups of six with nearly equal average body weights and serum cholesterol levels. Steroids were given orally for 3 days in 0.2 ml. cottonseed oil solution; the control group received oil vehicle alone. Three groups received graded doses of an appropriate standard steroid; test compounds were administered at a single 1-mg. dose level. On the morning of the fourth day the rats were weighed and serum cholesterol was determined. The average serum cholesterol values of the three standard groups, plotted against the log dose, gave a linear relationship from which the hypocholesterolemic activity of the test compounds were evaluated. Activity was expressed relative to the standard.

The effect of the test steroid on body weight was also evaluated by comparison with that of the standard. Since estrogen administration to a male rat will inhibit food consumption and retard body weight gain in a young animal or cause weight loss in an adult (11), a lack of effect of the steroid on body weight was accepted as an indication of a low degree of feminizing activity. Loss of weight, being nonspecific, could be considered as only suggestive evidence of feminization. The effect of the estrogenic steroids on body weight and food intake was roughly proportional to the log dose, particularly in young animals (Fig. 1). For semiquantitative evaluation the effects were graded 0 to +3, with 0 equal to the control value and +1 to that of the standard steroid. Pair-feeding experiments showed that the reduction in the intake of food (and cholesterol), comparable to that found in rats treated with estrogen for 3 days, had no appreciable effect on serum cholesterol concentration.

Fifteen days after the completion of a test, serum cholesterol was again determined and the rats regrouped for the next test which started 4 days later. Each group of hypercholesterolemic rats were routinely used for 4 to 6 assays.

The 3-day assay period followed by the 19-day rest period was selected after time studies revealed that serum cholesterol levels (*a*) decrease measurably about 5 hr. after oral administration, (*b*) drop to a constant value within 24–28 hr., and (*c*) return to pretest levels 5 days after the last dose of the steroid is given (Fig. 2). In earlier assays in which the steroids were administered for 3 weeks, no significant differences were found in the serum cholesterol levels determined at weekly intervals (Fig. 3).

The steroid used as a standard in all the tests reported in the present study was *dl*-13β-ethyl-3-methoxy-8α-gona-1,3,5(10)-trien-17β-ol (Wy-3359). This recently synthesized compound (12) has an antilipemic activity by subcutaneous injection 2.6

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[1] High nitrogen, Sheffield.
[2] Hydrol-100, Durkee.

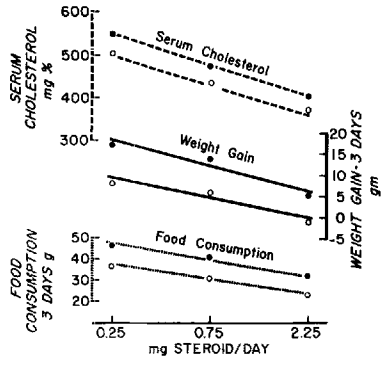

Fig. 1.—The effect of β-estradiol (○) and Wy-3359 (●) on serum cholesterol, weight gain, and food consumption of hypercholesterolemic rats.

times that of *d*-estrone but only 1% of its estrogenic activity (13). Oral administration of Wy-3359, 0.25 to 2.25 mg./day, resulted in a reasonably reproducible drop in serum cholesterol, 197 ± 10 mg./100 ml. (21 three-dose assays with six rats per group). This response, when expressed as per cent of the control, did not appear to be influenced either by the body weight in the 200–350-Gm. range, the initial serum cholesterol level, the season of the year, or the use of the animal in previous assays.

The confidence limits of the assay value could be estimated by any conventional procedure for a 4-point assay (three standards and one unknown) such as that of Gaddum (14). In practice, a convenient estimate of confidence limits was devised from the observation that the standard error was proportional to the average serum cholesterol level (9.2% ± 1.7). This value was calculated from the data obtained from 32 groups, six rats per group, which included controls and groups treated with a variety of steroids of different hypocholesterolemic activity. The 95% confidence limits were as follows: for compounds having activity near 25%, 7–75%; near 100%, 68–160%; and near 200%, 145–280%.

Serum Constituents.—Serum samples taken under routine assay conditions, *i.e.*, from unfasted rats after 3 days of oral steroid administration, were analyzed for a number of constituents in addition to cholesterol. Typical results are presented in Table I and Fig. 4. Compared with Purina chow-fed controls, the hypercholesterolemic rats had elevated serum proteins, phospholipids, cholesterol-phospholipid ratios, α and β lipoproteins, and β-to-α lipoprotein ratios. Serum triglycerides were slightly elevated with considerable individual variation presumably because of the unfasted state of the animal. The increase in the serum proteins was in the globulins, particularly in the β fraction (Fig. 4).

The oral administration of β-estradiol or Wy-3359 reduced all the lipid constituents (Table I); the decrease in cholesterol was relatively greater than that of phospholipids resulting in a reduced cholesterol–phospholipid ratio. Both α and β lipoproteins were reduced, the β considerably more than the α (Fig. 4). With increasing doses of Wy-3359, the β/α lipoprotein ratio of the hypercholesterolemic rat dropped from 1.08 to 0.59, approaching that of the chow-fed controls, 0.41. However, steroid administration had no effect on the elevated globulin fractions (Fig. 4); *i.e.*, only the cholesterol moiety of the globulin fractions was decreased.

Comparative Hypocholesterolemic Potencies.—Table II presents the hypocholesterolemic activity, as determined by the 3-day oral assay, of a number of natural and synthetic steroids. Many of these compounds have been studied previously by other investigators for antilipemic activity in the rat (see references in table). The most active compound by far was ethinyl estradiol, followed by estriol, β-estradiol, estrone, equilin, and equilenin, all with approximately equal activity. This order of activity is similar to that reported for oral estrogenicity (26). 17α-Estradiol was about half as active as the β isomer. The 3-methyl ethers of estrone and β-estradiol were slightly more active than the parent compounds. The Δ^4 steroids, methyltestosterone, progesterone, and norbolethone were inactive. The ineffectiveness of methyltestosterone corroborates the results obtained by Abel and Mosbach (20), who found that while the steroid had hypocholesterolemic activity in rats with normal or slightly elevated serum cholesterol, it was inactive in hypercholesterolemic animals.

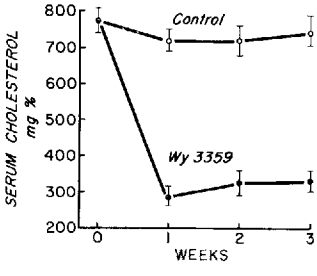

Fig. 3.—Effect of Wy-3359, 1 mg./day, 6 days a week for 3 weeks, on serum cholesterol.

Compared with the standard, Wy-3359, all of the natural steroid estrogens and diethylstilbestrol had a relatively greater adverse effect on body weight than on serum cholesterol reduction. The favorable separation of antilipemic and estrogenic activity found in the synthetic steroids, Wy-3271, Wy-3714, and Wy-6012, has previously been reported (21–23).

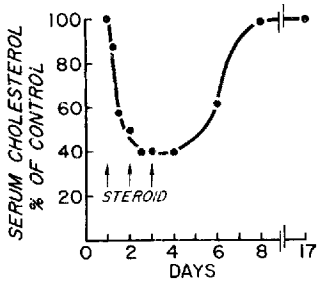

Fig. 2.—Time study of the effect of Wy-3359, 2.25 mg./day for 3 days, on serum cholesterol.

DISCUSSION

The hypocholesterolemic activity of a number of steroids, determined by this 3-day procedure with hypercholesterolemic rats, was in general agreement with values obtained by other procedures, indicating that a 3-day assay period is of adequate duration.

TABLE I.—EFFECT OF HYPOCHOLESTEROLEMIC STEROIDS ON SERUM CONSTITUENTS UNDER ASSAY CONDITIONS

Dietary Group[a]	Steroid[b]	Serum Constituent/100 ml. ± S. E. Protein, Gm.	Cholesterol,[c] mg.	Phospholipid, mg.	Chol./Phosp. Ratio	Triglyceride, mg.
Hypercholesterolemic	None	8.9 ± 0.3	643 ± 33	342 ± 4	1.87 ± 0.06	181 ± 36
Hypercholesterolemic	Wy-3359 0.75 mg.	8.8 ± 0.2	251 ± 16	235 ± 7	0.98 ± 0.06	70 ± 19
Hypercholesterolemic	β-Estradiol 0.75 mg.	8.9 ± 0.2	247 ± 37	267 ± 33	0.91 ± 0.05	39 ± 14
Chow	None	6.8 ± 0.1	73 ± 4	163 ± 10	0.46 ± 0.05	84 ± 21

[a] Six rats per group; average body weight 320 Gm. [b] Daily oral dose for 3 days. [c] Total cholesterol; the free cholesterol proportion of 20% was not affected by steroid administration.

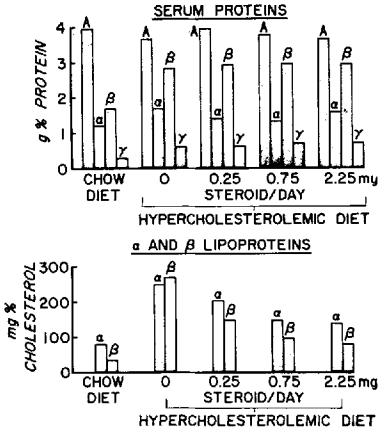

Fig. 4.—Effect of Wy-3359 on serum proteins and α and β lipoproteins. The β/α ratio was 0.41 for chow-fed rats and 1.08 for the hypercholesterolemic rats, but decreased in the latter to 0.87, 0.67, and 0.59 with the increasing doses of the steroid.

In addition to saving in time and material, the procedure has several other advantages over assays of longer duration with rats fed diets containing no added cholesterol. The small amount of hypercholesterolemic blood required for chemical analysis does not necessitate sacrifice of the animals; they can be used for a series of assays, and determinations can be run on the same animal before and after steroid administration, permitting a more precise measurement of effect. The decrease in food consumption, resulting from estrogenic steroid administration, did not lower serum cholesterol, presumably because of the high content of cholesterol in the diet and the brevity of the treatment.

While the rats varied in their response to the hypercholesterolemic diet, with serum values ranging from 400 to 1300 mg./100 ml., daily variations were small and the response to an antilipemic steroid was not related to the degree of existing hypercholesterolemia. The seasonal fluctuations in serum cholesterol in male rats fed diets without added cholesterol, noted by Thorp and Waring (24) and Edgren (27), has not been noted in our animals

TABLE II.—HYPOCHOLESTEROLEMIC ACTIVITY OF NATURAL AND SYNTHETIC STEROIDS

Common Name	Chemical Name	Hypocholesterolemic Activity	Body Wt. Effect	Ref.
Wy-3359 (std.)	*dl*-13β-Ethyl-3-methoxy-8α-gona-1,3,5(10)-trien-17β-ol	1.0	1	(12)
d-Estrone	*d*-3-Hydroxyestra-1,3,5(10)-trien-17-one	2.0	3	(15, 16)
β-Estradiol	*d*-Estra-1,3,5(10)-trien-3,17β-diol	1.8	>3	(17–19)
17α-Estradiol	*d*-Estra-1,3,5(10)-trien-3,17α-diol	1.0	2	...
Estriol	*d*-Estra-1,3,5(10)-trien-3,16α,17β-triol	3.5	>3	(2, 17)
Equilin	*d*-3-Hydroxyestra-1,3,5(10),7-tetraen-17-one	2.3	>3	...
Equilenin	*d*-3-Hydroxyestra-1,3,5(10),6,8-pentaen-17-one	2.3	>3	...
d-Estrone-3-methyl ether	*d*-3-Methoxyestra-1,3,5(10)-trien-17-one	2.5	3	...
β-Estradiol-3-methyl ether	*d*-3-Methoxyestra-1,3,5(10)-trien-17β-ol	3.8	>3	...
17α-Estradiol-3-methyl ether	*d*-3-Methoxyestra-1,3,5(10)-trien-17α-ol	0.8	1	...
Ethinyl β-estradiol	*d*-17α-Ethynylestra-1,3,5(10)-trien-3,17β-diol	65–90	>3	(19)
Methyltestosterone	*d*-17α-Methylandrost-4-en-17β-ol-3-one	0	0	(20)
Progesterone	*d*-Pregn-4-en-3,20-dione	0	0	(17)
Norbolethone	*dl*-13β,17α-Diethyl-17β-hydroxygon-4-en-3-one	0	0	...
Wy-3271	*dl*-13-Ethyl-3-methoxygona-1,3,5(10)-8,14-pentaen-17-one, cyclic ethylene acetal	1.2	1	(21)
Wy-3714	*dl*-13-Ethylgona-1,3,5(10)-triene-3,17β-diol	0.3	0.3	(22)
Wy-6012	*dl*-3-Methoxy-6-oxa-8α-estra-1,3,5(10)-trien-17β-ol	0.9	0.9	(23)
Diethylstilbestrol	4-4′-Dihydroxy-α,β-diethylstilbene	2.9	>3	(17, 19)
	Conjugated equine estrogens[a]	2.5	>3	...
	Ethyl(*p*-chlorophenoxy)isobutyrate[b]	0.007	0	(24)
	1-[*p*-(β-Diethylaminoethoxy)phenyl]-1(*p*-tolyl)-2-(*p*-chlorophenyl)ethanol[c]	Trace	2	(25)

[a] Marketed as Premarin by Ayerst. Activity relative to estrone sulfate content. [b] CPIB. Imperial Chemical Industries. Daily dose, 20–50 mg. [c] Trademarked as Triparanol by Merrell. Daily dose, 25 mg.

presumably because these changes are inconsequential in relation to the high concentration of serum cholesterol.

It should be noted that the above assay procedure is unsuitable for the screening of compounds that lower serum cholesterol by interfering with cholesterol biosynthesis, since this process is markedly reduced in rats fed high cholesterol diets (28, 29).

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BENZOIC ACID and its substituted isomers have been found to interact with a varied number of pharmaceutical compounds. Salicylic acid, *meta*, and *para* hydroxybenzoic acids have been shown to complex with polyethylene glycols (1) and with polyvinylpyrrolidone (2). In addition, the hydroxybenzoic acids have been found to interact with caffeine (3) and with theophylline and theobromine (4). Goodhart and Martin (5) reported that the solubilities of benzoic acid and its substituted isomers were greatest in the least hydrophilic of the polyethylene stearates. The solubilities of benzoic acid, salicylic acid, and *p*-hydroxybenzoic acid have also been found to increase in the presence of their sodium salts and the addition of a hydroxy group to the acid led to the formation of complexes (6).

Chelation of ferric and aluminum ions with salicylic acid and its amino, chloro, and nitro derivatives have been demonstrated by Foye and Turcotte (7). Results from the investigation of the action of Schardinger dextrins on benzoic acid and hydroxybenzoic acids did not seem to indicate a clear cut mechanism of the complexation involved. Apparently hydrogen bonding is an important factor in the cyclodextrin–hydroxybenzoic acid interaction (8). Schlenk and Sand (9) showed that the molecular ratio of cyclodextrin to certain benzoic acid derivatives was a function of the physical state of the complex. Monohalogenated benzoic acids and

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aminobenzoic acids have been reported to interact with cyclodextrins. The resultant complexes were considered to be due to inclusion formation and other attractive forces existing between host and guest molecules (10).

Higuchi and Drubulis (11) have attributed the interaction of hydroxyaromatic acids and their salts to a donor–acceptor type of mechanism. Urea has been shown to form complexes of low stability with benzoic acid while with salicylic acid it forms insoluble interaction products (12). Goudah and Guth (13) demonstrated that benzoic acid and its substituted compounds complexed with potato and arrowroot starch and postulated that attractive forces and inclusion formation were responsible for the interaction observed. Salicylic acid has been reported to exhibit the greatest binding tendency for polysorbate 80, followed by the *meta* and *para* isomers (14). This was identical with the findings of Blaug and Ebersman (15) for the behavior of hydroxybenzoic acids toward hyprose ester. Chakravarty, Lach, and Blaug (16) studied the interaction of hydroxybenzoic acids with polyoxyl 40 stearates based on solubility measurements.

It is the purpose of this investigation to study the interaction of hydroxybenzoic acids and the amino, chloro, and nitro derivatives of benzoic acid with cationic surfactants of the quaternary ammonium type as previous work has dealt with the interaction of pharmaceutical drugs with nonionic surfactants.

EXPERIMENTAL

Materials.—Recrystallized benzoic acid, m.p. 122–122.5° (Monsanto Chemicals Ltd.); recrystallized salicylic acid, m.p. 158–159°, recrystallized *m*-hydroxybenzoic acid, m.p. 205–205.5°, recrystallized *p*-hydroxybenzoic acid, m.p. 215–216°, recrystallized acetylsalicylic acid, m.p. 137–138°, recrystallized *o*-aminobenzoic acid, m.p. 145.5–146° (British Drug Houses Ltd.); recrystallized *m*-aminobenzoic acid, m.p. 174–175°, recrystallized *p*-aminobenzoic acid m.p. 187–188°, recrystallized *o*-chlorobenzoic acid, m.p. 140–141°, recrystallized *m*-chlorobenzoic acid, m.p. 155–156°, recrystallized *p*-chlorobenzoic acid, m.p. 240–241°, recrystallized *o*-nitrobenzoic acid, m.p. 146–147°, recrystallized *m*-nitrobenzoic acid, m.p. 140–141°, recrystallized *p*-nitrobenzoic acid, m.p. 241–242° (Hopkin and Williams Ltd.). The cationic surfactants were dodecyltrimethylammonium bromide,[1] tetradecyltrimethylammonium bromide,[1] and cetyltrimethylammonium bromide.[2] The anionic surfactant was sodium lauryl sulfate (Sipon Products Ltd.) and the nonionic surfactant was cetomacrogol 1000 B.P.C.[3] The melting points and the critical micelle concentrations of the surfactants are listed in Table I.

[1] Marketed as Morphan D and Morphan T, respectively, by Glovers Chemicals Ltd.
[2] Marketed as Cetrimide by Imperial Chemical Industries.
[3] Marketed as Texofor AIP by Glovers Chemicals Ltd.

TABLE I.—PHYSICAL CONSTANTS OF SURFACTANTS

Surfactant	M.p.[a]	CMC 25°[b] %w/v
Dodecyltrimethylammonium bromide	244–245	0.041
Tetradecyltrimethylammonium bromide	246–247	0,082
Cetyltrimethylammonium bromide	262–263	0.051
Sodium lauryl sulfate	204–206	0.014
Cetomacrogol 1000	37–38	0.0021

[a] Micro melting apparatus. [b] From surface tension measurements using the Du Nouy tensiometer.

Apparatus.—Portable Ferranti viscometer, model VL; Pye Tropical universal pH meter.

Measurement of the Solubilities of the Isomers of Hydroxy, Amino, Chloro, and Nitrobenzoic Acids in Surfactant Solutions.—Varying amounts of the organic acid were weighed into a series of 25-ml. graduated glass-stoppered cylinders containing the required concentration of surfactant. The cylinders were rotated in a thermostatically controlled water bath at 25 ± 0.1° for 24 hr. The end point was taken as the mean between a clear solution and one in which a slight excess of acid was present.

Measurement of Viscosity.—The required amounts of the organic acid were weighed into a series of 100-ml. volumetric flasks containing the required concentration of the surfactant. The flasks were rotated in a thermostatically controlled water bath at 25 ± 0.1° for 24 hr. The viscosities of the dispersions were measured using the portable Ferranti viscometer placed in a thermostatically controlled water bath at 25 ± 0.5°. The shear rates employed ranged from 483.0 to 78.56 sec. An interval of 30 sec. was allowed between any two readings.

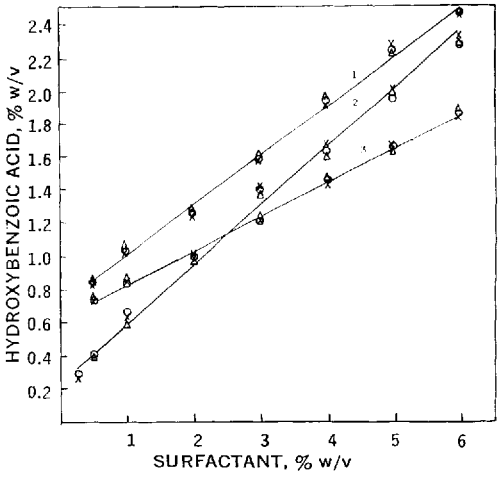

Fig. 1.—Solubilities of hydroxybenzoic acids in aqueous solutions of alkyltrimethylammonium bromide surfactants of varying chain length at 25°. Key: ×, dodecyltrimethylammonium bromide; O, tetradecyltrimethylammonium bromide; △, cetyltrimethylammonium bromide; *1*, *m*-hydroxybenzoic acid; *2*, salicylic acid; *3*, *p*-hydroxybenzoic acid.

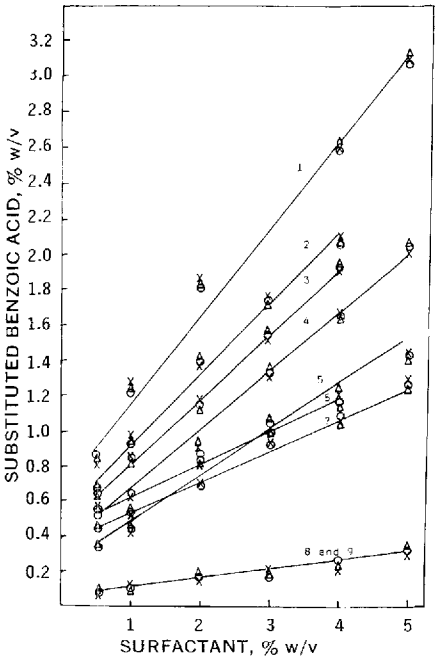

Fig. 2.—Solubilities of substituted benzoic acids in aqueous solutions of alkyltrimethylammonium bromide surfactants of varying chain length at 25°. Key: ×, dodecyltrimethylammonium bromide; O, tetradecyltrimethylammonium bromide; △, cetyltrimethylammonium bromide; *1*, *o*-nitrobenzoic acid; *2*, *m*-nitrobenzoic acid; *3*, *o*-aminobenzoic acid; *4*, *o*-chlorobenzoic acid; *5*, *m*-chlorobenzoic acid; *6*, *m*-aminobenzoic acid; *7*, *p*-aminobenzoic acid; *8*, *p*-nitrobenzoic acid; *9*, *o*-chlorobenzoic acid.

The shear rate was increased and then decreased for all viscosity measurements.

RESULTS AND DISCUSSION

Solubilities of Substituted Benzoic Acids in Surfactant Solutions.—Figures 1 and 2 show the solubilities of the isomers of hydroxy, amino, chloro, and nitrobenzoic acids in surfactant solutions. In all cases the solubilities of the acids are proportional to surfactant concentration. There is, however, no appreciable difference in the solubilities of the acids in the three surfactants. These solubility graphs seem to indicate that variation of the chain length of the lipophilic portion of the surfactant molecule has no effect on the solubilities of the acids suggesting that the solubilities of the various substituted benzoic acids studied are independent of the chain length of the surfactant molecule. Except for the hydroxy derivatives, the other acids show a decrease in solubility in the order of *ortho*, *meta*, and *para* isomers.

Viscosity Measurements.—Figures 3, 4, and 5 show the viscosities of dispersions containing varying amounts of salicylic acid. In general, there is an increase in viscosity with increasing concentration of salicylic acid in the surfactant solution up to approximately when the system becomes saturated with the acid, after which the viscosity remains constant or decreases. This behavior is seen with all the concentrations of surfactants studied, 2%, 3%, 4%, 5%, and 6% and in the range of shear rates used, the viscosity being greater at low rates of shear than at high rates of shear. These observations can be attributed to interaction of salicylic acid with the surfactants, particularly the tetradecyltrimethylammonium and cetyltrimethylammonium bromides. In the case of dodecyltrimethylammonium bromide a lesser degree of interaction with salicylic acid is apparent as no marked changes in viscosity have been observed. Probably it is due to the shorter chain length of the lipophilic portion of the surfactant molecule which may tend to discourage interaction, or it may be due to the resultant complex being smaller in size and hence is unable to produce the relatively higher viscosity seen with the longer chain surfactants. When the system becomes saturated with salicylic acid, the viscosity tends to remain constant with further additions of the acid and this is particularly noticeable at low surfactant concentration. These observations suggest that once interaction is complete the excess salicylic acid has no effect on the viscosity of the system. However, at high surfactant concentration the viscosity decreases at about the concentration at which the system becomes saturated with salicylic acid. This may be due to the unstable nature of the complex and which may manifest itself more predominantly in the presence of a large number of complexes which follows from an increased concentration of the surfactant.

When the concentration of salicylic acid in the surfactant solution is low, the system behaves like a Newtonian liquid. As the salicylic acid content is increased the system changes to a non-Newtonian liquid producing large differences in viscosities with varying rates of shear (Figs. 3, 4, and 5). It would appear that adequate salicylic acid must be present for interaction to take place to produce the viscosity effect. With a higher concentration of the surfac-

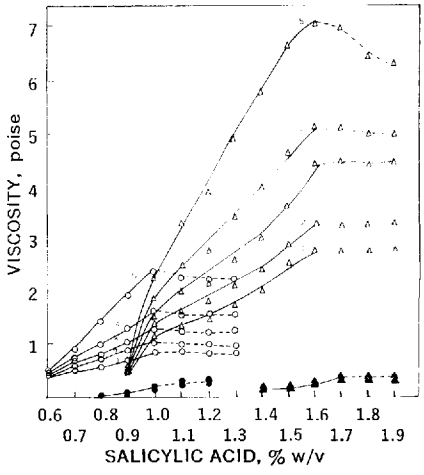

Fig. 3.—Effect of salicylic acid on the viscosity of alkyltrimethylammonium bromide solutions at 25°. Dodecyltrimethylammonium bromide: ●, 2%; ▲, 4%; tetradecyltrimethylammonium bromide: O, 2%; △, 4%; ——, below saturation; - - - -, above saturation. Shear rate: *1*, 234.6 sec.$^{-1}$; *2*, 195.9 sec.$^{-1}$; *3*, 155.1 sec.$^{-1}$; *4*, 117.35 sec.$^{-1}$; *5*, 78.56 sec.$^{-1}$.

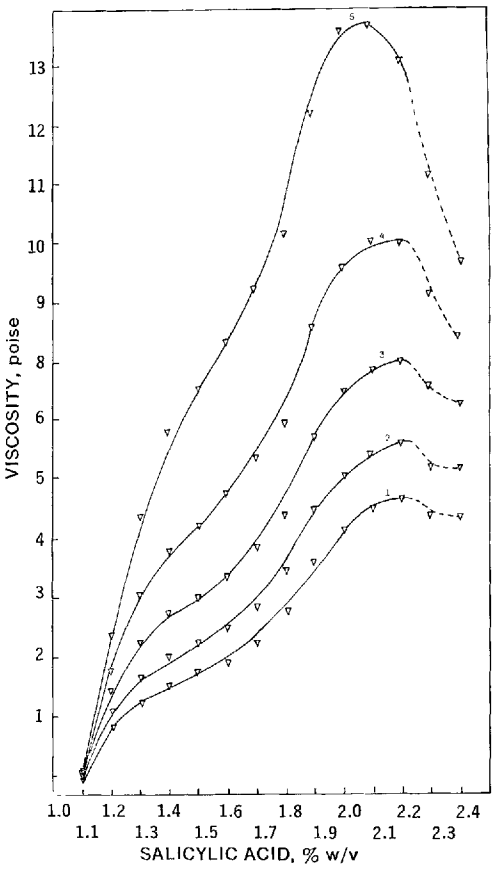

Fig. 4.—Effect of salicylic acid on the viscosity of tetradecyltrimethylammonium bromide solution (6%) at 25°. Key: ——, below saturation; - - - -, above saturation. Shear rate: *1*, 234.6 sec.$^{-1}$; *2*, 195.9 sec.$^{-1}$; *3*, 155.1 sec.$^{-1}$; *4*, 117.35 sec.$^{-1}$; *5*, 78.56 sec.$^{-1}$.

tant, more salicylic acid is required to initiate the non-Newtonian flow and this amount increases proportionately (Fig. 6). However, the ratio of salicylic acid concentration to surfactant concentration at which non-Newtonian flow commences decreases with the surfactant concentration as shown in Table II. As the ratio is probably linked up with the degree of interaction it may mean that a smaller degree of interaction is necessary for the system to exhibit non-Newtonian flow in the presence of a high concentration of the surfactant. At a low concentration of surfactant where there are less molecules of the surfactant a greater degree of interaction is required to initiate the non-Newtonian flow. In the case of dodecyltrimethylammonium bromide the viscosity effect is less marked, and the change to non-Newtonian flow is therefore less obvious.

The amounts of salicylic acid that have to be added to the tetradecyltrimethylammonium bromide solutions to produce the non-Newtonian flow initially are slightly lower than for the corresponding cetyltrimethylammonium bromide solutions as seen in Fig. 6. Furthermore, the viscosity of a tetradecyltrimethylammonium bromide solution containing the same amount of salicylic acid as in a cetyltrimethylammonium bromide solution is much higher (Figs. 3, 4, and 5) indicating that the chain length of tetradecyltrimethylammonium bromide is probably optimally favored for interaction. The maxi-

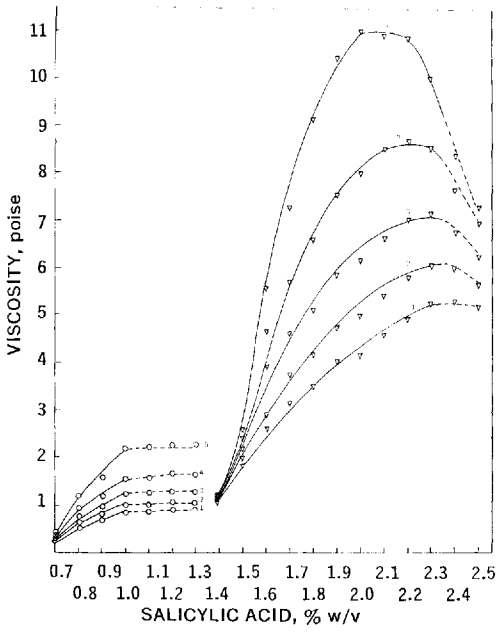

Fig. 5.—Effect of salicylic acid on the viscosity of cetyltrimethylammonium bromide solutions at 25°. Key: O, 2%; ▽, 6%; ——, below saturation; - - - -, above saturation. Shear rate: *1*, 234.6 sec.$^{-1}$; *2*, 195.9 sec.$^{-1}$; *3*, 155.1 sec.$^{-1}$; *4*, 117.35 sec.$^{-1}$; *5*, 78.56 sec.$^{-1}$.

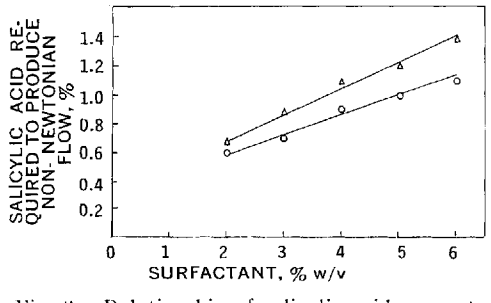

Fig. 6.—Relationship of salicylic acid concentration required to initiate non-Newtonian flow and surfactant concentration. Key: O, tetradecyltrimethylammonium bromide; △, cetyltrimethylammonium bromide.

Table II.—Ratios of Salicylic Acid Concentration to Surfactant Concentration at Which Non-Newtonian Flow is Initiated

Surfactant, %w/v	Salicylic Acid TTAB[a]	Salicylic Acid CTAB[b]
2.0	0.300	0.350
3.0	0.230	0.300
4.0	0.225	0.275
5.0	0.200	0.260
6.0	0.183	0.233

[a] TTAB, tetradecyltrimethylammonium bromide.
[b] CTAB, cetyltrimethylammonium bromide.

TABLE III.—pH OF SURFACTANT SOLUTIONS CONTAINING HYDROXYBENZOIC ACIDS

Salicylic Acid % in 2% DTAB[a]	pH	*m*-Hydroxybenzoic Acid % in 2% DTAB	pH	*p*-Hydroxybenzoic Acid % in 2% DTAB	pH
0.6	1.9	0.6	2.25	0.6	2.45
0.7	1.85	0.7	2.2	0.7	2.4
0.8	1.8	0.8	2.15	0.8	2.35
0.9	1.8	0.9	2.15	0.9	2.3
1.0	1.6	1.0	2.1	1.0	2.3
Salicylic Acid % in 2% TTAB[b]		*m*-Hydroxybenzoic Acid % in 2% TTAB		*p*-Hydroxybenzoic Acid % in 2% TTAB	
0.6	1.85	0.6	2.2	0.6	2.4
0.7	1.8	0.7	2.15	0.7	2.4
0.8	1.8	0.8	2.1	0.8	2.35
0.9	1.75	0.9	2.1	0.9	2.35
1.0	1.65	1.0	2.05	1.0	2.3
Salicylic Acid % in 2% CTAB[c]		*m*-Hydroxybenzoic Acid % in 2% CTAB		*p*-Hydroxybenzoic Acid % in 2% CTAB	
0.6	1.8	0.6	2.25	0.6	2.35
0.7	1.8	0.7	2.2	0.7	2.3
0.8	1.75	0.8	2.15	0.8	2.25
0.9	1.7	0.9	2.1	0.9	2.2
1.0	1.7	1.0	2.1	1.0	2.2

[a] DTAB, dodecyltrimethylammonium bromide. [b] TTAB, tetradecyltrimethylammonium bromide. [c] CTAB, cetyltrimethylammonium bromide.

mum viscosity of salicylic acid in the tetradecyltrimethyl and cetyltrimethylammonium bromide solutions occurs in about the same concentration due to the fact that the solubility of the acid in both the surfactants are practically identical. In dodecyltrimethylammonium bromide solutions containing salicylic acid this is not clear as the viscosity effect is not marked.

m-Hydroxybenzoic and *p*-hydroxybenzoic acids were included in the investigation, but both failed to show the viscosity effect observed with salicylic acid. The viscosities of both these acids remain unchanged when compared with the corresponding concentrations of surfactant in the absence of the acid. Benzoic acid and acetylsalicylic acid also failed to show any viscosity changes. The interaction of salicylic acid with the quaternary type of surfactants is highly specific as further investigations with the *ortho*, *meta*, and *para* substituted aminobenzoic, chlorobenzoic, and nitrobenzoic acids produced no viscosity change. In addition, salicylic acid did not exhibit this viscosity effect when dispersed with an anionic surfactant such as sodium lauryl sulfate or with a nonionic surfactant such as cetomacrogol 1000. The viscosities of dodecyltrimethylammonium, tetradecyltrimethylammonium, and cetyltrimethylammonium bromide solutions are independent of the concentration of the surfactant and practically independent of each other.

The viscosity effect is not related to pH. Table III shows the pH of surfactant solutions containing hydroxybenzoic acids. There is a small change in pH with increasing concentration of the acid. The viscosity of a surfactant solution whose pH is adjusted to that of a corresponding surfactant solution containing salicylic acid remains unaffected. This is true for all the three surfactants.

The mechanism of interaction is complicated and perhaps not yet fully understood. Suggestions for interactions of compounds with surfactants of the polyether type have been made by Higuchi and Lach (1) as due to the formation of molecular complexes and by Goodhart and Martin (5), Blaug and Ebersman (15), and Autian and Shaikh (17) as due to micellar solubilization. The results from this investigation indicate that the interaction of salicylic acid with surfactants of the quaternary type involves complex formation which leads to a marked change in viscosity and is only possible with the *ortho* substituted hydroxybenzoic acid while the *meta* and *para* compounds possess structures which are unfavorable to interaction. Besides, other attractive forces are likely to play a part as it is not only the *ortho* substituent that effects a viscosity change; a hydroxy group is essential, since the amino, chloro, and nitrobenzoic acids do not exhibit this viscosity effect.

The above-mentioned complexation is of importance to pharmaceutical formulation. It can be made to stabilize emulsified preparations such as creams, ointments containing the quaternary ammonium type of surfactants, and salicylic acid due to the high viscosity resulting from the interaction.

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Phosphorus–Nitrogen Compounds VI

Some Phenethylamine Derivatives

By L. A. CATES, W. H. LAWRENCE*, and R. J. MCCLAIN†

Eight diphenyl or diethyl phosphoramidates and one phenyl phosphorodiamidate containing phenethylamine moieties were synthesized for pharmacological testing. The derivatives were screened for their effect on the rabbit eye and isolated intestine, on dog blood pressure, and locomotor and anorexigenic activity in mice. The anorexigenic properties exhibited by some of these compounds may be related to their sedative effects. Three of the compounds inhibited spontaneous duodenal contractions at concentrations of 10 p.p.m. followed by tissue recovery. This antispasmodic effect was not reversed by acetylcholine or barium chloride.

THE EFFECT of *N*-substitution on the pharmacological activities of adrenergic amines has been extensively investigated and much of the work in this area has been directed toward determining the influence of various *N*-alkyl modifications on the cardiovascular and central nervous systems. Some attention has, however, been given to acyl derivatives of phenethylamines, principally in efforts to reduce cardiovascular side effects of the parent compound and to prolong the duration of adrenergic activity. One extensive synthetic program involving amides of dextroamphetamine and phenylpropanolamine resulted in the testing of an orally effective locomotor stimulant without significant cardiovascular effects (1). An attempt in protracting sympathomimetic activity is exemplified by the acetylation of epinephrine (2).

In view of these previous studies, the effect of phosphorylation on the pharmacological properties of phenethylamines was considered worthy of investigation and amphetamine, methamphetamine, ephedrine, phenylpropanolamine, and homoveratrylamine were selected as representative adrenergic agents which readily lend themselves to phosphorylation. Testing of compounds which have been previously reported in this series indicates that conversion of various amines to phosphoramidates reduces the biological activities of the parent compounds in that most of the derivatives possess low toxicities. The majority of *p*-toluidine (3), 2-aminopyridine (4), and guanidine and 2-aminopyrimidine (5) derivatives displayed little or no toxicity when administered to mice for 7 to 11 days at dosage levels of 100 to 500 mg./Kg./day.[1] These derivatives may be excreted unchanged and not undergo the desired *in vivo* enzymatic hydrolysis to liberate the physiologically active amines. To test this supposition, potent central nervous system stimulants were phosphorylated and the resulting phosphoramidates screened for their ability to increase locomotor activity in mice. Acute toxicity studies were made on all but one of the derivatives and most of the compounds were also screened for anorexigenic activity and their effects on the eye and isolated intestine. In addition, five of the derivatives were tested for their acute effects on blood pressure in the anesthetized dog.

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EXPERIMENTAL

Chemical Synthesis

Phenethylphosphoramidates (Table I, Compounds I–VIII).—Compounds I–VI were prepared by refluxing the appropriate amine (2 moles) and diethyl or diphenyl phosphorochloridate (1 mole) in anhydrous benzene for 1–4 hr.

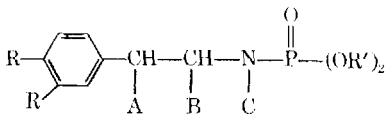
In compounds II–VI the resulting amine hydrochloride was removed by filtration and the filtrate spin-evaporated over a steam bath to yield an oil. The oil was washed with petroleum ether to give a white solid which was washed with dilute hydrochloric acid and water. The product was crystallized from ethanol (compound II) or petroleum ether (compounds III–VI).

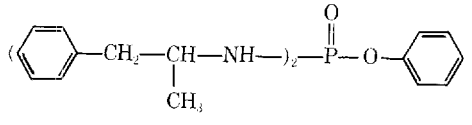
Compound I was isolated from the precipitate formed in the reaction mixture which was washed with water and crystallized from ethanol–water as the monohydrate. The ephedrine required for this synthesis was dried over alumina prior to its use in the reaction.

Reagent chloroform was employed as the reaction solvent for compounds VII and VIII. Following a reflux period of 8 hr. the phenylpropanolamine hydrochloride was removed by filtration and the filtrate was spin-evaporated to yield an oil. The oil was converted to a white solid when crystallized from ethanol–dilute hydrochloric acid. Hemihydrolysis occurred with the more labile aryl ester

[1] Testing results furnished by the Cancer Chemotherapy National Service Center, Bethesda, Md.

TABLE I.—PHENETHYLPHOSPHORAMIDATES



Compd.	R	A	B	C	R′	M.p.° C.[a]	Formula	Anal., % Calcd.	Found
I	H	OH	CH_3	CH_3	Phenyl	134–135	$C_{22}H_{24}NO_4P \cdot H_2O$	C, 63.6 H, 6.3 N, 3.4	63.8 6.3 3.4
II	H	H	CH_3	H	Phenyl	101–102	$C_{21}H_{22}NO_3P$	C, 68.6 H, 6.0 N, 3.8	68.3 5.9 3.7
III	H	H	CH_3	H	Ethyl	57–58	$C_{13}H_{22}NO_3P$	C, 57.5 H, 8.2 N, 5.2	57.0 8.6 4.9
IV	H	H	CH_3	CH_3	Phenyl	63–65	$C_{22}H_{24}NO_3P$	C, 69.3 H, 6.3 N, 3.7	69.4 6.4 3.5
V	CH_3O	H	H	H	Ethyl	63–64	$C_{14}H_{24}NO_5P$	C, 53.0 H, 7.6 N, 4.4	52.9 7.9 4.4
VI	CH_3O	H	H	H	Phenyl	88–89	$C_{22}H_{24}NO_5P$	C, 63.9 H, 5.8 N, 3.4	63.6 5.9 3.4
VII	H	OH	CH_3	H	Ethyl	81–83	$C_{13}H_{22}NO_4P$	C, 54.3 H, 7.7 N, 4.9	54.4 7.4 4.8
VIII	H	OH	CH_3	H	Phenyl hydroxyl	243–245 dec.	$C_{15}H_{18}NO_4P$	C, 58.6 H, 5.9 N, 4.6	58.1 6.1 4.6
IX						76–77	$C_{24}H_{29}N_2O_2P$	C, 70.6 H, 7.2 N, 6.9	70.3 7.2 6.7

[a] All melting points are uncorrected.

product (compound VIII) during this purification step as shown by its solubility in sodium hydroxide solution, high melting point, infrared spectra, and microanalysis. The phenylpropanolamine required in this synthesis was prepared by treatment of the hydrochloride salt with an equal molar amount of sodium hydroxide.

Phenethylphosphorodiamidate (Table I, Compound IX).—Dextroamphetamine (0.1 mole) and phenyl phosphorodichloridate (0.025 mole) were refluxed 12 hr. in anhydrous benzene. The reaction mixture was filtered and the filtrate spin-evaporated over a steam bath to yield a viscous oil. The oil was dissolved in ether and dried over anhydrous sodium sulfate. Spin-evaporation of the ethereal solution gave an oil which developed crystals after 3 days. Washing of the crystals with petroleum ether yielded phenyl-*N*,*N*′-bis-(1-methyl-2-phenylethyl)-phosphorodiamidate as a white, crystalline solid.

Infrared Spectra

All starting materials and products were examined by means of a Beckman IR-8 spectrophotometer using a Nujol mull. The new derivatives showed the following characteristic absorptions, γ in cm.$^{-1}$: 1238–1250 (P=O in compounds I–VIII), 1220 (P=O in compound IX), 1140–1160 (POEt in compounds III, V, and VII), and 1070–1100 and 1200 (POCaryl in compounds I, II, IV, VI, VIII, and IX).

Derivatives of primary amines showed the N–H stretching vibration of the intermolecularly hydrogen-bonded form at 3210–3260 cm.$^{-1}$ (6), while compounds I and IV gave no bands in this region. Compound VIII showed a weak absorption at 2550 cm.$^{-1}$ which can be assigned to P–OH (7).

Pharmacological Screening

The compounds were subjected to screening tests for anorexigenic properties; central nervous system effects, as indicated by spontaneous locomotor activity; toxicity; stimulation or inhibition of isolated rabbit intestine; and pupillary dilation or constriction, topical anesthesia, and irritating effects in the rabbit's eye. Five of the nine compounds were also tested for their acute effect on blood pressure in anesthetized dogs, when administered intravenously.

Anorexigenic Screening Test.—Food was withheld from mice for 20–24 hr. prior to the test but water was

provided *ad libitum*. Each test compound was administered in three dose levels, 4, 8, and 16 mg./Kg., and each dose level was given to a group of three mice of approximately equal weight. Appropriate dilutions were prepared so a consistent volume of solution was injected into each mouse (0.01 ml./Gm.).

Each mouse was weighed and injected intraperitoneally (i.p.) with the appropriate solution and placed back in its individual cage for 30 min., after which it was permitted free access to a food pellet for exactly 10 min. The food pellet was accurately weighed to one-tenth of a milligram (0.1 mg.) using a model H-6 Mettler balance, before and after the feeding period. Control mice were employed which received a similar volume of saline and others which received 0.5, 1, and 2 mg./Kg. of dextroamphetamine.

CNS Activity. Spontaneous Locomotor Activity, 24 hr.—Each mouse was injected i.p. with 0.01 ml./Gm. of 1% propylene glycol in saline and placed in an actophotometer (Metro Scientific) for 24 hr. The instrument was located in a windowless room free of external noise and a dim light was maintained during the test period. Each breaking of the beams of infrared light by the mouse was recorded by means of a digital counter. The mouse had free access to food and water during this time. The count obtained was used as the control for this particular mouse and each animal was used at 7-day intervals. The mouse received 5 mg./Kg. of the test compound i.p. and the activity was determined for 24 hr. under the same environmental conditions.

CNS Activity. Spontaneous Locomotor Activity, 5-min. Test.—The method used was essentially that of Dews (8) as modified by Moffett and Seay (9). Five mice of approximately equal weight comprised an experimental group. Each mouse was weighed, administered 4 mg./Kg. i.p. of the compound, and returned to his cage for 30 min. The animal was then placed in an actophotometer for exactly 5 min. Solutions were prepared so each mouse received 0.01 ml./Gm.

Irritancy, Local Anesthesia, and Pupillary Reactions in the Rabbit Eye.—These three tests were run simultaneously. All compounds tested were 1% solutions (w/v) in propylene glycol. Healthy rabbits were selected and, after their careful examination, 2 drops of the test solution were placed in one eye, and 2 drops of propylene glycol in the other eye as a control. The animals were observed at 10-min. intervals during the next hour for evidence of corneal anesthesia, pupillary dilation, or constriction and irritation.

Isolated Rabbit Intestine.—A piece of rabbit small intestine, about 2 to 3 cm. long, was suspended in aerated Tyrode's solution in a constant-temperature bath. The tissue was anchored at the bottom, and the top was attached *via* a lever system to a Grass force-displacement transducer. Contractions of the isolated intestine were recorded with a Grass model 7, ink-writing polygraph. Quantities of compounds administered are all expressed in terms of final concentrations of the derivative in Tyrode's solution.

Toxicity Studies.—Acute toxicity tests were conducted according to the method of Thompson and Weil (10, 11) for estimating the LD_{50}. Four mice were used for each dose level, and four dose levels were employed for each compound. The dose levels selected represent a geometric progression in that the logarithm of adjacent doses differed by a factor of 0.176. Solutions of the compounds were prepared to give the desired quantity when 0.01 ml./Gm. was injected. The mice were weighed, injected i.p., and observed for 1–4 hr. Following this they were returned to the animal room where another check was made at the end of 24 hr.

Our definition of "acute toxicity" includes any deaths which occur within 24 hr. after administration of the compound. Since few compounds exhibited significant toxicity within the 24-hr. period, observation of these animals was continued for an additional 6 days. No further administration was given. Any deaths which occurred during these additional 6 days is reported as subacute toxicity (7-day toxicity).

Blood Pressure Effects in Dogs.—Mongrel dogs were anesthetized with sodium pentobarbital, 30 mg./Kg., intravenously, and supplemented as needed to maintain the desired level of anesthesia. The carotid artery was cannulated and attached to a Statham pressure transducer, the femoral vein was cannulated for intravenous injections, and the trachea was cannulated to permit free air flow. Arterial pressure responses were recorded using a Grass model 7 polygraph.

RESULTS AND DISCUSSION

Anorexigenic Activity.—Since some of these compounds are derivatives of potent anorexigenic drugs, amphetamine and methamphetamine, it was deemed advisable to see if these agents would exhibit any effect on food consumption of mice. As noted (Table II), most of these compounds produced some decrease in food consumption during the feeding period. Food consumption was determined for each mouse during its 10-min. feeding period; this was converted to grams of food consumed per kilogram of body weight of the mouse for uniformity in comparing one animal, or group of animals, with another. Saline-treated animals served as controls in determining relative food consumption for the various treatments. Each value presented in Table II represents the mean response of three animals.

The inconsistency of these data is probably a reflection of the variability of response as seen in the small size of animal groups (three mice). The decreased food consumption by many of these animals was apparently due to varying degrees of hypoactivity and sedation of the animals which was noted

Table II.—Anorexigenic Activity

Compd.	Food Consumption, as % of Control (Control = 100%) 4 mg./Kg.	8 mg./Kg.	16 mg./Kg.
I	107.4	40.1	102.3
II	22.5	16.5	36.9
III	12.2	14.2	21.0
IV	3.2	3.8	3.3
V	63.7	95.4	78.0
VI	23.3	29.0	48.9
VII	104.4	120.1	24.7
VIII	64.6	69.2	77.1
IX	63.7	15.7	20.2
Dextroamphetamine[a]	51.9	54.0	17.9

[a] Dose levels of dextroamphetamine were 0.5, 1, and 2 mg./Kg., respectively.

TABLE III.—SPONTANEOUS LOCOMOTOR ACTIVITY

Compd.	5 min. Test[a] Drug, 4 mg./Kg.	Control (Saline)	% of Control[c]	t Values	24 hr. Test[b] Drug, 5 mg./Kg.	Control	% of Control[d]
I	75.6	142.2	53.2	3.437[e]	4908	9101	53.9
II	99.4	105.2	94.5	0.132	8507	17,382	48.9
III	41.8	105.2	39.7	2.065[f]	15,824	18,867	83.9
IV	112.4	105.2	106.8	0.226	8286	10,852	76.4
V	5.8	142.2	4.1	10.10[e]	12,866	10,513	122.4
VI	53.0	105.2	50.4	1.259	3963	6503	60.9
VII	106.0	105.2	100.8	0.040	16,877	18,867	89.5
VIII	110.0	105.2	104.6	0.141	...	...	...
IX	101.6	105.2	96.1	0.118	15,522	15,817	98.1

[a] Mean value of five animals. [b] Value for one animal. [c] Drug counts/control counts ×100. [d] Drug counts/control of same animal (1% propylene glycol in saline) ×100. [e] Significant at 5% level. [f] Significant at 10% level.

TABLE IV.—EFFECT ON ISOLATED RABBIT INTESTINE

Compd.	Concn.	Effect	Response to Acetylcholine (1:100,000)	Response to Barium Chloride (1:10,000)	Response to Adrenaline (1:1,000,000)
I	1:5000	Inhibition	NSR[a]	NSR	...
II	1:100,000	Inhibition	NSR	NSR	...
IV	1:100,000	Inhibition	NSR	NSR	...
V	1:10,000	Stimulation	...	...	Inhibition
VI	1:25,000	Inhibition	NSR	NSR	...
VII	1:10,000	NSR	Stimulation[b]	...	...
VIII	1:10,000	NSR	Stimulation[b]	...	...
IX	1:100,000	Inhibition	NSR	NSR	...

[a] No significant response. [b] Acetylcholine concentration = 1:1,000,000.

during the period of the experiment. In a few instances this was sufficient to cause the animal to lose its righting reflex. It was thought that the 5-min. locomotor activity tests would clarify this aspect, but the results of the actophotometer tests do not correlate well with the decreased food consumption.

Spontaneous Locomotor Activity.—Initially it was thought these compounds would probably increase locomotor activity, with a slow onset and long duration of effect. Consequently, spontaneous motor activity determinations were made over a 24-hr. observation period (Table III). As testing proceeded, it became obvious that at least some of the compounds exerted their action rather promptly; therefore, spontaneous motor activity was again determined 30 min. after intraperitoneal injection. This time the observation period was reduced to 5 min., thus minimizing acclimatization of the mouse to the environment of the actophotometer.

Since the 24-hr. test was discontinued in favor of a 5-min. test, the results presented represent the response of a mouse after receiving the designated compound and comparing this response to that obtained from the same mouse after receiving 0.01 ml./Gm. of 1% propylene glycol in saline.

The 5-min. locomotor activity test utilized groups of five animals for each compound and for the saline controls. The mean for each five-animal group was used to compare the counts for the experimental animals with the controls and to compute the per cent of control values. A comparison between the means of each series of five animals and its control was made by calculating Student t values. Two compounds (I and V) showed a decrease in locomotor activity which was significant at the 5% level and compound III gave a t value which would be significant at the 10% level. None of the compounds produced any statistically significant increase in such activity, which was somewhat unexpected, particularly for the amphetamine and methamphetamine derivatives.

Fig. 1.—Typical response of isolated small intestine to compounds I, II, IV, VI, and IX. Key: A, compound (in this case, VI, 1:25,000); B, acetylcholine br, 1:1,000,000; C, acetylcholine br, 1:250,000; D, acetylcholine br, 1:100,000.

Topical Anesthesia, Pupillary Reaction, and Irritation in the Rabbit's Eye.—No local anesthesia, pupillary dilation, or constriction was noted during the period of observation (1 hr.). The solvent used (propylene glycol) irritated the eyes, as indicated by redness of the cornea. The solutions of the compounds did not appear to produce any greater irritation than the solvent, although this was difficult to assess.

Isolated Rabbit Intestine.—Three of the eight compounds tested produced a marked decrease or complete inhibition of spontaneous activity and tonus of the isolated small intestine at a concentration of 1:100,000 (compounds II, IV, and IX). While a 1:1,000,000 concentration of acetylcholine was sufficient to produce spasm of the normal intestinal strip, following administration of each of these three compounds, concentrations of acetylcholine as great as 1:100,000 failed to produce any response (Table IV). A typical response of this type is shown

TABLE V.—ACUTE AND SUBACUTE TOXICITIES[a]

Compd.	Dose,[b] mg./Kg.	24 hr. 100	150	225	337	7 Day 100	150	225	337
I		0	2	3	2	2	2	3	2
II		0	0	0	0	1	2	1	2
IV		0	0	0	0	0	2	3	0
V		0	1	0	0	0	2	4	3
VI[c]		0	0	0	0	2	3	4	4
VII		1	0	0	0	1	0	0	0
VIII[d]		0	0	2	3	0	0	3	4
IX		1	0	0	0	1	0	0	0
Propylene glycol, 0.01 ml./Gm.		0	0	0	0	0	0	0	0

[a] Number of deaths out of four animals. [b] mg./Kg. in propylene glycol given i.p. [c] Dose levels = 66.7, 100, 150, and 225 mg./Kg. [d] In propylene glycol/NaOH. Test of this solvent system without compound VI resulted in three deaths in 24 hr. and four deaths in 7 days.

in Fig. 1. In addition, barium chloride in concentrations up to 1:10,000 failed to contract the isolated intestine following these drugs. Thus, these compounds relaxed the intestinal strip and prevented it from responding to the neurogenic stimulation of acetylcholine and the myogenic stimulation of barium chloride.

Two other compounds (I and VI) produced a similar response but a higher concentration of the compound was required for this effect (1:25,000 for VI; 1:5000 for I).

One compound (V) produced stimulation of the isolated gut at a concentration of 1:10,000. This stimulation was counteracted by 1:1,000,000 adrenaline, and prevented by prior addition of 1:1,000,000 atropine.

The other two compounds (VII and VIII) did not produce any significant response upon the intestine in concentrations up to 1:10,000.

Three diethyl phosphoramidates were synthesized in order to give an indication of what different effect, if any, an alkyl ester produces compared to an aryl ester. One obvious difference in properties is the much greater water solubility exhibited by the ethyl esters. The pharmacological activity most affected by this difference in solubility appears to be that on the isolated gut. It is noted that the four compounds (II, IV, VI, and IX) most active as antispasmodics were insoluble in water, while only one water-soluble compound (I) exhibited this activity, and quantitatively it was much weaker than the others, and another water-soluble compound (V) produced intestinal stimulation. It is also interesting to note that compound VI, which produced inhibition of the intestine is the diphenyl phosphate derivative of homoveratrylamine, while compound V, which produced stimulation of the intestine, is the diethyl phosphate derivative of homoveratrylamine.

Toxicity Studies.—These tests indicate that most of the compounds are much less toxic when considered in terms of acute toxicity (24 hr. or less) than when considered in terms of subacute toxicity (7 days or less). Thus, the data suggest that most of the compounds exert their toxic effect indirectly by adversely affecting a vital organ or function (*e.g.*, possibly renal or hepatic function) or else they are retained in the body and slowly converted into some more toxic substance. No attempt was made to determine the cause of death for the mice that succumbed to these compounds. This pattern of delayed death is readily illustrated by compounds V and VI (Table V). Only one of the 16 mice which received V died within 24 hr., however, by the end of the 7th day, nine of the 16 mice were dead; compound VI produced no deaths during the first 24 hr., however, by the end of the 7th day, 13 of the 16 mice which had received the drug were dead.

Fig. 2.—Effect on arterial blood pressure of anesthetized dog from slow intravenous injection of 1.6 mg./Kg. of compound I.

Some of the other compounds did not exhibit significant toxicity at the doses utilized. Compounds VII and IX produced only one fatality each during the 7-day period, and the rapidity of death in both cases (less than 4 hr.), and the fact that both deaths were from the lowest dose, would lead one to suspect something, other than drug toxicity, as a possible cause of these deaths. Two groups of four mice each were administered an equivalent volume of propylene glycol; none of these eight mice died within the 7-day observation period.

Compound VIII presents a different problem. It is not soluble in water or propylene glycol but does form the water-soluble sodium salt. Sodium hydroxide was used to solubilize the compound; the result was seven deaths out of the 16 injected. The same quantity of sodium hydroxide as used to solubilize VIII was added to propylene glycol and an equivalent volume of this sodium hydroxide-propylene glycol solution was injected into four mice; three of these died within 24 hr. and the other died before the 7th day. This would represent the sodium hydroxide concentration in the strongest solution (337 mg./Kg.), but since the solutions for the lower doses were prepared by diluting some of this solution with propylene glycol, the lower dose levels contained correspondingly less caustic.

Blood Pressure in Anesthetized Dogs.—Five of

the nine compounds were administered intravenously to anesthetized dogs to determine their effect on systemic arterial pressure. Compounds I, III, IV, V, and VII were administered in various dosage schedules ranging from 0.5 to 10.0 mg./Kg. Only compound I produced a marked change in blood pressure. Figure 2 shows the alteration in carotid arterial pressure produced by 1.6 mg./Kg. of compound I. It will be noted that there is an increase in both systolic and diastolic pressure, however, systolic pressure increased more than diastolic, thus increasing the pulse pressure too.

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A Potent α-Receptor Blocking Agent, SU-14542

By T. F. BURKS* and J. P. LONG

SU-14542 was studied in isolated cat and rabbit hearts and in *in situ* dog hearts for possible β-adrenergic receptor stimulating activity. It was determined that this compound has no demonstrable action on β receptors. Investigation of its effects on isolated mesenteric arteries demonstrated powerful α-adrenergic receptor blockade; SU-14542 was found to be 2–7 times more potent than phentolamine as an α-receptor blocking agent. The ID_{50} for SU-14542 in blocking epinephrine was determined to be $6.6 \times 10^{-9} M$.

IT HAS BEEN reported by Barrett *et al.* (1) that SU-14542, which is 3′-methoxy-4′ [(4-phenyl-1-piperazinyl)-butoxy]-acetophenone monohydrochloride, decreases arterial blood pressure in both anesthetized normotensive dogs and unanesthetized renal hypertensive dogs. Decreases in pressor responses produced by epinephrine, norepinephrine, and amphetamine were observed following oral administration of 0.20 and 1.80 mg./Kg. of SU-14542. These workers reported that the experimental compound does not possess ganglionic blocking activity but, since tachycardia was observed following administration of SU-14542, that it does possess β-adrenergic receptor stimulating activity.

Povalski *et al.* (2) reported that while 5.0 mg./Kg. SU-14542 given orally to anesthetized dogs produced a decrease in mean arterial blood pressure, cardiac output was not significantly altered. Rutledge *et al.* (3) found that SU-14542 increased femoral arterial blood flow in dogs but did not significantly increase renal blood flow.

In this communication evidence will be presented demonstrating that SU-14542 is a potent α-adrenergic receptor blocking agent that has no β-adrenergic receptor stimulating properties on dog, cat, or rabbit hearts.

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EXPERIMENTAL

Cat and Rabbit Hearts.—Dutch rabbits of either sex, weighing from 1.5–2 Kg. were sacrificed by cervical dislocation, and the beating hearts were removed and flushed through the aorta with a heparin–saline solution. An aortic cannula was tied into place and the hearts perfused in the usual Langendorf preparation (4) with Locke Ringer solution for isolated hearts warmed to 35–37° and aerated by bubbling 95% O_2–5% CO_2. Aortic pressure was maintained at 40–50 mm. Hg to ensure adequate coronary perfusion. Drugs were injected in 0.5–1 ml. vol. into the aortic cannula. Force of contraction was measured from a Grass Instrument Co. force - displacement transducer (FTO3C) and recorded on a Gilson (GME) polygraph. Heart rates were obtained by direct observation of recorder pen movement.

Cat hearts were prepared in a similar manner from cats of either sex weighing 1.5–3 Kg., anesthetized by intrathoracic administration of 30 mg./Kg. sodium pentobarbital.

Dog Hearts.—Mongrel dogs of either sex, weighing 10–12 Kg., were anesthesized by 15 mg./Kg. sodium thiopental and 250 mg./Kg. sodium barbital administered intravenously. Systemic blood pressure was measured from a carotid artery cannula by a Statham pressure transducer (P23AA) and recorded by an Offner type RS Dynograph. Drugs, in volumes of 0.1–1 ml., were injected through a cannula placed in a femoral vein. Both vagus nerves were sectioned in the cervical region and each animal was administered 20 mg./Kg. hexamethon-

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TABLE I.—EFFECTS OF SU-14542 AND EPINEPHRINE ON HEARTS (MEANS ± S.E.)

Species	N[a]	Rate/min. Control	Epi[b]	P	Contractile Force, g. Control	SU[c]	P[d]	Control	Epi[b]	P	Control	SU[c]	P[d]
Rabbit	5	168 ± 14	202 ± 16	<0.01	164 ± 13	148 ± 11	<0.01	7 ± 0.9	14 ± 1.1	<0.01	7 ± 1.0	4 ± 1.0	<0.01
Cat	5	90 ± 8	138 ± 7	<0.01	94 ± 15	77 ± 12	<0.05	9 ± 1.6	24 ± 3.7	<0.01	8 ± 1.3	5 ± 1.5	<0.01
Dog	5	108 ± 7	144 ± 11	<0.01	106 ± 6	104 ± 5	N.S.[e]	123 ± 16	267 ± 47	<0.01	118 ± 18	111 ± 12	N.S.[e]

[a] N, number of animals used per experiment. [b] Epinephrine, 1 mcg./dose in cat and rabbit hearts; 1 mcg./Kg. in dogs. [c] SU-14542, 100 mcg./dose in cat and rabbit hearts; 1.0 mg./Kg. in dogs. [d] Analysis for decrease in response. [e] Not statistically significant.

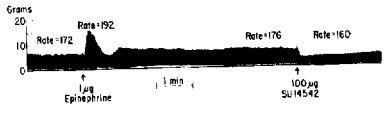

Fig. 1.—Effects of the standard β-receptor stimulant, epinephrine, and SU-14542 on rate and force of contraction of isolated rabbit heart. Note that epinephrine produced increases in both contractile force and rate. SU-14542 decreased both contractile force and rate.

ium bromide. The heart was exposed by a midline thoracic incision, the pericardium opened, and a Walton-Brodie strain gauge arch sewn directly on the myocardium. Contractile force was recorded by the Offner Dynograph. Heart rates were obtained by direct observation of recorder pen deflection.

Isolated Mensenteric Arteries.—Isolated arteries were prepared in the manner described by Rogers *et al.* (5). Dogs of either sex were anesthetized as above and the small intestine exposed *via* midline abdominal incision. Mesenteric arteries with branching fans of smaller resistance arteries and periarterial sympathetic nerves were removed from the animals and mounted in an organ bath where they were perfused with Krebs bicarbonate solution. The Krebs solution was aerated with 95% O_2–5% CO_2 and maintained at 37°. The arterial segments were immersed in a 150-ml. recirculating bath and perfused by use of a Sigmamotor model T-8 peristaltic infusion pump. Since flow was held constant, changes in perfusion pressure were directly proportional to changes in arterial resistance. Perfusion pressure was measured from a T tube between the pump and the artery by a Statham pressure transducer (P23AA) and recorded on an Offner Dynograph.

Stimulation of the periarterial sympathetic nerves was accomplished with a Grass Instrument Co. model S4 stimulator. Parameters of stimulation were within the following ranges: frequency 20–30 c.p.s., duration 5–20 msec., and at 6–15 v. for 1–10 sec. Epinephrine and norepinephrine dissolved in purified water were injected into the arterial cannula in volumes of 0.01–0.05 ml. and the blocking agents were injected directly into the bath fluid. Several test doses of the agonists were given and several stimulations performed until repeated challenge produced reproducible responses between 50–100 mm. Hg above the baseline pressure which was maintained at 80–120 mm. Hg.

After establishment of control responses, the antagonists were added to the bath and the agonists reapplied; this was repeated for a second dose of antagonists.

Chemicals.—Chemicals employed were *l*-epinephrine HCl, *l*-norepinephrine HCl (calculated as the base), phentolamine HCl, SU-14542, and hexamethionium bromide.

The isolated heart experiments were so designed that each heart served as its own control and the data were analyzed with the Student t test, paired comparisons (6). The data from the isolated arteries were analyzed by analysis of variance and a 2 × 2 parallel line bioassay (7). ID_{50} doses for the isolated arteries were calculated by the method of Litchfield and Wilcoxon (8). In all experiments a P value equal to or less than 0.05 was considered significant.

RESULTS

Hearts.—As can be seen from perusal of Table I, administration of epinephrine resulted in increases in both rate and force of contraction of the hearts in all species investigated. This is of course consistant with its well-known ability to stimulate β-adrenergic receptors. SU-14542 produced decreases in rate and/or force of contraction (Fig. 1). The effects of SU-14542 on the hearts were dose-related and doses ranging from 1–300 mcg. were applied to the cat and rabbit hearts; a 100-mcg. dose was chosen for the study. In the dogs, doses from 0.01–5.0 mg./Kg. were employed and 1.0 mg./Kg. was chosen for the study, this being a dose comparable to those used by other workers. In one instance a dog was prepared as described above except the vagi were left intact and hexamethonium was not administered. In this case, administration of 1.0 mg./Kg. SU-14542 resulted in increases in both heart rate and contractile force, the onset corresponding to the fall in arterial blood pressure. It was also interesting to note that when epinephrine was administered to the isolated hearts after SU-14542 there was no indication of β-receptor blockade when the response was compared to that produced by epinephrine before SU-14542.

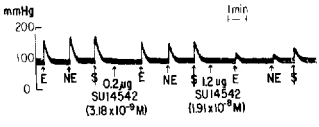

Fig. 2.—Demonstration of α-receptor blocking action of SU-14542. The stimuli applied were epinephrine (E), 0.1 mcg.; norepinephrine (NE), 0.2 mcg.; and sympathetic nerve stimulation (S with an arrow) 25 c.p.s., 5 msec./pulse, 10 v. for 3 sec. The mcg. doses and M concentrations of SU-14542 represent total bath concentration.

TABLE II.—EFFECTS OF SU-14542 AND PHENTOLAMINE ON ISOLATED MESENTERIC ARTERIES, INCREASE IN PERFUSION PRESSURE, mm. Hg

	N^a	Mean Response: Control	Low Dose[b]	High Dose[c]	R[d]
Epinephrine: phentolamine	8	74	56	14	...
SU-14542	8	59	45	8	2.27 (2.05–2.50)
Norepinephrine: phentolamine	8	74	54	10	...
SU-14542	8	63	30	6	2.40 (2.07–2.77)
Nerve stimulator: phentolamine	8	56	42	37	...
SU-14542	8	73	54	24	7.02 (6.34–7.84)

[a] N, number of animals per experiment. [b] Low dose of phentolamine was $8.41 \times 10^{-9}M$; SU-14542 was $3.18 \times 10^{-9}M$. [c] High doses (representing total concentration): phentolamine was $5.05 \times 10^{-8}M$; SU-14542 was $1.91 \times 10^{-8}M$. [d] Potency ratio (standard substance was phentolamine) with 95% fiducial limits as determined by parallel line bioassay.

TABLE III.—ANALYSIS OF VARIANCE–SU-14542 AND PHENTOLAMINE *Versus* EPINEPHRINE (RANDOMIZED COMPLETE BLOCK DESIGN)

Source of Variation	d.f.[a]	SS[b]	MS[c]	*F*[d]	*Pe*
Preparation	1	95	95	<1	NS[f]
Regression	1	12,207	12,207	89.10	<0.01
Parallelism	1	63	63	<1	NS[f]
Treatments (doses)	(3)	(12,364)	...	...	...
Animals (blocks)	7	2,711	387	2.82	<0.1 >0.05
Error	21	2,868	137	...	...
Total	31	17,943			

[a] Degrees of freedom. [b] Sum of squares. [c] Mean square. [d] F ratio. [e] Level of significance. [f] Not statistically significant.

Isolated Arteries.—SU-14542 was found to be a potent α-adrenergic receptor blocking agent, being some 2–7 times more potent than phentolamine (Table II). Preliminary experiments determined that $3.18 \times 10^{-9}M$ and $1.91 \times 10^{-8}M$ concentrations of SU-14542 were approximately comparable to $8.41 \times 10^{-9}M$ and $5.05 \times 10^{-8}M$ concentrations of phentolamine in blocking the responses to epinephrine and norepinephrine. It was also determined that the dose-response curve to SU-14542 was relatively flat, the optimal log interval between the high and low doses was found to be 0.8 (Fig. 2). In later experiments SU-14542 was determined to be some 7 times more potent than phentolamine in antagonizing responses to sympathetic nerve stimulation. As was expected, and as can be seen from Fig. 2 and Table II, both α-receptor blocking agents were more effective antagonists of exogenously administered catecholamines than in antagonizing sympathetic nerve stimulation.

Median inhibitory doses (ID_{50}) calculated for each of these α-receptor blocking agents against epinephrine with their 95% confidence limits were as follows: SU-14542, $6.6 \times 10^{-9}M$ ($2.3–19.1 \times 10^{-9}M$); phentolamine, $16.5 \times 10^{-9}M$ ($5.9–46.2 \times 10^{-9}M$).

Analysis of variance and parallel line bioassay provided evidence for good regression lines for each agent at the 0.8 log interval of doses, no deviation from parallelism and good matching of doses. A typical table of analysis of variance is provided in Table III.

DISCUSSION

From the data presented it may be concluded that SU-14542 is a potent antagonist of the α-adrenergic receptor stimulation resulting either from addition of exogenous catecholamines or from stimulation of sympathetic nerves in isolated mesenteric arteries. SU-14542 was determined to be some 2–7 times more effective than phentolamine as an α-receptor blocking agent, and, therefore, could well find use in experimental and perhaps in clinical situations where the use of such a property might be indicated. The relatively flat dose-response curve demonstrated for this agent may be beneficial to its employment in diverse circumstances.

It is evident, however, that this compound is not a stimulant of the β-receptors of cat, rabbit, or dog hearts. It is suggested that the heart stimulation observed by other investigators following administration of SU-14542 was mediated reflexly as compensation for the fall in arterial blood pressure resulting from the intense α-receptor blockade produced by this compound. The single experiment described above in which a dog with intact cardiovascular reflexes responded to SU-14542 by heart stimulation may be contrasted to the other animals with reflexes blocked by vagatomy and hexamethonium that failed to show any such heart stimulation.

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Comparison of an Immunoassay and the U.S.P. Bioassay for Determining Potency of Extracted Insulin

By G. W. PROBST, W. F. BROWN, and H. J. HENRY

An immunoassay procedure is described for determining the potency of extracted insulin. The method, based on salt precipitation for the separation of antibody bound and free insulins originally described by Grodsky and Forsham, was modified and simplified to obtain greater precision and reliability of results. Repetitive immunoassay results, conducted on different days with pork, beef, and mixed source zinc insulins, commercial insulin products, and insulin preparations from different stages of manufacture, were compared with the corresponding U.S.P. bioassay results. Statistical analysis of the immunoassay data revealed a confidence coefficient of ±11.2 per cent ($p = 0.95$) for a single immunoassay. This value is within the limits established by the U.S.P. for insulin potency bioassay. A desired variation of ±5–6 per cent is obtained by statistical combination of several bioassay results. Similar variation is achieved by repeating the immunoassay on 4 different days. An analysis of variance indicated that the immunoassay, under the conditions employed, is not subject to variations due to species specific insulins if the antigen used to produce antiserum is a mixed species insulin. The beef and pork zinc insulins, originally selected to serve as standards for immunoassay, were found to have a significantly different potency by immunoassay when compared to the potency established by bioassay. In order to establish an immunoassay secondary reference standard equivalent to the U.S.P. reference standard, the reaction of three lots of pooled insulin was determined repetitively at all standard curve values. Statistical analysis by least squares, of the data obtained at 20, 30, and 40 milliunits/ml. (munits/ml.) insulin concentrations, yielded a calculated common slope which lies within the computed individual slope confidence interval. These data further support the validity of the immunoassay in establishing the potency of extracted insulin and was given credence by comparing immunoassay and bioassay results obtained on large manufactured lots of crystalline zinc insulins.

FOR MORE than two decades the potency of extracted insulin has been determined by the official U.S.P. bioassay (1). Like all bioassays, the "twin crossover" rabbit blood sugar-lowering assay, used as the official method for determining insulin potency, is beset with many shortcomings. These include inherent biological variation, high cost of facilities, considerable time consumption, and lack of sensitivity. The development of specific and sensitive immunological assays (2–6) provided rapid, accurate, and economic methods for the quantitative determination of insulin. These methods are based on isotope dilution as a quantitative index for measuring the competitive reaction of labeled and unlabeled insulins for specific antibody. The separation of the antigen–antibody complex from the free antigen in the immunological reaction is achieved by different means in these reported methods. A sensitivity great enough for measuring microunit quantities of insulin found in plasma and serum is a cardinal feature of the immunoassay. However, for control of insulin development and production, milliunit sensitivity is satisfactory. In the present study, the immunoassay method of Grodsky and Forsham (3) as modified by Baum *et al.* (7) was further explored and appropriately modified to determine the best conditions for reproducible results. Immunoassays possess the potential capability of determining insulin with an accuracy equivalent to that obtained with the bioassay. Although the immunoassay and bioassay measure quite different properties of the insulin molecule, attainment of analytical equivalence makes the immunoassay attractive for establishing insulin potency followed, when indicated, by a simplified animal response as a means of confirming the hormonal activity of extracted insulin.

Experiments were designed to determine the precision of the immunoassay under specifically defined conditions, utilizing a series of insulin preparations. The results of the immunoassay were compared with the results of the U.S.P. bioassay for these same insulin samples. Statistical analysis of the results reveal that the immunoassay exhibits precision and reliability for establishing insulin potency well within the confidence limits for computed potency defined in the U.S.P.

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MATERIALS AND METHODS

Antibody Production.—Mixed source insulin (75% beef crystalline zinc insulin and 25% pork crystalline

zinc insulin, by weight) is used as the antigen in the production of antibody used in routine analysis.

The antigen is prepared as follows. To a sterilized 250-ml. (Virtis 45) homogenizer cup add 250 mg. of heat killed *Mycobacterium tuberculosis* (BP008), cells (Biological Production, Eli Lilly and Co.), 50 Gm. of a mixture of 45 Gm. mineral oil[1] plus 5 Gm. sorbitan ester,[2] and 0.23 ml. of liquefied phenol. Sterilize the mixture of mineral oil and the sorbitan ester separately and weigh directly into the homogenizing cup. Add 50 ml., 80 units/ml., of mixed source insulin solution and homogenize the mixture for 5 min. at full speed. Prepare 80 units/ml. of insulin by weighing an appropriate mixed beef-pork insulin (potency previously established by U.S.P. bioassay) and dissolve the solid in insulin diluent (1).

Use the resulting emulsion of "complete" antigen immediately for immunization. If injection delay is encountered, repeat the homogenizing step. A "modified" antigen is also prepared using 40 units/ml. of mixed source insulin in the same manner but without the heat killed *M. tuberculosis* cells. Smaller or larger quantities of the emulsion can be prepared using proportional quantities of the components in the mixture.

Inject mongrel guinea pigs (500–600 Gm. each) subcutaneously with 1.0 ml. of the "complete" antigen divided into 0.2-ml. increments at five hind quarter sites on the initial day of immunization. Repeat same treatment with "complete" antigen on the 15th day. On the 30th day of the immunization regimen and every 30 days, thereafter, further stimulate the animals with a 0.5 ml. intraperitoneal injection of the "modified" antigen. Seven days after antigenic stimulation; namely, the 37th, 67th, 97th, etc., day of the immunization regimen, recover 10 ml. of blood from each animal by cardiac puncture. Use clear antiserum, obtained in the conventional manner, immediately for determining insulin antibody titer or freeze and store at −25° for future antibody determination.

The presence of antibody in the individual guinea pig antisera is readily detected by substituting 100 μl. of 160 munits/ml. insulin standard, 20 μl. of antiserum and 1.0 ml. of immuno-diluent into the standard immunoassay procedure. An antiserum is considered to have suitable antibody concentration if 50% or more of the labeled insulin in the system is bound. Those antisera exhibiting suitable antibody concentration are pooled and freeze-dried. This freeze-dried antiserum is stored at −25° indefinitely and serves as a uniform source of antibody for extended periods of routine analysis.

Prior to use in the immunoassay, a given lot of freeze-dried antiserum is carefully titered against insulin concentrations (10–50 munits/ml. range) chosen from the standard reference curve at three selected antibody concentrations. Experience indicates that antisera, selected on the basis of the detection assay, should be diluted in a range of 1:1000–1:2000 to provide a linear relationship when per cent radioactivity remaining in the supernatant liquid is plotted as a log function of insulin concentration, as shown in Fig. 1. In this manner, a five-point reference curve is prepared for each antibody concentration. A visual inspection of the linearity of the three resulting curves permits a selection of the proper antibody concentration to be used in the immunoassay system in the range of the selected insulin concentrations.

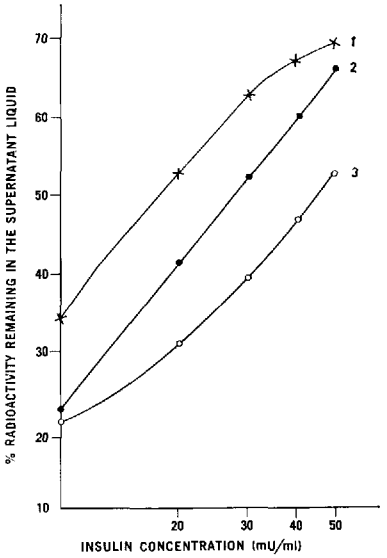

Fig. 1.—A typical insulin antibody titer determination and standard reference curve obtained with pork zinc insulin (lot PJ-5682) and guinea pig antiserum. Key: curve 1, antiserum diluted 1:1000 (1.0 μl./ml.), exhibits the effect of excess antibody; curve 2, a typical standard reference curve for insulin immunoassay is obtained with antiserum diluted 1:1500 (0.67 μl./ml.); curve 3, antiserum diluted 1:2000 (0.50 μl./ml.), shows the effect of insufficient antibody in the immunological reaction.

Due to inherent biological variation in antibody production from individual animals, the results obtained in this type antibody titer determination may indicate a repetition of the determination at higher or lower antibody concentrations. In addition to determining antibody titer, this assay adjustment system is also used to check new lots of human serum albumin, γ-globulin, or other reagents and standards.

REAGENTS

Immuno-Diluent.—*0.25% Serum Albumin-Borate Buffer.*— The immuno-diluent (pH 8.2, 0.1 ionic strength) is prepared with 6.18 Gm. of boric acid, 5.00 Gm. of sodium tetraborate, 4.62 Gm. of sodium chloride, and 10 ml. of 25% salt-poor human serum albumin (Cutter Laboratories) per liter. Stock of 10–20 L. can be prepared and stored at room temperature for 2 months.

Urea–γ-Globulin Reagent.—Urea solution (17%) containing 0.1% bovine γ-globulin (Cohn, fraction II) is prepared by dissolving 510 Gm. of urea, 3.0 Gm. of bovine γ-globulin, and diluting to 3 L. with immuno-diluent. The solution is adjusted to pH 8.2 with 5.0 *N* HCl. The reagent is stored under refrigeration (5°) when not in use and is stable for 6 weeks.

[1] Marketed as Drakseol-6VR by Pennsylvania Refining Co., Butler, Pa.
[2] Marketed as Arlacel A by Atlas Chemical Industries, Inc., Wilmington, Del.

Labeled Insulin.—Insulin labeled with ^{131}I or ^{125}I is satisfactory as a tracer in the immunoassay. ^{131}I insulin is obtained from Abbott Laboratories with a specific activity of 250–300 mc./mg. The concentrate, supplied in 1% human serum albumin with glycine buffer at pH 8.2, is diluted for use with immuno-diluent to 0.266 μc./ml. ^{125}I insulin is prepared at Eli Lilly and Co. with a specific activity of 6–12 mc./mg. and is diluted for use with immuno-diluent to 0.133 μc./ml. The diluted ^{125}I insulin is portioned into 25-ml. aliquots. A fresh aliquot of labeled insulin is used each week, the remainder is stored at −25°. A lot of ^{131}I insulin can be used for a 2-week period and then must be replaced with a freshly prepared material. On the other hand, a lot of ^{125}I insulin,[3] when frozen in aliquots as described, can be used for 3 months. The weekly supply of ^{125}I insulin is stored under refrigeration (5°) when not in use.

Insulin Standards.—Two reference standards used in the present study were selected at random; namely, pork zinc insulin, lot No. PJ-5682 (U.S.P. bioassay: 23.9 unit/mg. ±5.51%) and beef zinc insulin, lot No. 836550 (U.S.P. bioassay: 25.4 units/mg. ±5.59%). Fifty units/ml. stock solution of the reference standards are prepared in insulin diluent and carefully diluted (two steps) to 0.25 units/ml. with insulin diluent. In the final dilution of the 0.25 unit/ml. solution to 10–50 munits/ml. solutions used for the preparation of the standard reference curve, immuno-diluent is used for the dilutions. The mixed source insulin reference is prepared by mixing the 50 units/ml. stock reference standards in the ratio of 25% pork, 75% beef zinc insulin, and appropriately diluting the mixed stock for use. Fresh reference standards are prepared weekly.

Protein Precipitant.—A 1.25 *M* sodium citrate dihydrate solution is prepared by dissolving 367.6 Gm. in distilled water and diluting to 1 L. It is essential that analytical reagent grade sodium citrate be used in the preparation of this salt solution.

INSULIN IMMUNOASSAY METHOD

Sample Preparation.—In order to minimize volumetric error, samples are carefully diluted with conventional laboratory pipets and volumetric flasks; or, micro dilutions are prepared by employing a syringe microburet (Micro-metric Instruments Co.). If micro dilutions are utilized in sample preparation, volumetric error is avoided if the delivery volume from the syringe microburet is not less than 50 μl. with highly concentrated insulin solutions. The first step dilution is performed to about 50 units/ml. using insulin diluent. The final dilutions are made with immuno-diluent. After the final dilution, the samples are conveniently stored overnight at 5°.

Assay Procedure.—Clean all glassware with detergent (Alconox), rinse with 2% (w/v) HCl, then deionized water, and dry in an oven before use. Number a set of seventy-two 10-ml. conical flasks, 12-ml. double strength centrifuge tubes, and plastic counting tubes in sequence. Add 100 μl. of 0.133 μc./ml. ^{125}I insulin (by syringe microburet) and 1.8 ml. urea–γ-globulin reagent to all flasks. Flasks 1 and 2 serve as control blanks for labeled insulin; add 1.10 ml. immuno-diluent to each in place of the sample and antibody. Flask 3 (reference zero) serves as a guide in measuring the binding capacity of the antibody with the labeled insulin. It contains 100 μl. of immuno-diluent in place of the sample. The insulin reference standard (flasks 4–18) and the samples (flasks 19–72) are run in triplicate. Using the syringe microburet, deliver 100-μl. aliquots of each of the insulin reference standards (10, 20, 30, 40, and 50 munits/ml., respectively) and aliquots of the unknown samples into the appropriate flask. Initiate the immunological reaction by the sequential addition of 1.0 ml. of antibody solution (flasks 3–72). Momentarily agitate the mixture by hand and continue gentle agitation on a rotary shaker (A. H. Thomas No. 3623) for 15 min. at room temperature. Commence all timing sequences after additions are made to the last flask. Precipitate the antigen–antibody complex (bound form) by adding 6.0 ml. of 1.25 *M* sodium citrate into each flask. Again agitate this mixture on the rotary shaker for 15 min., then transfer the contents to the appropriate numbered centrifuge tube, and centrifuge in a refrigerated angle head centrifuge at 2400 × g for 45 min. at 15°.

Following centrifugation, carefully remove 5 ml. of the supernatant liquid and transfer the liquid to the appropriately numbered plastic tube.

Radioactivity Measurement.—Measure the radioactivity of the sample in the plastic tube in an automatic gamma spectrometer (model 410A, Packard Instrument Co.) precalibrated for a peak emission of ^{125}I or ^{131}I. The counting efficiency of the instrument is measured with a γ-ray emission reference source (^{133}Ba) lot No. B-508 (Abbott Laboratories).

Prepare a radioisotope control tube for monitoring a given lot of labeled insulin during the entire period of time that the particular lot is used for routine assay. Dilute a 100-μl. aliquot of diluted labeled insulin (0.133 μc./ml.) with 8.9 ml. of immuno-diluent and transfer a 5-ml. aliquot of this mixture to a plastic tube. Tightly seal the tube with a rubber stopper and tape.

The above control tube is followed by an empty plastic tube used to measure background radiation. Counting time is usually 5 min./tube. A radioactivity concentration is selected such that the 5-min. count of the "reference zero" tube is never less than 5000.

Calculation of Potency.—By convention, correct the individual tube counts for background and decay. Calculate the amount of radioactivity remaining in the supernatant liquid and express as per cent (average corrected sample count × 100 divided by average corrected blank count). Plot a five-point curve of the standard reference insulin concentration, 10, 20, 30, 40, and 50 munits/ml., respectively, on semilog graph paper. Curve 2, Fig. 1, represents a typical standard response curve exhibited by a plot of these concentrations. Sample concentration is estimated from this graph or calculated on a point-to-point linear assumption as a logarithmic function of the insulin concentration. For greater reliability, the calculation is limited to three center standard reference points; namely, 20, 30, and 40 munits/ml. of insulin. For sample values (per cent radioactivity in the supernatant) equal to

[3] ^{125}I insulin was prepared by Dr. R. E. Crabtree, Analytical Research, Eli Lilly and Co., Indianapolis, Ind.

or less than the midpoint of the standard curve, the following equations are used to calculate insulin concentration in the sample. (Assuming 20, 30, and 40 munits/ml. = standard curve.)

$$\text{slope} = \log 30 - \log 20/(\%\text{ at } 30) - (\%\text{ at } 20)$$
$$\log \text{sample} = \log 30 - (\text{slope})(\%\text{ at } 30 - \%\text{ sample})$$
$$\text{antilog sample} = \text{sample concentration}$$
$$\%\text{ estimate} = (\text{sample concentration/midpoint standard concentration}) \times 100$$
$$(\%\text{ estimate})(\text{estimate}) = \text{unknown insulin concentration}$$

For sample values (per cent radioactivity in the supernatant) equal to or greater than the midpoint of the standard curve, the equations are the same as above except for calculation of the slope, which is:

$$\text{slope} = \log 40 - \log 30/(\%\text{ at } 40) - (\%\text{ at } 30)$$

Immunoassay results are reported with their 95% confidence interval values. The variance of the indicated sample potency is calculated as described by Baum *et al.* (7). For routine immunoassay of insulin, a digital computer (IBM 360) is programed to perform the calculations.

Insulin Bioassay Method.—The potencies of all standard insulins and insulin samples used in this study were established in accordance with the official U.S.P. bioassay (1) utilizing the U.S.P. zinc insulin reference standard for comparison. Sufficient individual bioassay results were combined to yield 95% fiducial limit in the range of 5–8%.

Insulin Testing Samples.—Triplicate sample of regular pork, beef, mixed source master lots of crystalline zinc insulin, NPH (isophane) insulin, and protamine zinc insulin were selected at random for establishing assay precision. The samples, to be subjected to repetitive immunoassay, were the same trial dilutions of the respective insulin master lots used previously in establishing their insulin potency by the official U.S.P. bioassay. In addition to the commercial insulin products, several process samples from insulin manufacture were obtained and assayed with both beef and pork insulin standards. Two of these samples, 174-A and 9CR40 + 41-A, were subjected to U.S.P. bioassay to establish the biological potency for comparison with the immunoassay results.

The initial aspects of the study revealed the desirability of having a secondary reference standard which is equivalent to the U.S.P. standard. For this purpose three pooled lots of pork, beef, and mixed source insulin were carefully selected from a number of lots which had previously been thoroughly tested by U.S.P. bioassay. Lot 14GP-257, pork zinc insulin crystals, was obtained from two pooled lots of pork insulin, and the average biopotency value was used as a basis of preparing a solution of this pork zinc insulin at 40 units/ml. Similarly, lot 14GP-258, beef zinc insulin crystals, was obtained from six different beef lots of established potency and lot 14GP-259, a mixed source zinc insulin, was a pool of 32 lots of mixed pork and beef zinc insulin crystals with potency also established by bioassay. The average potency value of these pooled lots of insulin should be closely related to the potency of the U.S.P. reference standard. Therefore, the lot, which under conditions of the immunoassay, gave results equivalent to the U.S.P. reference standard, would provide an insulin standard that permits immunoassay results to be expressed in terms of the U.S.P. standard.

Requirements for Immunoassay Precision.—The experimental plan was designed to yield results which were considered as "maximum capability" of the immunoassay under presently recognized conditions. "Maximum capability" is defined as those conditions of procedural operations which would yield the smallest possible variation from one daily assay to another. These conditions place special demand on the technical operators which would not be imposed on the regular daily "routine" testing.

Samples were prepared for each day's assay by a two-step dilution from the concentrated sample. The same pipet was used with a given sample each time that sample was diluted. In order to achieve more accuracy throughout, each technical operator used only one syringe for the ^{125}I insulin and only one syringe for the sample in the micrometric delivery. The sample syringe was carefully rinsed each time with the sample to be delivered into the reaction mixture. Every effort was made to keep dilutions and volumetric errors to a minimum. All reagents utilized throughout the testing sequence were prepared, as necessary, from the same lots of antibody, γ-globulin, human serum albumin, urea, sodium citrate, and borate buffer.

In all experimental testing, each sample was evaluated 10 times on different days by each of two technical operators. The experiments in each sequence were carried out over a period of a month with daily runs scheduled not to interfere with the routine insulin immunoassays also being performed. The tabulated results were submitted for statistical calculation and an analysis of variance.

RESULTS AND DISCUSSION

Comparative Assay Results.—*Regular Insulin.*—Table I shows the comparative results of the U.S.P. bioassay and the immunoassay of three lots each of pork, beef, and mixed zinc insulin. Both the bioassay and the immunoassay were conducted on a 40 units/ml. sample solution of each lot of insulin. The results were calculated in terms of units/mg. so that both assay systems could be directly compared. The 95% confidence limits of the immunoassay results are expressed for 4 and 10 daily repetitive tests. The insulin test samples were measured against the randomly selected pork standard, beef standard, and the mixed standard to determine the degree, if any, of species specificity affecting the immunoassay. The results reveal that the deviation of the immunoassay values for 4 repetitive tests is generally less than that of the corresponding bioassay and is always less in the case of 10 repetitive immunoassays. The test sample potency values obtained with the immunological assay using the pork insulin reference standard closely coincides with the bioassay results which were established with the U.S.P. insulin standard. On the other hand, the immunological data obtained with the beef insulin reference standard are consistently higher than the corresponding bioassay results. The statistical analysis indicates a significant difference in the assigned bioassay potencies of the two randomly selected reference standards. The overall results revealed that the beef zinc insulin refer-

TABLE I.—COMPARATIVE RESULTS OF THE U.S.P. BIOASSAY AND THE IMMUNOASSAY

U.S.P. Bioassay			Immunoassay								
				Pork Std.			Beef Std.			Mixed Std.	
Sample	units/mg.	$\%2\sigma$	Animals No.	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
Regular Pork Zinc Insulin												
PJ5682	23.9	±5.51	240	27.56	±3.99	±2.55	30.25	±4.85	±3.07	30.18	±3.11	±1.97
W-3789	25.6	±6.59	216	25.02	±3.89	±2.46	28.23	±3.95	±2.50	26.62	±2.47	±1.56
W-3864	25.2	±5.46	144	25.26	±3.99	±2.52	27.04	±4.71	±2.98	26.46	±4.65	±2.94
Regular Beef Zinc Insulin												
W-3879	24.64	±7.06	192	24.71	±4.61	±2.92	26.78	±2.00	±1.27	25.65	±4.57	±2.89
W-3912	24.60	±5.11	192	23.69	±4.10	±2.59	26.65	±6.31	±3.99	25.57	±5.67	±3.59
W-3905	25.47	±5.77	168	23.95	±4.43	±2.80	26.13	±6.37	±4.03	25.58	±5.74	±3.63
Regular Mixed Zinc Insulin												
W-3885	25.69	±5.43	240	24.78	±5.17	±3.27	26.82	±2.80	±1.77	25.45	±6.33	±4.00
W-3867	24.80	±5.92	192	25.45	±6.43	±4.07	27.66	±7.80	±4.93	25.76	±5.35	±3.38
W-3846	24.72	±5.18	192	24.07	±5.68	±3.59	26.54	±5.42	±3.42	25.96	±6.65	±4.21

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays respectively.

ence standard (lot No. 836550) gave consistent values which were higher than expected in the immunoassay when compared to the bioassay. This suggests that the original bioassay of this lot of beef zinc insulin crystals, although within the bioassay fiducial limit, was unrealistic as an estimate of the true potency; hence, in preparing the beef reference standard based on the mean biopotency, more insulin was introduced into the standard than would normally be expected. This was further supported by the analysis of variance and cross checking the reference standards in the immunoassay. Of the two immunoassay reference standards, randomly chosen, the pork zinc insulin (PJ-5682) compares most favorably with the U.S.P. reference standard.

The effect of species specificity on immunoassay reliability could be determined by an analysis of variance of the data obtained in Table I. The analysis of variance revealed no interaction of the groups studied under the conditions employed in the immunoanalysis.

Therefore, immunoassay, performed with antiserum obtained from guinea pigs immunized with mixed pork and beef insulin as the antigen, can be used with either pork or beef insulin as a standard in the immunoassay of beef, pork, or mixed source insulin samples. The fact that species specificity has no effect upon the method employed in this study is important for a satisfactory method for control of insulin development and manufacture.

Immunoassay precision is influenced from two principal sources: the variation encountered within a single day's testing and the variation experienced between days. The within-day assay variation is largely dependent upon sample replication, instrumentation, etc. Differences between days which exceed the within-day variation, are less amenable. The result of these two types of variation often produced deviations as large as ±15–25% in the immunoassays obtained by the salt precipitation procedure as described by Baum *et al.* (7). By carefully modifying the procedure, standardizing reagents, practicing rigid analytical techniques, and strict attention to details, the assay variation was reduced to ±11.2% when expressed as a 95% con-

TABLE II.—COMPARISON OF CONFIDENCE INTERVALS OF THE INSULIN IMMUNOASSAY CONDUCTED BY "MAXIMUM" AND "ROUTINE" CAPABILITY IMMUNOASSAY WITH PORK INSULIN STANDARD

	Max. Capability			Routine Capability	
Sample	units/mg.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
Regular Pork Zinc Insulin					
PJ-5682	27.56	±3.99	±2.52	±4.90	±3.10
W-3789	25.02	±3.89	±2.46	±1.98	±1.25
W-3864	25.26	±3.99	±2.52	±1.90	±1.20
Regular Beef Zinc Insulin					
W-3879	24.71	±4.61	±2.92	±5.49	±3.47
W-3912	23.69	±4.10	±2.59	±1.76	±1.11
W-3905	23.95	±4.43	±2.80	±8.12	±5.14
Regular Mixed Zinc Insulin					
W-3885	24.78	±5.17	±3.27	±6.10	±3.86
W-3867	25.45	±6.43	±4.07	±6.31	±3.99
W-3846	24.07	±5.68	±3.59	±8.88	±5.62

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays, respectively.

TABLE III.—COMPARISON OF CONFIDENCE INTERVALS OF THE IMMUNOASSAY OF NPH AND PZ INSULIN BY "MAXIMUM" AND "ROUTINE" CAPABILITY

				Immunoassay					
				Max. Capability			Routine Capability		
	U.S.P. Bioassay			Mixed Insulin Std.			Pork Insulin Std.		
Sample	Type	units/mg.[b]	$\%2\sigma$	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
W-3891[c]	NPH	25.4	±5.43	41.92	±9.45	±5.98	38.24	±6.08	±3.85
W-3871[c]	NPH	24.8	±5.92	41.23	±4.24	±2.68	37.00	±5.50	±3.48
W-3858[c]	NPH	24.9	±5.18	41.47	±6.02	±3.81	37.40	±6.52	±4.12
W-3857[c]	PZI	24.9	±5.18	44.91	±6.35	±2.01	39.56	±6.35	±2.01
W-3870[c]	PZI	24.8	±5.92	42.19	±6.62	±2.09	40.16	±5.56	±3.54
W-3892[c]	PZI	25.4	±5.43	42.81	±4.65	±1.47	39.10	±5.65	±3.57

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays, respectively. [b] All samples were prepared at 40 units/ml. based on the insulin potency established by bioassay. [c] Acidified with 30 µl. concentrated HCl/10 ml. of mixture for solution prior to dilution with immuno-diluent.

fidence limit for a single assay. The data in Table I were used to calculate such a generalized confidence interval of 88.8–111.2%.

The U.S.P. regulates the potency of insulin with a 95% fiducial limit of 87–115%. However, specifications for establishing insulin potency at Eli Lilly and Co. require a 95% fiducial limit of ±5–6% for the U.S.P. insulin bioassay. The number of daily immunoassays which give equivalent results is predicted from the average variation observed in the data summarized in Table I. Under the assumption that the daily immunoassays of a single sample are normally distributed, the required sample size (repetitive immunoassays conducted on different days, n) is calculated as:

$$\pm 5.5 = \pm 11.2\sqrt{\frac{}{n}}$$

$$n = 4.15$$

Hence, four daily immunoassays must be conducted to be within the Eli Lilly and Co. specifications.

The coefficient of variation (±5.6%) established from the data in Table I was obtained under conditions of "maximum capability." These rigid analytical techniques are impractical in the routine immunoassay for insulin. Therefore, the same series of insulin samples was re-evaluated under conditions defined as "routine capability." "Routine capability" permits the technician to utilize different syringes and pipets for all analytical measurements. Table II shows a comparison of the confidence limits of the U.S.P. bioassay and the insulin immunoassay conducted under conditions of "maximum" and "routine" capability. In this series of immunoassays, only the pork insulin standard was used since it has a potency very similar to that of the U.S.P. reference standard. Visual inspection of the "routine capability" results reveals little difference from the results of "maximum capability." The results further support the validity of the calculated coefficient of variation (±5.6%) as originally determined and indicate that the between-days variation encountered in the immunoassay is not due to operator error or analytical measurement.

Immunoassay of Some Commercial Insulin Products and Manufacturing Intermediates.—The reliability of the immunoassay for determining insulin concentrations in commercial products containing protamine is shown in Table III. NPH (isophane) insulin and protamine zinc insulin were from lots with potency established previously by bioassay. The immunoassay results under "maxi-

TABLE IV.—IMMUNOASSAY OF SAMPLES FROM DIFFERENT STAGES OF INSULIN MANUFACTURE

						Immunoassay					
	U.S.P. Bioassay				Animals,	Pork Std.			Beef Std.		
Sample	Species	Type	units/ml.	$\%2\sigma$	No.	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]	units/ml.	$\%2\sigma_4$[a]	$\%2\sigma_{10}$[a]
9CR-48 2nd Ch.	Beef	Cr. ext.	Not possible	...	...	6.23	±1.30	±0.83	6.68	±2.58	±1.63
9CR-48 3rd Ch.	Beef	Cr. ext.	Not possible	...	...	6.39	±1.65	±1.04	6.72	±2.10	±1.33
9CR-48 4th Ch.	Beef	Cr. ext.	Not possible	...	...	6.97	±3.90	±2.47	7.60	±5.11	±3.23
9CR40-41A	Pork	pH 5.6 ppt.	363.4 ± 6.92		144	387.4	±4.66	±2.95	397.0	±8.60	±5.44
9CR40-41B	Pork	pH 5.6 ppt.	...	..	...	405.0	±2.27	±1.44	422.9	±2.48	±1.57
9CR40-41C	Pork	pH 5.6 ppt.	...	..	...	404.5	±3.65	±2.31	...	...	...
L-174A	Pork	1st iso.	667.3 ± 7.87		144	666.5	±1.61	±1.02	713.0	±6.10	±3.86
L-174B	Pork	1st iso.	...	...	...	658.4	±2.88	±1.82	703.1	±2.09	±2.32
L-174C	Pork	1st iso.	...	...	...	660.0	±6.03	±3.81	692.8	±7.54	±4.77

[a] The symbols indicated as $2\sigma_4$ and $2\sigma_{10}$ represent the 95% confidence limit of the mean of 4 and 10 daily immunoassays, respectively.

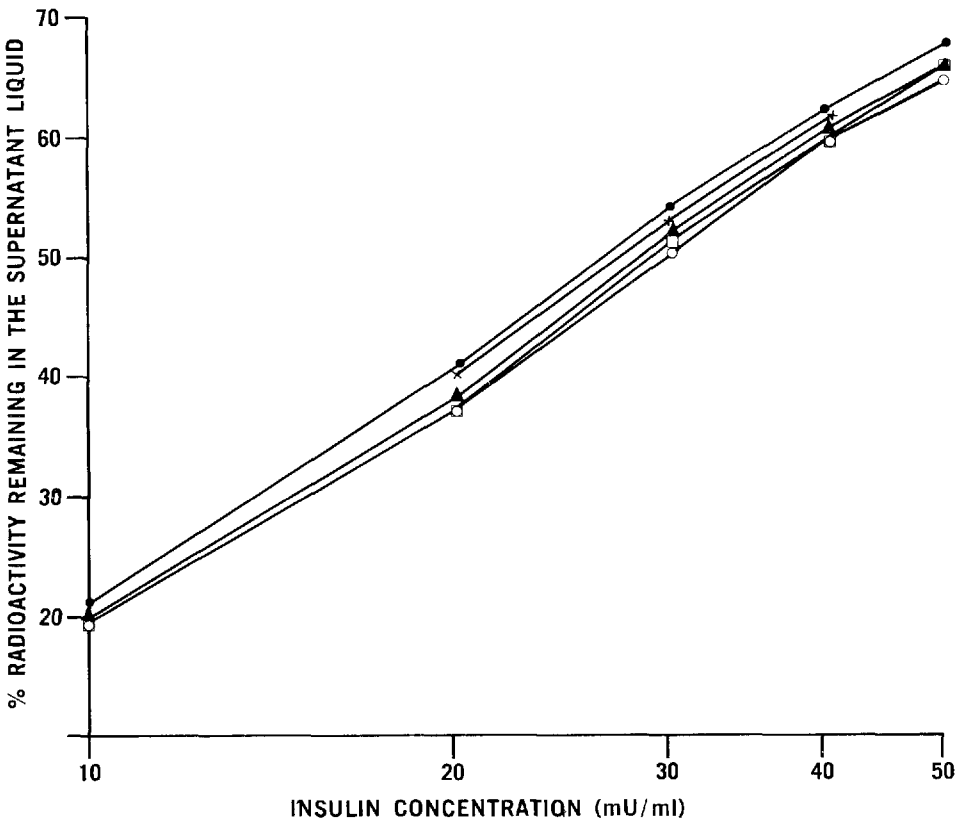

Fig. 2.—Full-scale response curves of the mean value of 10 immunoassays conducted on different days. Key: ●, pork zinc insulin, lot PJ-5682 (served as the reference standard); ○, pooled samples of pork insulin, 14GP-257; □, beef insulin, 14GP-258; ▲, mixed pork and beef insulins, 14GP-259, diluted based on the calculated mean of the individual bioassay result; ×, U.S.P. reference standard, W-3930, diluted based on the established biopotency of 24 units/mg. and determined at three of the five standard concentrations.

mum capability" conditions were obtained with a mixed source insulin reference standard having the same ratio of pork to beef insulin as that of the sample. The immunoassay values are slightly high compared to the bioassay figures. This is accounted for by the fact that the beef reference standard, lot 836550, which constitutes 75% of the mixed source reference standard, was found to give consistently high results, as previously noted. The unrealistic high results obtained with the mixed standard and the similarity of the pork insulin standard with the U.S.P. reference standard suggested that pork insulin standard should be used in the series conducted under "routine capability." The results readily reflect the difference in the absolute potency of these two randomly selected reference standards. The confidence limits, expressed for 4 and 10 repetitive tests, are slightly greater than the values presented in Tables I and II, but are within the expected range of the coefficient of variation. The results in Table III support the view that commercial insulin products can be analyzed effectively with this immunoassay.

Table IV provides evidence that the immunoassay can be used in monitoring insulin manufacture. Immunoassay results on samples selected at different stages of the insulin process show a variation that is within the limits of the coefficient of variation and agree satisfactorily with the U.S.P. bioassay. The immunoassay possesses a distinct advantage over the U.S.P. bioassay in its ability to determine insulin concentration in crude samples that cannot be satisfactorily handled under the conditions of the bioassay system.

The pooling of many lots of crystalline zinc insulin of established biological potency would seem to provide a material with a mean biopotency which would closely correspond to the U.S.P. reference standard. Three types of pooled samples were prepared by mixing aliquots of zinc insulin crystals from lots that were thoroughly assayed in the U.S.P. method; namely, 14GP-257 from two lots of pork zinc insulin crystals with an average potency of 25.45 units/mg., 14GP-258 from six lots of beef zinc insulin crystals with an average potency of 25.50 units/mg., and 14GP-259 from 32 lots of mixed source zinc insulin crystals with an average potency of 24.79 units/mg. The average potency value was used to prepare 40 units/ml. insulin solutions of each for 10 repetitive immunoassays on different days. To further substantiate and extend the findings of the initial study, these samples were tested at all concentrations used

TABLE V.—SIMILARITY OF THE INDIVIDUAL COMPUTED OF POOLED ZINC INSULIN SAMPLES

Sample	Type	Range, munits/ml.	Slope[a]	95% Confidence Interval of the Slope
PJ5682	Pork	20–40	69.57	66.96–72.18
14GP-257	Pork	20–40	70.05	67.44–72.66
14GP-258	Beef	20–40	73.60	70.99–76.21
14GP-259	Mixed	20–40	73.36	70.75–75.98
W-3930	U.S.P.	20–40	69.93	67.32–72.54
Common slope—all species from statistical analysis.		20–40	71.30[b]	

[a] Individual slopes by least squares. [b] Least squares common slope lies within each of the computed individual slope confidence intervals.

in the standard reference curve. According to the procedure described, a sample for immunoassay is diluted to a concentration which is estimated to be equivalent to that of the midpoint standard (30 munits/ml.). The statistical analysis and the interpretation of results are limited to this single point. On the other hand, a comparison of the slopes of the curves obtained from determining these pooled samples at five different concentrations would avoid this limitation. If the slopes of the curves obtained from the pooled samples are identical, within the experimental limits, with that of the randomly chosen standard (in this case pork zinc insulin PJ-5682), the original interpretation would be confirmed.

Figure 2 shows a plot of the mean values of 10 repetitive immunoassays of the pooled samples, standard PJ-5682, and three concentrations of the U.S.P. reference standard. Visual inspection indicates the curves to have similar slopes in the range of 20–40 munits/ml. insulin concentrations, respectively. In view of the fact that this immunoassay has been limited to the determination of potency of extracted insulin by definition, only the most sensitive portion of the standard curve was subjected to statistical analysis to substantiate the use of one sample concentration for routine analysis.

Statistical analysis, by least squares, of the data obtained at 20, 30, and 40 munits/ml. insulin concentrations gave 95% confidence limits for the individual slopes as shown in Table V. The value of the calculated common slope, 71.30, lies within the limits of the individual slopes. Furthermore, by a more refined technique of regression analysis, no significant differences in slopes were detected. Figure 3 shows the dose response curves of these

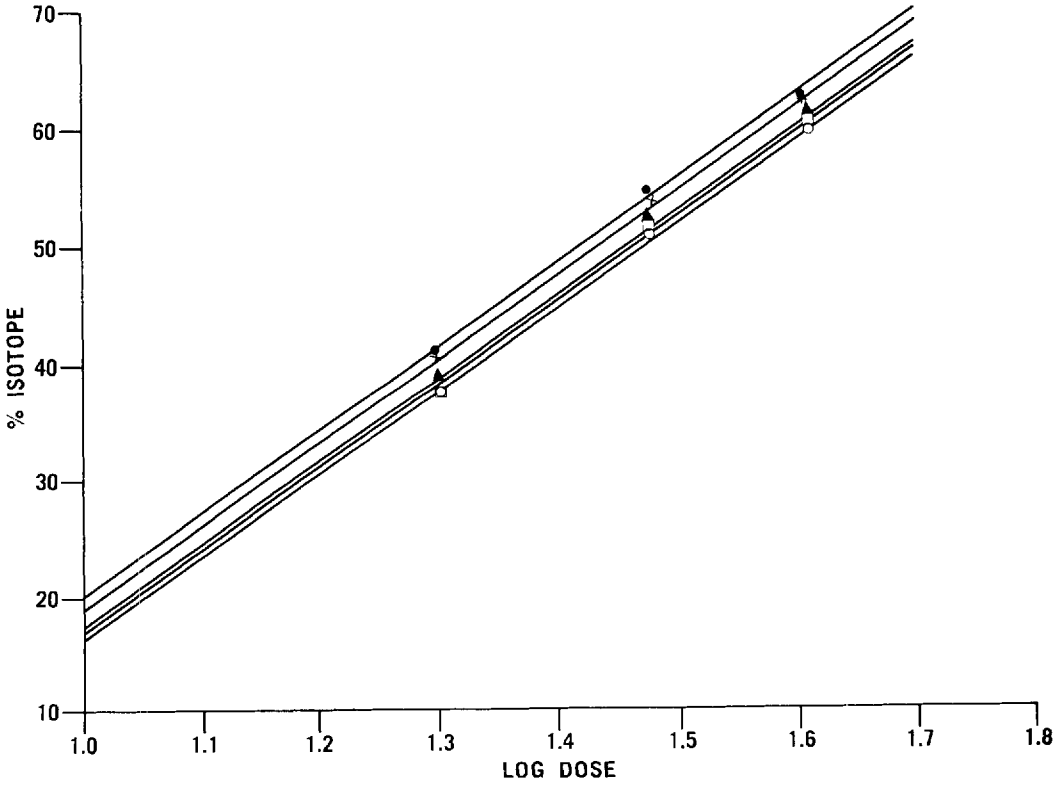

Fig. 3.—Dose response curves based on the common slope calculated by least squares for the selected concentrations at 20, 30, and 40 munits/ml. Key: ●, PJ-5682, ○, 14GP-257; □, 14GP-258; ▲, 14GP-259; ×, U.S.P. reference standard W-3930 as in Fig. 2.

TABLE VI.—COMPARATIVE POTENCY RESULTS OF INSULIN MASTER LOTS DETERMINED BY U.S.P. BIOASSAY AND IMMUNOASSAY

Sample	Estimated Potency, units/mg.	% Estimate	Potency, units/mg.	Variation, %	Rabbits, No.	Estimate, %	Potency, units/mg.	% S. D.	Replications
M.L. 47 (W-3927) Beef	25.0	99.06	24.76	5.71	192	98.75	24.69	6.3	5
M.L. 48 (W-3934) Beef	25.0	100.41	25.10	5.94	192	102.00	25.50	11.4	5
M.L. 9BV49 (W-3929) Mixed	24.5	102.86	25.20	5.46	240	102.67	25.15	4.5	5
M.L. 9GZ30 (W-3940) Mixed	25.0	101.28	25.32	5.90	192	101.44	25.36	5.6	4

[a] Pork zinc insulin (lot PJ-5682) used as the immunoassay reference standard.

insulin samples plotted with the common slope. Consequently, species specificity does not affect the validity of the immunoassay result if mixed antigen is used in production of antibody in guinea pigs. The individual regression lines for the different samples, although parallel, are not superimposable, thus revealing slight errors in the assigned potency of these samples. In order to express insulin immunoassay results in terms of the U.S.P. reference potency, a secondary reference standard would need to be experimentally adjusted to the potency of the U.S.P. reference standard. Any of these pooled insulin samples can serve as a secondary reference after appropriate matching with the U.S.P. reference by repetitive immunoassay.

Application of the Immunoassay for Establishing the Potency of Manufactured Insulin.—The information obtained in this investigation was applied to the determination of potency of several lots of manufactured insulin. Table VI compares the U.S.P. bioassay and the immunoassay of four lots of zinc insulin crystals. Instead of four repetitive immunoassays on different days as required under "maximum capability" conditions, five immunoassays were conducted (except on ML-9GZ30) under routine conditions. The mean value of insulin potency established by immunoassay compares favorably with the bioassay result being well within the limits defined in the U.S.P. (1). However, the standard deviation of the mean of the individual values, particularly with ML 48, varied somewhat more than would be predicted. The cause of this excessive variation could not be readily determined, but suggests the necessity of vigilant attention to detail in immunoassay conduct.

The results of this investigation indicate that the insulin immunoassay, in accordance with the described procedure, is a rapid and economic method, with satisfactory precision and sensitivity for establishing the potency of extracted insulin. The use of this immunoassay for extracted insulin fills an important need in monitoring the manufacture of insulin and assessing insulin concentration in commercial insulin products. The information provided in this investigation can serve to promote interest and support in establishing the immunoassay as a U.S.P. method for determining insulin potency.

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Irreversible Enzyme Inhibitors LXVII

2-Amino-6-(*p*-bromoacetamidophenylbutyl)-5-phenylbutyl-4-pyrimidinol, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

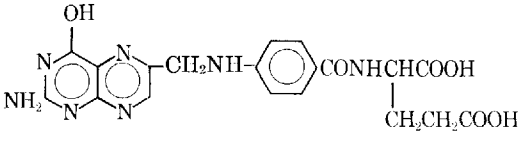
By B. R. BAKER and JOHANNES H. JORDAAN

2-Amino-5-phenylbutyl-4-pyrimidinols substituted by a *p*-bromoacetamidophenylbutyl group (XIII) or a *p*-bromoacetamidophenethyl group (XIV) on the 6-position can inactivate dihydrofolic reductase with a half-life of 18 and 12 min. at 37°, respectively. These inactivations have the kinetic parameters expected for enzyme inactivation by the active-site-directed mechanism, that is, an inactivation proceeding by a neighboring group reaction within the reversible complex formed between dihydrofolic reductase and XIII or XIV. Neither iodoacetamide nor *p*-bromoacetamidophenylbutyric acid showed any inactivation of dihydrofolic reductase at a concentration equal to XIII under conditions where XIII gave 65–70 per cent inactivation. The rate of inactivation of dihydrofolic reductase by XIII and XIV was slowed by the reversible inhibitors, folic acid (I) and 2,4-diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine (XV). The inactivation was also slowed by TPNH, but not by DPNH or adenosine diphosphate.

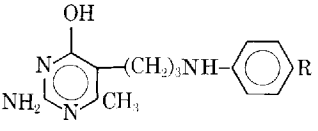
BEFORE THE strong hydrophobic bonding to dihydrofolic reductase was discovered (1)—with its conformational implications (2–8)—more than 30 potential active-site-directed irreversible inhibitors for this enzyme had been synthesized and evaluated on the premises discussed below.

THEORETICAL

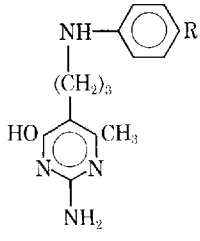
It should theoretically be possible to modify folic acid (I) with a chloromethyl ketone group in place of one of the carboxyls in order to obtain an active-site-directed irreversible inhibitor that would operate by the endo-mechanism (9) by alkylation of the enzymic binding point for one of the carboxyl groups. The synthesis of such a pteridine is fraught with monumental incompatibilities of functional groups. Since the anilino propylpyrimidine (II*a*) (10) without a carboxy-L-glutamate moiety was still about one-eighth as good an inhibitor as II*b* (10, 11), compounds II*c* (n = 0,2,4,6) and II*d* were synthesized as potential active-site-directed irreversible inhibitors of dihydrofolic reductase (11). Although these five compounds were good reversible inhibitors, they failed to show irreversible inhibition. In retrospect, these failures were most probably due to the possibility that the anilinopropyl group of II*a*, *c*, and *d* was not complexed to the enzyme at the *p*-aminobenzoyl locus for folic acid, but was complexed in the hydrophobic region in a conformation such as III where the pyrimidine moiety has been twisted 60° with respect to the "normal" complexing of the pteridine of folic acid in conformation I. A conformation such as II would destroy the juxtaposition of the nucleophilic groups on the enzyme—which complex the glutamate carboxyls—with the alkylating functions of III*c* and *d;* that is, if the anilinopropyl side chains of III*c* and *d* are complexed in a hydrophobic region, then by definition there are not apt to be hydro-



I



II



III

a, R = H
b, R = CONHCHCOOH
|
CH_2CH_2COOH
c, R = $-(CH_2)_nCCH_2Cl$
||
O
d, R = $-CH{=}CHCCH_2Cl$
||
O

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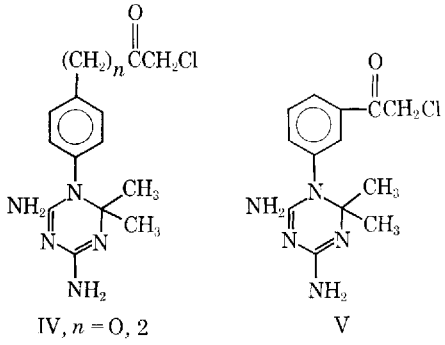
For a preliminary announcement of this work see *Reference 7*.

Previous paper: Baker, B. R., Santi, D. V., Coward, J. K., Shapiro, H. S., and Jordaan, J. H., *J. Heterocyclic Chem.*, to be published.

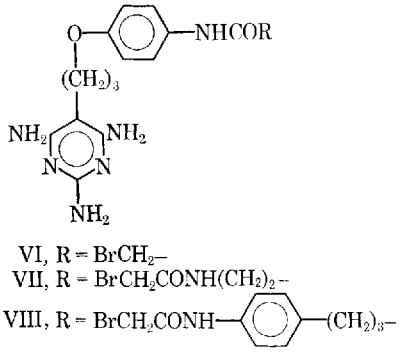
The previously published papers on "Nonclassical Antimetabolites" and on "Analogs of Tetrahydrofolic Acid" have been combined into one series, since they have the common objective of the design of active-site-directed irreversible enzyme inhibitors. A collected list of references on "Irreversible Enzyme Inhibitors" will be sent on request.

philic nucleophilic groups on the enzyme in this hydrophobic area needed for covalent bond formation in a neighboring group reaction within the enzyme–inhibitor complex.

Some related types of potential active-site-directed irreversible inhibitors were synthesized and evaluated—namely, the 1-phenyl dihydro-*s*-triazine derivatives, IV and V (12). These compounds were excellent reversible inhibitors but failed to show irreversible inhibition since the phenyl moiety is complexed in a hydrophobic region (3). Similarly, compounds V–VIII were good reversible inhibitors, but failed to show irreversible inhibition (13).

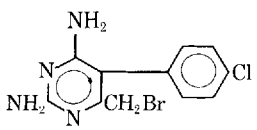


IV, $n = 0, 2$ V

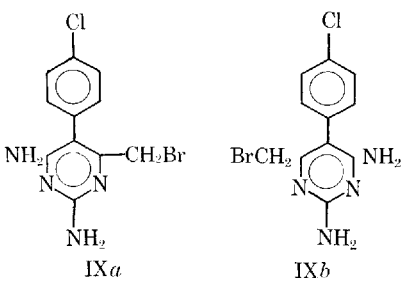


VI, R = $BrCH_2$–
VII, R = $BrCH_2CONH(CH_2)_2$–
VIII, R = $BrCH_2CONH$–C$_6$H$_4$–$(CH_2)_3$–

A different type of potential active-site-directed irreversible inhibitor was constructed on the proposition that there might well be an enzymic proton donor, such as imidazole, near the 8-position of folic acid when it is complexed to the enzyme; such a proton donor could aid in the TPNH reduction of folic acid. The pyrimethamine-type analog (IX) was synthesized and found to be an excellent reversible inhibitor, but not an irreversible inhibitor of dihydrofolic reductase (14). Again, in retrospect, it is probable that the 5-phenylpyrimidine (IX) binds its phenyl to the hydrophobic region in a "twist" conformation as depicted in IX*a*; if such were the case, then IX*a* would not have its bromomethyl in close enough proximity to the supposed enzymic proton donor. A similar conclusion could be drawn if IX complexed to dihydrofolic reductase in a "twist-flip" conformation (IX*b*).

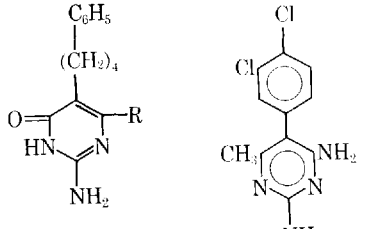


IX



IX*a* IX*b*

As a result of a study on possible pyrimidine conformations when these inhibitors are complexed to dihydrofolic reductase, it was suggested that a pyrimidine such as X would have the conformation shown (with respect to conformation I for folic acid), since the strong hydrophobic bonding could be determinant for the binding conformation (7). Such a conformation for X suggests that chain lengthening of the R group should project it into a hydrophilic region. That this might be the case was supported by the observation that the *n*-propyl group caused XI to be a thirtyfold less effective inhibitor than the 6-methylpyrimidine (X) (7); this result could be due to the repulsion of a hydrophobic group from a hydrophilic area on the enzyme or to a steric interaction unfavorable to complex formation between the enzyme and the inhibitor. That steric interaction was less likely was indicated by the increase in the size of the R group to phenylbutyl (XII) (15); XII was a fivefold better inhibitor of dihydrofolic reductase than XI, also indicating that the phenyl group on the 6-side chain was complexed to the enzyme. Therefore, the 6-bromoacetamidophenyl butyl pyrimidine (XIII) was selected as a likely candidate for an active-site-directed irreversible inhibitor of dihydrofolic reductase with the premise that the 5-phenylbutyl group would complex in the hydrophobic area, and the 6-phenylbutyl group could project into a hydrophilic area on the enzyme; the corresponding 6-phenethyl derivative (XIV) was also investigated.



X, R = CH_3
XI, R = C_3H_7-*n*
XII, R = –$(CH_2)_4C_6H_5$
XIII, R = –$(CH_2)_4C_6H_4(NHCOCH_2Br)$-*p*
XIV, R = –$(CH_2)_2C_6H_4(NHCOCH_2Br)$-*p*

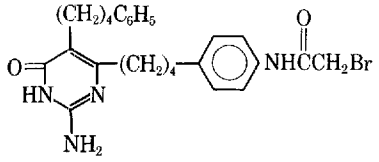
XV

RESULTS

The bromoacetamido pyrimidine (XIII) was about equal to XII when assayed (16) as a reversible inhibitor of dihydrofolic reductase; that is, XIII showed 35% inhibition at a concentration of 100 μM—the maximum solubility in 10% *N*,*N*-dimethylformamide—in the presence of 6 μM di-

hydrofolate. It was estimated (1) that the K_i was about 4×10^{-5} M. When XIII was incubated with dihydrofolic reductase at 37° in the absence of TPNH, inactivation occurred with a half-life of about 18 min. When XIII, iodoacetamide, and *p*-bromoacetamidophenylbutyric acid (17), all at 40 μM, were simultaneously incubated with the enzyme at 37° for 25 min., XIII gave 65% inactivation, but the other two compounds gave only 2–3% inactivation (Table I, *A*); thus, the random bimolecular inactivation of dihydrofolic reductase was ruled out (21).

TABLE I.—INACTIVATION OF DIHYDROFOLIC REDUCTASE[a] BY



XIII

Expt.	Incubation Mixture	Inactivation, %
A	40 μM XIII	65
	40 μM ICH_2CONH_2	3
	40 μM *p*-$BrCH_2CONHC_6H_4(CH_2)_3COOH$	2
B	40 μM XIII	73
	40 μM XIII + 15 μM Folic acid	50
C	40 μM XIII	68
	40 μM XIII + 0.15 μM XV	32
D[b]	40 μM XIII	62
	40 μM XIII + 12 μM TPNH	23
	40 μM XIII + 60 μM TPNH	0
F	40 μM XIII	76
	10 μM XIII	43

[a] Dihydrofolic reductase from pigeon liver in 0.05 M Tris buffer (pH 7.4) plus 10% *N*,*N*-dimethylformamide was incubated in the absence of TPNH at 37° for 25 min. with the additions indicated; the amount of remaining enzyme was then assayed (14). In each experiment an enzyme control was run simultaneously with the two or three other solutions; only 0–4% thermal inactivation of the enzyme occurred. [b] In similar simultaneous experiments, adenosine diphosphate or DPNH at 36 μM showed no protection, whereas 36 μM TPN protected about half as well as 36 μM TPNH.

Table I summarizes experiments on the protection against this inactivation. The reversible inhibitors, folic acid (18) and 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine (XV) (19), slowed the rate of inactivation, evidence that the active site was involved in the inactivation (20, 21). Surprisingly, TPNH also protected against inactivation; since the reversible inhibition by XIII was found to be independent of TPNH concentration, the protective effect of TPNH was not due to the complexing of the bromoacetamidophenyl moiety of XIII to the enzymic region normally complexing TPNH. Two other possible explanations are that (*a*) the complex between XIII and dihydrofolic reductase juxtaposes the bromoacetyl group to a TPNH binding point or (*b*) TPNH causes a conformational change in the enzyme which moves the enzymic nucleophilic group from juxtaposition with the bromoacetyl group in the XIII–enzyme complex. These two possibilities cannot as yet be distinguished.

It was noted that 36 μM adenosine diphosphate or DPNH showed no protection. TPN at 36 μM showed half the protection given by 36 μM TPNH at this pH of 7.4. These results exclude the destruction of the inhibitor by the phosphate mono ester group. DPNH binds poorly to dihydrofolic reductase at pH 7.4 (22); the binding of TPN is quite good at pH 5.5 (22), but its binding at pH 7.4 has not been measured. Thus, these protection experiments are best explained on the relative binding of the various phosphates to dihydrofolic reductase.

Increasing incubation time did not lead to total inactivation, but inactivation leveled off at 80–90%. This result is similar to that observed (23) with the action of *p*-nitrophenyl α-bromoacetamidoisobutyrate on chymotrypsin where 80–90% inactivation occurs; the remaining activity was shown to be due to a modified enzyme where K_m was increased, but $V_{max.}$ remained nearly the same (23, 24). It will be of interest in the future to determine if $V_{max.}$ or K_m has changed for dihydrofolate or TPNH or both after dihydrofolic reductase has been inactivated with XIII.

If the inactivation of dihydrofolic reductase proceeds through an obligatory complex between the enzyme and the inhibitor (XIII), then a "rate-saturation" effect should be observed, that is, the rate of inactivation is dependent upon the concentration of enzyme–inhibitor complex, EI (21), as shown in Eq. 1.

$$[EI] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad \text{(Eq. 1)}$$

The amount of reversible complex can be calculated from Eq. 1, previously derived (21), where [EI] = concentration of complex, $[E_t]$ = total active enzyme, and [I] = inhibitor concentration. Since XIII has $K_i = 4 \times 10^{-5}$ M and the incubation is performed with 4×10^{-5} M of XIII, then $[EI] = 0.5\,[E_t]$. If $[I] = 1 \times 10^{-5}$ M, as in experiment *F* (Table I), then $[EI] = 0.2\,[E_t]$. Note that XIII at 4×10^{-5} M gave 69% inactivation in 25 min., but that XIII at 1×10^{-5} M gave 43% inactivation. It can be calculated that 1×10^{-5} M of XIII would have given 23% inactivation in 25 min. if the reaction were proceeding by a bimolecular reaction, but 40% if inactivation occurred through the EI complex as in Eq. 1. Thus, the observed 43% at 25 min. with 1×10^{-5} M represents a rate-saturation effect as a first approximation.

The 5-phenylbutyl-4-pyrimidinol (XIV) with the shorter phenethyl side chain containing a *p*-bromoacetamido group was also found to be an active-site-directed irreversible inhibitor of dihydrofolic reductase. XIV was a reversible inhibitor with $K_i = 3 \times 10^{-5}$ M; when incubated with dihydrofolic reductase at 37°, inactivation occurred with a half-life of about 12 min. With extended time the enzyme became 80–90% inactivated, but did not inactivate completely, as also noted with XIII. The phenethyl pyrimidine (XIV) showed the same protection pattern as observed with XIII in Table I.

Whether XIII and XIV attack the same amino acid on dihydrofolic reductase must await inactivation experiments with the pure enzyme; that the same amino acid would be attacked is unlikely, due

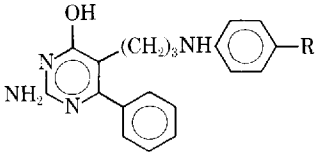
to the conformational changes that either XIII or the enzyme would have to undergo. Such conformational changes on XIV would most likely be energetically unfavorable, but the energetics of such a conformational change in the enzyme are unknown.

DISCUSSION

An active-site-directed irreversible enzyme inhibitor theoretically operates by first forming a complex with the enzyme, then the complex undergoes a facile neighboring group reaction between an enzymic nucleophilic group and a leaving group on the inhibitor (9, 25, 26). There are two types of these irreversible inhibitors: (*a*) those that operated by covalent bond formation inside the active-site—the so-called endo-mechanism—and (*b*) those that operate by covalent bond formation outside of the active-site—the so-called exo-mechanism. Examples of both types are known (9, 25, 26).

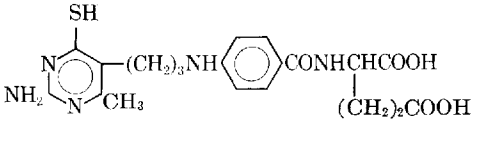
The endo-type of irreversible inhibitor is preferred by protein structure chemists since they wish to "label" amino acids in the active-site by attachment of a covalently linked moiety that can be identified; the specific "labeled" amino acid is determined by total hydrolysis of the protein and its position in the amino acid sequence by partial hydrolysis (26). It has been anticipated (26) that the endo-type would be much less effective for chemotherapy than the exo-type since the active site of an enzyme can vary only little from species to species and still be operational. In contrast, the exo-type could be expected to be far more species and tissue specific since covalent bond formation is taking place outside the active site where numerous changes can be made without disrupting the protein's function (26, 27).

At the time our program on active-site-directed irreversible inhibitors for dihydrofolic reductase was initiated in 1961, the factors in the design of irreversible inhibitors for enzymes was just beginning to emerge (21, 25, 28); since then the field has developed considerably in both concept and practice (9, 26). Therefore, at the start we set goals toward both the endo-type and exo-type of irreversible inhibitors (25) for dihydrofolic reductase. It soon became apparent that the active-transport system for cell wall penetration by folic acid and its derivatives (10, 29) was sensitive to the wrong positioning of bulky groups. It was noted that the folic acid analog (XVIII) (30), was cytoxic to S-180 cells in culture, but the 6-phenylpyrimidine analog of folic acid (XVI) (31) was inert; however, when the carboxy-L-glutamate moiety of XVI was not present, as in XVII (31), XVII was cytoxic.[1]



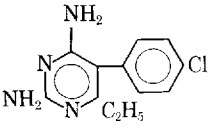
XVI, R = $-CONHCHCOOH$ with CH_2CH_2COOH

XVII, R = H



XVIII

The carboxy-L-glutamate moiety has been implicated as being essential for active transport of folic acid and its close analogs (10), but less related dihydrofolic reductase inhibitors such as pyrimethamine (XIX) entered cells by passive diffusion (10, 29). It then follows that perhaps XVI was inert because the 6-phenyl group was too bulky to be



XIX

accommodated by the active transport system for folic acid, but, in addition, the ionized carboxy-L-glutamate aborted any possible passive diffusion. For this reason all of our subsequent work avoided the presence of the carboxy-L-glutamate moiety so that the inhibitors would be able to penetrate the cell membrane by passive diffusion. Furthermore, certain 2,4-diamino heterocycles without the carboxy-L-glutamate moiety can complex to dihydrofolic reductase even better than folic acid (1) (16, 19, 26, 29), and such molecules without carboxy-L-glutamate moiety are considerably easier to synthesize and purify.

The removal of the carboxy-L-glutamate moiety led to the discovery of the useful hydrophobic bonding area on dihydrofolic reductase (1) with its species differences in conformational tolerances (4, 8, 29). Although the presence of the hydrophobic region on dihydrofolic reductase aborted the success of more than 30 candidate active-site-directed irreversible inhibitors, knowledge now developed on the hydrophobic region (1–8) should be usable for the design of species- or tissue-specific irreversible inhibitors of this enzyme.

Note that the irreversible inhibition of dihydrofolic reductase by XIII (Table I) is slowed by the presence of TPNH. If XIII is alkylating a point on the enzyme that normally complexes TPNH, then by definition XIII is operating by the endo-mechanism; if XIII is alkylating some other point not essential for enzyme activity, but TPNH causes a conformational change in the enzyme which removes this attackable function on the enzyme from juxtaposition with the alkylating function of XIII, then the exo-mechanism is operating. In either case the hydrophobic region can be used to introduce a new parameter for tissue- or species-specificity.

The maximum specificity should be observed if an alkylating function on the inhibitor can be bridged back from the hydrophobic region on the enzyme to the more polar nucleophilic region. In this way the ability of an irreversible inhibitor to bridge between the pyrimidine locus in the active-site and the enzymic nucleophilic site can be controlled by the nature of the hydrophobic site, which is outside the active site and where the greatest

[1] The authors thank Dr. M. Hakala for these assays.

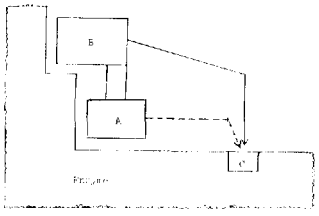


Fig. 1.—Schematic representation of an active-site-directed irreversible inhibitor that utilizes hydrophobic bonding for specificity. Key: A, the pyrimidine of an inhibitor; B, the hydrophobic group of an inhibitor; C, an enzymic nucleophilic group. The solid arrow represents a covalent-forming group on one inhibitor that can bridge properly from the hydrophobic bonding region to a nucleophilic site on the enzyme; the dotted arrow represents a covalent-forming group on a second inhibitor that can bridge from the pyrimidine region to a nucleophilic site on the enzyme (see *Discussion*).

evolutionary differences can occur (26, 27) (Fig. 1). It should also be possible to obtain specificity by varying the group that complexes in the hydrophobic region; this could alter the position of the alkylating function (dotted arrow, Fig. 1) by positioning the pyrimidine slightly differently; this can be likened to a fulcrum where a slight shift on the hydrophobic side will shift the alkylating side in the opposite direction where a part of the pyrimidine is the axis of the fulcrum.

Although studies of this type for tissue specificity could be pursued with the 2-amino-4-pyrimidinol type of inhibitors such as XIII, an additional important problem should be solved before such studies are initiated. Since XIII and XIV have K_i's near 3×10^{-5} M, it is unlikely that these inhibitors would be effective below 3×10^{-6} M since the amount of intracellular enzyme in the enzyme–inhibitor complex would only be about 10% of the total enzyme. There is a definite practical limitation on the intracellular concentration of inhibitor that can be obtained in a whole animal system, which is in turn partially dependent upon dosage. A reasonable maximum limit for intracellular inhibitor concentration is 10^{-6} M; the more dilute the inhibitor can be and still form 50% reversible complex with the available enzyme, the more potent the inhibitor will be irreversibly (32) and the less dosage will have to be used. 2,4-Diaminopyrimidines are 300–1000-fold more potent reversible inhibitors than the corresponding 2-amino-4-pyrimidinols (1, 16). For example, pyrimethamine (XIX) has $K_i = 7 \times 10^{-9}$ M with the folic reductase from rat liver (10); therefore, an active-site-directed irreversible inhibitor of dihydrofolic reductase that has a K_i approaching the 10^{-8} M of pyrimethamine (XIX) would at 10^{-8} M complex half the enzyme. Since the inactivation rate is dependent upon the concentration of reversible enzyme–inhibitor complex, such a hypothetical irreversible inhibitor should be effective at 10^{-8} M or less; such a concentration is readily achieved within a cell by passive diffusion.

The solution to such a problem may not be simply the conversion of XIII or XIV[2] to the corresponding 2,4-diamino pyrimidines since there seems to be some conformational differences in the manner in which 2,4-diaminopyrimidines and 2-amino-4-pyrimidinols complex to dihydrofolic reductase (7). The vigorous pursuit of the 2,4-diaminopyrimidine and 4,6-diamino-1,2-dihydro-*s*-triazine types of active-site-directed irreversible inhibitors for dihydrofolic reductase is continuing in this laboratory.

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[2] The synthesis of XIII and XIV have been described previously (15, 17, 33).

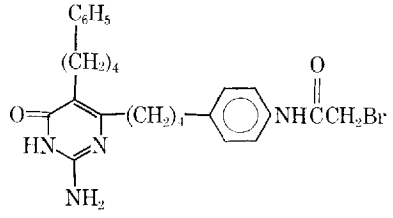
Irreversible Enzyme Inhibitors LXVIII

2-Amino-5-(*p*-bromoacetamidophenoxypropyl)-6-phenyl-4-pyrimidinol, an Active-Site-Directed Irreversible Inhibitor of Dihydrofolic Reductase

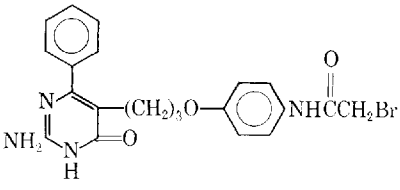
By B. R. BAKER and HOWARD S. SHAPIRO

2-Amino-5-(*p*-bromoacetamidophenoxypropyl)-6-phenyl-4-pyrimidinol (II), when incubated with dihydrofolic reductase at 37°, inactivated the enzyme with a half-life of about 45 min. In contrast, iodoacetamide and *p*-bromoacetamidophenylbutyric acid at the same concentration showed no inactivation of the enzyme in the same time. An interesting contrast to II was the 6-methyl analog (III) of II which inactivated the enzyme at about one-seventh the rate of II. This result gives unequivocal support for a previous suggestion that 6-methylpyrimidines and 6-phenylpyrimidines do not reversibly complex with dihydrofolic reductase in the same manner, else II and III should have inactivated the enzyme at the same rate at equal concentrations of reversible complex. These experiments are best explained on the basis of active-site-directed irreversible inhibition of dihydrofolic reductase.

OVER THIRTY attempts[1] to create an active-site-directed irreversible inhibitor (1) of dihydrofolic reductase were aborted prior to the discovery of the strong hydrophobic region on the enzyme adjacent to the active-site (2–8) and the realization that the pyrimidine type of inhibitor may have any one of several possible rotational conformers when complexed to the enzyme (8–10). Attention was then turned to the synthesis of candidate active-site-directed irreversible inhibitors that were substituted with one group on the 5- or 6-position that could complex to the hydrophobic region but also had a 6- or 5-substituent that could project into the hydrophilic region of the enzyme such as I and II. Efforts to synthesize the 5-phenylbutyl pyrimidine (I) and the 6-phenyl pyrimidine (II) started about the same time; I was found to be the first example of the long-sought active-site-directed irreversible inhibitors of dihydrofolic reductase, and II was found to be an irreversible inhibitor shortly thereafter. The synthesis and enzymic evaluation of I was presented in the previous paper of this series (11); the synthesis and enzymic evaluation of II is the subject of this paper.



I



II

DISCUSSION

Due to the lack of solubility, the maximum concentration in 10% *N,N*-dimethylformamide that could be reached with the 6-phenylpyrimidine (II) was 200 μM; at this concentration II showed 20% reversible inhibition in the presence of 6 μM dihydrofolate (12). From these data the 50% inhibition concentration was estimated to be 800 μM, and the K_i was estimated (3) to be $1 \times 10^{-4} M$. From Eq. 1, it could be estimated that at a concentration of 40 μM, 25% of the enzyme was in the form of an enzyme–II complex. It is the concentration of this complex that determines the rate of irreversible inhibition with an active-site-directed irreversible inhibitor (13–15) where [EI] = the enzyme–inhibitor reversible complex, E_t = the total active enzyme, [I] = the inhibitor concentration, and K_i = the reversible dissociation constant of EI (14, 15).

$$[EI] = \frac{[E_t]}{\frac{K_i}{[I]} + 1} \qquad \text{(Eq. 1)}$$

When dihydrofolic reductase was incubated in the absence of TPNH at 37° with 40 μM II at pH 7.4 in 10% *N,N*-dimethylformamide by the procedure previously described (9, 11), the enzyme was inactivated with a half-life of about 50 min.; thus, cor-

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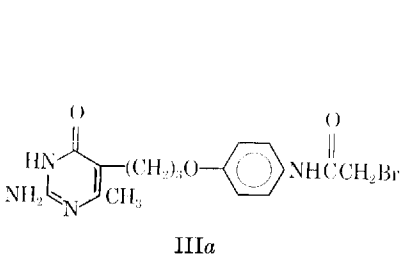
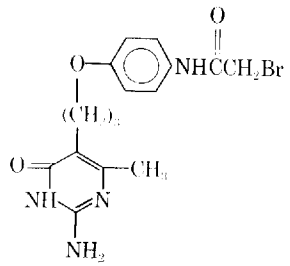
For a preliminary announcement of this work see *Reference 8*.

Previous paper: Baker, B. R., and Jordaan, J. H., *J. Pharm. Sci.*, **55**, 1417(1966).

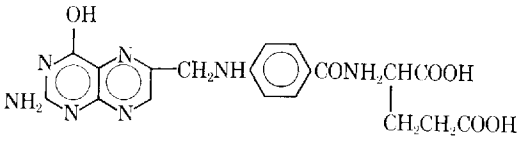
[1] For a listing of some of these unsuccessful candidate irreversible inhibitors see *References 11* and *15*.



IIIa



IIIb



IV

rected for equal concentrations of reversible E · · · I complex, II inactivates dihydrofolic reductase about one-half the rate of I. When 40 μM each of II, iodoacetamide, and *p*-bromoacetamido phenylbutyric acid (16) were incubated with dihydrofolic reductase simultaneously for 60 min. at 37°, only II showed irreversible inhibition; thus, the bimolecular mechanism of inactivation was readily eliminated (14, 15). Protection against inactivation was shown by the reversible inhibitors, folic acid (IV) and 2,4-diamino-5-(3,4-dichlorophenyl)-6-methylpyrimidine, as noted with the inactivation of dihydrofolic reductase by I (11).

An interesting comparison as irreversible inhibitors are the 6-phenylpyrimidine (II) and the 6-methylpyrimidine (III), both having the identical *p*-bromoacetamidophenoxypropyl side chain at the 5-position. From previous studies on hydrophobic bonding (8), it was suggested that the data were best rationalized if a 6-phenylpyrimidine, such as II, has a conformation as depicted in II when compared to an assigned conformation of the pteridine ring in folic acid indicated in IV. In contrast, it was suggested that a 6-methylpyrimidine, such as III, would have its large 5-group complexing with the hydrophobic region as in conformation III*b*, but III would not complex in conformation III*a* or the conformation taken by II (8, 11). If II and III assumed identical conformations when complexed to the enzyme, then the alkylating function of both would be positioned in the enzyme complex in an identical fashion and both should attack an enzymic nucleophilic group at the same rate. The 6-methylpyrimidine (III) slowly inactivated dihydrofolic reductase with a half-life of about 220 min. at 37°, compared to II with a half-life of 45 min. Since III has K_i about $8 \times 10^{-5} M$, 40 μM III would convert 33% of the total enzyme to a reversible E · · · I complex; thus compared to 40 μM II—which gives 25% E · · · I complex—II inactivates dihydrofolic reductase about seven times the rate of III. This sevenfold difference in rate proves unequivocally that the main mode of reversible complexing to dihydrofolic reductase by II and III is different as previously suggested for 6-phenylpyrimidines (8). This slow inactivation by III is probably not due to a random bimolecular reaction (1, 14, 15); a more plausible explanation is that one out of seven times III complexes in the same conformation as II—then inactivates through the reversible complex—although this mechanism is not certain.

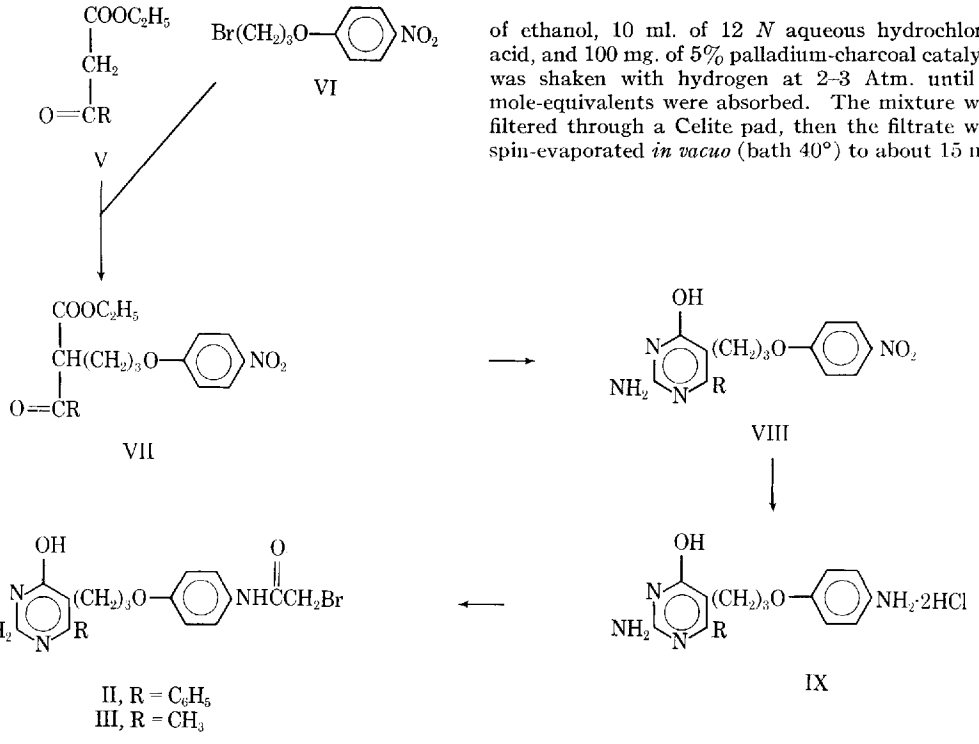
CHEMISTRY

Methods.—The general method of synthesis of 2-amino-6-R-4-pyrimidinols bearing a functionalized phenyl in a 5-side-chain developed earlier in this program (17) worked smoothly for the synthesis, the requisite precursors bearing a terminal nitrophenoxy group on the 5-side-chain (VIII). (Scheme I.) Alkylation of ethyl benzoylacetate (V*a*) with *p*-nitrophenyl-3-bromopropyl ether (VI) (18) in dimethylsulfoxide in the presence of sodium hydride gave the crude β-keto ester (VII*a*); condensation of VII*a* with guanidine carbonate in *tert*-butyl alcohol (19) gave the 4-pyrimidinol in 31% over-all yield of pure material. Earlier ethyl acetoacetate (V*b*) had been converted to VIII*b* *via* VII*b* (20).

Hydrogenation of the 5-nitrophenoxypropyl pyrimidinols (VIII) in ethanolic hydrochloric acid with a palladium-charcoal catalyst afforded pure IX*a* and IX*b* in 69 and 64% yields, respectively. The selective bromoacetylation of IX with bromoacetic anhydride in *N*,*N*-dimethylformamide has been previously described in a paper from this laboratory on selective bromoacylation methods for a variety of types of side-chain amino groups on a variety of 2-aminopyrimidines (16).

Synthesis.—Melting points were determined in capillary tubes on a Mel-Temp block, and those below 230° are corrected. Ultraviolet spectra were determined in 10% ethanol with a Perkin-Elmer 202 recording spectrophotometer. Infrared spectra were determined in KBr pellet with a Perkin-Elmer 137B recording spectrophotometer.

2 - Amino - 5 - (p - nitrophenoxypropyl) - 6-phenyl-4-pyrimidinol (VIIIa).—To a magnetically stirred solution of 3.84 Gm. (20 mmoles) of ethyl benzoylacetate in 10 ml. of reagent dimethylsulfoxide protected from moisture was added 0.866 Gm. of 55.6% sodium hydride dispersed in mineral oil. When hydrogen evolution had ceased, 4.70 Gm. (18 mmoles) of VI (18) was added in one portion. After being stirred at ambient temperature for 18 hr., the



a series, R = C_6H_5
b series, R = CH_3

Scheme I

mixture was heated on a steam bath for 1 hr., then acidified with acetic acid, and poured into 100 ml. of 1:1 benzene–water with good stirring. The separated aqueous phase was extracted with 50 ml. of benzene. The combined benzene solutions were washed with two 50-ml. portions of ice cold 3% aqueous sodium hydroxide to remove unchanged V*a*, then washed with water. Dried with magnesium sulfate, the solution was spin-evaporated *in vacuo* leaving 6.3 Gm. of crude VII*a* as an oil.

The oil was dissolved in 50 ml. of *tert*-butyl alcohol, 1.8 Gm. (10 mmoles) of guanidine carbonate was added, then the mixture was gently refluxed with magnetic stirring for 48 hr. The cooled mixture was filtered and the product was washed with cold ethanol; yield, 2.65 Gm. By dilution of the filtrate with water an additional 0.90 Gm. (total 54%) of crude product was obtained. Two recrystallizations of the combined crude products from aqueous 2-methoxyethanol gave 2.05 Gm. (31%) of pure product as white crystals, m.p. 277–278°; $\lambda_{max.}$ (pH 1): 242 (ϵ 17,200), 290 mμ (ϵ 16,800); (pH 7): 242 (ϵ 17,800), 310 mμ (ϵ 21,900); (pH 13): 242 (ϵ 15,800), 310 mμ (ϵ 16,800); $\lambda_{max.}$ 2.92 (NH); 6.08, 6.31, 6.67 (NH, C=O, C=C, C=N); 6.72, 7.50 (NO_2); 7.93 (ether C—O—C); 11.88 (*p*-C_6H_4); 13.33, 14.40 μ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{18}N_4O_4$: C, 62.3; H, 4.92; N, 15.3. Found: C, 62.3; H, 5.12; N, 15.1.

2 - Amino - 5 - (p - aminophenoxypropyl) - 6-phenyl-4-pyrimidinol Dihydrochloride (IXa).—A mixture of 500 mg. (1.37 mmoles) of VIII*a*, 90 ml. of ethanol, 10 ml. of 12 *N* aqueous hydrochloric acid, and 100 mg. of 5% palladium-charcoal catalyst was shaken with hydrogen at 2–3 Atm. until 3 mole-equivalents were absorbed. The mixture was filtered through a Celite pad, then the filtrate was spin-evaporated *in vacuo* (bath 40°) to about 15 ml. The solution was diluted with ether to turbidity, then chilled at 3° for about 18 hr. The product was collected on a filter and washed with ether; yield, 473 mg. (93%), m.p. 185–190°. Recrystallization from absolute ethanol–ether with the aid of decolorizing carbon gave 315 mg. (69%) of pure product as nearly white crystals, m.p. 187–190°; $\lambda_{max.}$ 2.99 (NH); 5.98 (C=NH^+), 6.15, 6.50 (NH, C=O, C=C, C=N); 8.04 (ether C—O—C); 12.23 (*p*-C_6H_4); 13.35, 14.29 μ (C_6H_5).

Anal.—Calcd. for $C_{19}H_{20}N_4O_2 \cdot 2HCl \cdot H_2O$: C, 53.5; H, 5.63; N, 13.3. Found: C, 53.7; H, 5.77; N, 13.3.

2 - Amino - 5 - (p - aminophenoxypropyl) - 6-methyl-4-pyrimidinol Dihydrochloride (IXb).—Reduction of 304 mg. (1 mmole) of VIII*b* (20), as described for the preparation of IX*a*, gave 288 mg. (83%) of product, m.p. 265–268°. Recrystallization from 2-methoxyethanol–ether with the aid of decolorizing carbon afforded 222 mg. (64%) of pure product as white crystals, m.p. 271–272°; $\lambda_{max.}$ 2.98, 3.10 (NH); 5.94 (C=NH^+); 6.03, 6.31, 6.66 (NH, C=O, C=N, C=C); 8.00 (ether C—O—C), 11.85, 12.05 μ (*p*-C_6H_4).

Anal.—Calcd. for $C_{14}H_{18}N_4O_2 \cdot 2HCl$: C, 48.4; H, 5.76; N, 16.1. Found: C, 48.3; H, 5.88; N, 16.0.

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Chemistry and Pharmacology of a Glycoside of *Vallaris solanacea*

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One of the glycosides of *Vallaris solanacea* has been identified as *O*-acetyl-solanoside (*O*-acetyl acofreosyl-digitoxigenin). It possesses potent cardiotonic activity. Its pharmacological properties have been compared with those of lanatoside C and digoxin.

In a previous communication (1) it was reported that the glycoside mixture obtained from the leaves of *Vallaris solanacea* Kuntze (N.O. *Apocynaceae*) possesses powerful digitalis-like activity. Two recent publications by Kaufmann *et al.* (2, 3), describing the isolation and determination of the structure of six new glycosides from the seeds of *V. solanacea*, have prompted the authors to report the observation of glycoside B, one of the glycosides obtained from the leaves.

EXPERIMENTAL

Chemistry

Shade-dried leaves of *V. solanacea*, collected from Kashmir, India, were percolated with ethanol and the percolate was concentrated to about one-fifth its volume. The concentrate was diluted with an equal volume of water and extracted with benzene. The aqueous phase was concentrated *in vacuo* and extracted with chloroform. The residue from the chloroform extract was taken up in chloroform and chromatographed on a column of silica gel (E. Merck, fine grade), and the column developed with chloroform containing increasing proportions of methanol when three major glycoside fractions A, B, and C were obtained. On thin-layer chromatography [Silica Gel G; solvent system, ethyl methyl ketone–cyclohexane (1:1)] fractions A and C showed up as two spots, while fraction B gave only one spot (spots detected by spraying with water). Preparative thin-layer chromatography of fraction B [Silica Gel G; solvent system, chloroform–isopropyl alcohol (19:1)] gave glycoside B, which was crystallized from chloroform–hexane and ethanol–water mixture, m.p. 137–139° (Kofler block). The angle of rotation was $[\alpha]_D^{20} - 16$ (±2) (c, 1 in methanol). The homogeneity of the glycoside was established by thin-layer chromatography (Silica Gel G) in four solvent systems: ethyl acetate; chloroform–isopropyl alcohol (19:1); ethyl methyl ketone–cyclohexane (1:1); benzene methanol (3:1).

Anal.—Calcd. for $C_{32}H_{48}O_9$: C, 66.6; H, 8.39. Found: C, 67.05; H, 8.75.

Mannich hydrolysis of glycoside B gave a single sugar which was identified as acofreose by paper chromatographic comparison with an authentic sample [solvent systems: methyl ethyl ketone–*n*-butanol (1:1)/borate buffer; toluene–*n*-butanol (1:1)/water], while Killiani hydrolysis yielded digitoxigenin. The highly nonpolar character of glycoside B suggested the possibility of one of its hydroxyl groups being blocked. Its I.R. absorption spectrum and color reactions indicated its identity with *O*-acetyl-acofreoside of digitoxigenin (*O*-acetyl-solanoside) (3). This was confirmed by comparison with an authentic sample of *O*-acetyl-solanoside using thin-layer chromatography as described above.

A thin-layer chromatographic comparison kindly carried out by Reichstein of fractions A and C with the glycosides obtained from the seeds (2, 3), showed that the glycosides contained in the leaves are very similar to those present in the seeds.

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Materials and Methods.—A stock solution of glycoside B containing 500 mcg./ml. was prepared

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Fig. 1.—Isolated papillary muscle of cat. Muscle stimulated supramaximally (12 v., 30/min., 1 msec. duration). At arrow glycoside B (10 mcg./ml.) was added to the bath. Key: 1, normal amplitude of contraction; 2, typodynamic state; 3, onset of improvement in the amplitude of contraction after 8 min. of addition of glycoside B; 4, complete restoration of the amplitude of contraction 40 min. after the addition of the glycoside; 5, decrease in the amplitude of contraction 60 min. after the addition.

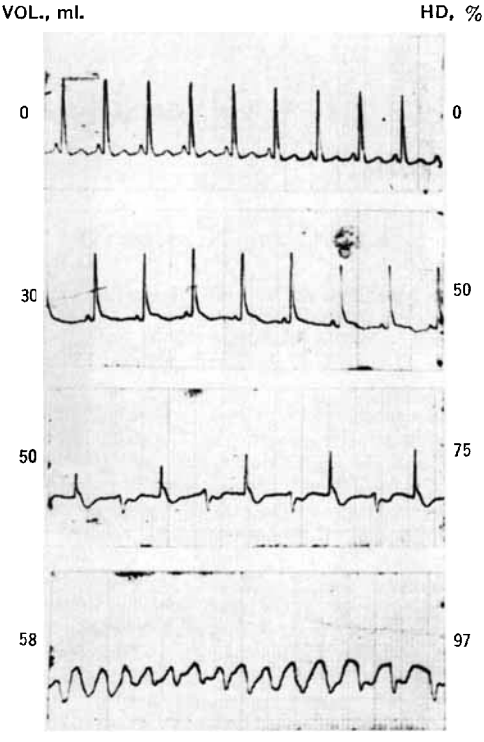

Fig. 2.—Electrocardiographic changes in an anesthetized cat (3.2 Kg.) recorded on standard lead II. Glycoside B was perfused (i.v.) at the rate of 20 mcg./ml./min. Key: 0 ml., normal record, sinus rhythm, T-wave upright, heart rate 210/min.; 30 ml., sinus rhythm, reverted biphasic T-wave, heart rate 180/min.; 50 ml., irregular ventricular extra systoles; 58 ml., ventricular tachycardia and fibrillation. Numbers on the right side denote the percentage of Hatcher dose against volume perfused shown on the left side.

by dissolving 10 mg. in 1 ml. of 90% alcohol and diluting with 19 ml. of normal saline. Further dilutions were made as required just before the experiment.

Cardiotonic Activity.—Direct proof of the cardiotonic activity of glycoside B was obtained by (*a*) perfusing the frog heart (*Rana tigrina*) through the inferior vena cava, (*b*) perfusing isolated hypodynamic guinea pig heart, prepared according to the method of Vick and Kehn (4), and (*c*) studying its action on the hypodynamic papillary muscle of cats (5).

Indirect evidence of the cardiotonic activity of the glycoside was obtained by (*a*) recording electrocardiographic (ECG) changes on standard lead II in anesthetized cats following continuous perfusion, as well as after single 10–50% of Hatcher dose (HD), (*b*) studying its effects on guinea pig or rabbit ileum *in vitro* and on intestinal movements of anesthetized cats.

Intensity of Biological Activity.—This was determined in chloralosed cats (80 mg./Kg. i.v.) by infusing continuously a solution of 25 mcg./ml./min. The concentration was so adjusted as to cause cardiac arrest within 50–60 min. of perfusion. The British Pharmacopoeia (1958) method was employed for the assay in guinea pigs. A concentration of 50 mcg./ml. of glycoside B was used which brought about cardiac arrest in 20–40 min.

Absorption, Persistence, and Cumulative Toxicity.—Its absorption from the gastrointestinal tract after oral feeding was measured in unanesthetized cats by the method of Purdum (6), and in anesthetized cats by the method of Dille and Whatmore (7). The persistence of the glycoside was studied in cats after intravenous administration of 40% HD. Cumulative toxicity was determined by the method described earlier (8) by daily subcutaneous injections of 20 and 40% HD for 21 days, or less if death took place earlier. For comparative purposes digoxin and lanatoside C were used in most of these studies.

RESULTS

Cardiotonic Activity.—*Direct Evidence.*—(*a*) Perfusion of the frog heart with glycoside B in concentrations of 5–30 mcg./ml. produced an initial increase in the amplitude of contraction and a slowing of the heart rate. This was followed by partial A-V block and systolic standstill of the heart.

(*b*) Perfusion of the hypodynamic guinea pig heart with 5 and 10 mcg./ml. concentrations of glycoside B produced a marked positive inotropic effect within 5–7 min. Further perfusion resulted in cardiac irregularities followed by cardiac arrest in 15–20 min.

(*c*) Glycoside B, in a concentration of 0.4–0.5 mcg./ml., restored the contractility of the hypodynamic papillary muscle of the cat. At low concentrations (0.04–0.1 mcg./ml.) improvement in the amplitude of contraction appeared within 5–7 min. of addition, the prefatigue amplitude being attained in 20–30 min. and being well maintained for about 2 hr. At high concentration (0.5 mcg./ml.) the restored contractility lasted for 45–60 min.; there-

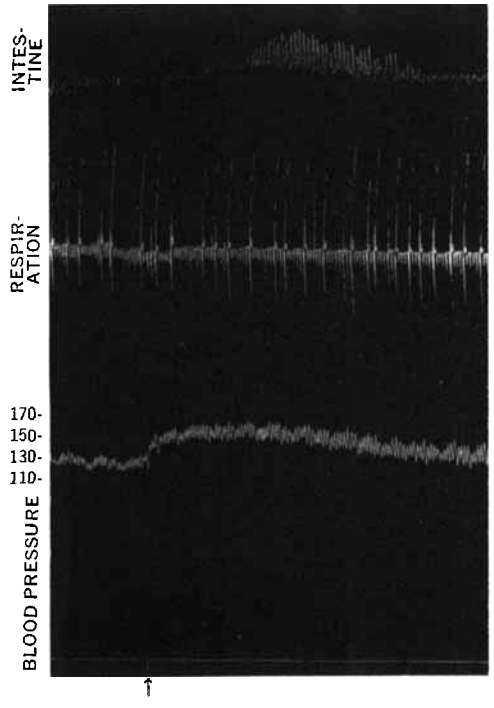

Fig. 3.—The effect of 25% Hatcher dose (arrow) of glycoside B in an anesthetized cat.

TABLE I.—MEAN LETHAL DOSES OF GLYCOSIDE B, LANATOSIDE C, AND DIGOXIN IN CATS AND GUINEA PIGS

Drug	Mean Lethal Dose (mcg./Kg.) Mean ± S.E. Cat	Guinea Pig
Glycoside B	388.2 ± 10.85 (8)[a]	1093.0 ± 29.05 (6)
Lanatoside C	200.2 ± 8.75 (5)	483.9 ± 22.00 (6)
Digoxin	278.0 ± 7.56 (4)	838.17 ± 31.67 (7)

[a] Figures in parentheses indicate the number of animals used.

after, the amplitude of contraction declined steadily and ultimately the muscle became unresponsive to the stimuli (Fig. 1). Occasionally, this concentration increased the irritability of the preparation.

Indirect Evidence.—(*a*) The ECG record following continuous infusion with glycoside B showed bradycardia, prolongation of PR interval, sagging of ST segment, PR dissociation, ventricular extra systoles followed by irregular ventricular rhythm, and cardiac standstill (Fig. 2), typical effects of a cardenolide.

The therapeutic index of glycoside B was calculated from the ECG records. Bradycardia, prolongation of PR interval, inversion of T wave, or sagging of ST segment were considered as the therapeutic stage, the appearance of extra systoles and bundle block as the early toxic stages, and ventricular tachycardia and fibrillation as the severe toxic stage. Results showed that 25–50% of the lethal dose was required to produce the therapeutic stage while 60–90% of the same produced the severe toxic stage. In comparative studies with ouabain (10 mcg./ml.) these changes were produced with 30–35 and 55–75% of the lethal dose, respectively.

A study of the effect of single intravenous doses of glycoside B on the ECG changes in anesthetized cats revealed that 10 and 25% HD produced prolongation of PR from 0.066 to 0.0825 sec., 10–14% bradycardia, and slight increase in QRS within 5–10 min. of administration; change in the T wave was also noticed. On the other hand 50% HD produced inversion of T wave and sagging of ST segment followed by ventricular tachycardia after 25 min. and death after 40 min.

(*b*) In pigeons, intravenous doses of 100, 150, 200, and 300 mcg./Kg. of glycoside B produced 16, 42, 71, and 100% emesis, respectively. The lag period decreased as the dose was increased.

(*c*) Glycoside B in a concentration of 3×10^{-5} produced a marked contraction of isolated segments of guinea pig ileum and increased the tone and amplitude of contraction of isolated rabbit ileum.

Intravenous administration of 25% HD of glycoside B in anesthetized cats produced a slight rise in blood pressure and an increase in the tone and

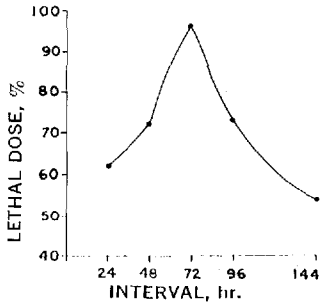

Fig. 4.—Persistence of 40% Hatcher dose of glycoside B administered intravenously denoting time in hours at which the animals were perfused to determine the persistence and the percentage of lethal dose needed to kill the treated animals

TABLE II.—ABSORPTION IN UNANESTHETIZED CATS AFTER 5 hr. AND IN ANESTHETIZED CATS AFTER 1 hr. FOLLOWING THE ADMINISTRATION OF 1 HATCHER DOSE OF GLYCOSIDE B, LANATOSIDE C, AND DIGOXIN

Drug	Hatcher Doses in Unanesthetized Cats (mcg./Kg.) ± S.E. Control	Treated	% Absorption	Hatcher Doses in Anesthetized Cats (mcg./Kg.) ± S.E. Control	Treated	% Absorption
Glycoside B	288.20 ± 6.85 (8)[a]	140.00 ± 4.46 (7)	51.3	329.40 ± 3.87 (3)	0.00 (6)	100.00
Lanatoside C	200.20 ± 8.75 (5)	169.10 ± 2.05 (5)	15.2	183.70 ± 2.50 (3)	157.20 ± 3.25 (3)	14.42
Digoxin	...	...	...	254.20 ± 2.30 (2)	180.00 ± 5.02 (2)	29.13

[a] Figures in parentheses indicate the number of animals used.

TABLE III.—CUMULATIVE TOXICITY OF GLYCOSIDE B, LANATOSIDE C, AND DIGOXIN

Drug	Cats, No.	Hatcher Dose Administration Subcutaneously, %	Deaths, No.	Av. Day of Death	Observations
Lanatoside C	3	20	3/3	12	1 died on 9th day, 1 on 11th day, and 1 on 15th day
		40	3/3	7	2 died on 7th day, and 2 on 9th day
Glycoside B	3	20	2/3	8	2 died on 8th day
	5	40	5/5	4	2 died on 2nd day, 1 on 3rd day, 1 on 5th day, and 1 on 9th day
Digoxin	2	20	2/2	6	1 died on 5th day, and 1 on 7th day

amplitude of contraction of the intestines. The latter effect lasted for 5–10 min. (Fig. 3).

Intensity of Biological Activity.—Table I shows that glycoside B is more active in cats than in guinea pigs, that its potency is three-fourths that of digoxin and one-half that of lanatoside C, both in cats and guinea pigs.

Absorption.—The comparative absorption of glycoside B and of lanatoside C was studied in unanesthetized cats following oral administration of one HD of each. The results, calculated in terms of the absolute absorption, are shown in Table II. Glycoside B was found to be very well absorbed (63.9%) from the gastrointestinal tract, while the absorption of lanatoside C was only 15.2%. Furthermore, the oral absorption of the former was found to be consistent and dependable since it varied only from 52.9 to 70.0% in a series of six animals.

The absorption of the glycoside from the small intestine was studied in five anesthetized cats by administering one HD to the intestine isolated between two ligatures. All animals died between 20–55 min. after administration, indicating that the main site of absorption is the intestine. In these experiments lanatoside C and digoxin showed 14.4 and 29.13% absorption, respectively.

Persistence.—The persistence of glycoside B showed a biphasic phenomenon (Fig. 4). At 72 hr. the glycoside appeared to have been completely eliminated from the system as shown by the percentage of HD required to kill the treated animals. However, after 96 hr. this dose decreased, so much so that at 144 hr. the dose was even lower than that required at the beginning of the experiment. The implication of this observation is discussed later.

Cumulative Toxicity.—The results are shown in Table III along with those obtained with lanatoside C and digoxin. The average number of days taken for the animals to die indicate that the cumulative toxicity of glycoside B lies between that of digoxin and lanatoside C. All the treated animals showed marked emaciation, apparently due to loss of appetite and body weight.

DISCUSSION

Glycoside B obtained from the leaves of *V. solanacea* has been identified as *O*-acetyl-solanoside which is present in the seeds.

Glycoside B is found to possess marked cardiotonic activity which is one-half that of digoxin and one-half and one-fourth that of lanatoside C in cats and guinea pigs, respectively. Its therapeutic index is the same as that of ouabain. It has a quick onset of action like that of ouabain and lanatoside C which may be an advantage over digoxin. This may be attributed to its highly nonpolar character. Its good, consistent, and dependable absorption after oral administration resembles digoxin more than lanatoside C.

The fact that administration of one HD into the intestine invariably produced cardiac standstill within 20–25 min., while the same dose given orally failed to produce cardiac arrest within 6 hr. would indicate that glycoside B is inactivated to some extent by the gastric juices.

In persistence studies glycoside B has been found to possess medium duration of action as the effect of a single dose lasts for 72 hr. In this respect it resembles lanatoside C more than digoxin. However, a peculiar behavior of glycoside B has been observed. At 72 hr. the effect of a single dose was found to have disappeared, but observations carried out at 96 and 144 hr. indicated the reappearance of the effect so much so that at the latter interval it was even greater than the effect produced initially when the glycoside was administered. Obviously, this phenomenon cannot be explained on the basis of the actual reappearance of the glycoside B itself in the system, but must be due to the sensitization of the receptors to the action of the drug so that at 96 and 144 hr. a lesser dose of the glycoside can produce the same effect. Rothlin (9) has reported the phenomenon of sensitization and desensitization, tachyphylaxis, and tolerance with digoxin, digilanid A, and digilanid C in cats. The observation regarding the medium duration of action of glycoside B is also supported by the cumulative toxicity data which show that it possesses a low toxicity compared to digoxin but a high value as compared to lanatoside C.

It may be concluded that glycoside B is a potent cardenolide with a therapeutic index similar to that of the cardiac glycosides used clinically. It has a quick onset, medium duration of action, and shows consistent and dependable oral absorption with low cumulative toxicity.

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Interaction of a Group of Weak Organic Acids and Phenols with a Polyamide

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A drug–plastic sorption study was conducted using six compounds of interest to pharmaceutical and medical scientists. The compounds studied were (*a*) benzoic acid, (*b*) salicylic acid, (*c*) *p*-hydroxybenzoic acid, (*d*) methyl *p*-hydroxybenzoate, (*e*) propyl *p*-hydroxybenzoate, and (*f*) butyl *p*-hydroxybenzoate, while the plastic material was an insoluble polyamide (nylon-6). Sorption and diffusion experiments were conducted at several original concentrations and at three different temperatures, from which it was possible to calculate a number of constants for each agent. Evaluation of the various constants indicated that drug–plastic interactions were due to hydrogen bonding of the agents to the polyamide, but that secondary valence forces of van der Waals type most likely play a predominate role in the interaction or binding mechanism for the more hydrophobic molecules studied. The apparent diffusion coefficients were of the order of 10^{-8} cm.²/sec., while activation energies of diffusion fell within a range of 13.2 Kcal./mole for benzoic acid to 15.5 Kcal./mole for butyl *p*-hydroxybenzoate. Permeation constants had values approaching 10^{-7} cm.²/sec.

In the past this laboratory has reported the interaction of a number of weak organic acids or compounds acting as weak organic acids with several insoluble polyamides (1–5). This report is a continuation of these studies. Six compounds and one specific polyamide[1] were employed in this specific study. Sorption and permeation experiments were conducted on all the compounds at several concentrations and at several temperatures. The data permitted the evaluation of a number of constants, both of a thermodynamic and kinetic nature which in turn helped to propose the mechanism of interaction.

EXPERIMENTAL

Equipment and Supplies.—A Beckman DB spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), benzoic acid (A. R., J. T. Baker Chemical Co., Phillipsburg, N. J.), salicylic acid (A. R., Mallinckrodt Chemical Works, St. Louis, Mo.), *p*-hydroxybenzoic acid (A. R., Eastman Organic Chemicals, Rochester, N. Y.), methyl *p*-hydroxybenzoate (A. R., Eastman Organic Chemicals, Rochester, N. Y.), propyl *p*-hydroxybenzoate (A. R., Eastman Organic Chemicals, Rochester, N. Y.), butyl *p*-hydroxybenzoate (A. R., Eastman Organic Chemicals, Rochester, N. Y.), and nylon-6[2] were employed.

Method of Analysis of Solutions.—The assay for each compound was based upon a spectrophotometric method, using enough diluent to give a final concentration of solute in the range of 2×10^{-3} mg./ml. to 6×10^{-3} mg./ml. for spectrophotometric examination. The absorbance was determined at the optimum wavelength for each compound, using matched 1-cm. silica cells as the sample and reference holders. Table I presents the wavelength of maximum absorbance and diluting vehicle for each compound.

Equilibrium Sorption Studies.—Solutions of benzoic, salicylic, and *p*-hydroxybenzoic acid, as well as methyl *p*-hydroxybenzoate, were prepared in five concentrations, ranging from 0.05 to 0.025%.[3] Due to limited solubility in water, five concentrations of propyl- and butyl *p*-hydroxybenzoate were made in ranges of 0.005 to 0.025%. In all cases, freshly prepared distilled water was utilized as the solvent medium.

Exactly 100 ml. of each solution was pipeted into specially constructed cylindrical glass tubes, measuring 12 in. in length and 1.5 in. in diameter. The tubes were fitted with ground glass stoppers, attached by metal or rubber springs, allowing for total immersion into a water bath at the desired temperature. After a period of time, adequate for equilibrium temperature to be reached in the tubes, the tubes were lifted from the bath, and strips of polyamide (accurately weighed to a total of 2 Gm.) were placed into each tube. The tubes were once again stoppered and carefully placed back into the water bath. Determinations of solute concentrations were made on each solution after sufficient time (approximately 14 days) had elapsed to ensure that equilibrium had been reached. Since blank solutions, exposed to similar temperatures, showed no loss of solute for the time period under consideration, any loss of solute in the test solutions was assumed to have been sorbed by the polyamide. These experiments were conducted at three different temperatures (50 ± 0.02°, 60 ± 0.02°, and 70 ± 0.02°).

The data obtained from these sorption experiments were then treated to determine the solubility coefficient or distribution ratio for each individual compound in the polyamide at the various temperatures under consideration. This solubility coeffi-

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[1] The polyamide employed is known as nylon-6.

[2] Marketed as Capran 77-C by Allied Chemical, Morristown, N. J.

[3] All per cent values expressed as w/v.

TABLE I.—WAVELENGTH OF MAXIMUM ABSORPTION

Compd.	Diluent	Optimum Wavelength, mμ
Benzoic acid	0.1 *N* HCl	230
Salicylic acid	0.1 *N* HCl	236
p-Hydroxybenzoic acid	0.1 *N* HCl	255
Methyl *p*-hydroxybenzoate	Distilled water	255
Propyl *p*-hydroxybenzoate	Distilled water	255
Butyl *p*-hydroxybenzoate	Distilled water	255

TABLE II.—SOLUBILITY COEFFICIENTS OF COMPOUNDS IN POLYAMIDE AT THREE TEMPERATURES

Compd.	S (moles/Kg./moles/L.) 50°	60°	70°
Benzoic acid	19.08	15.51	12.97
Salicylic acid	33.23	26.81	19.97
p-Hydroxybenzoic acid	19.44	15.96	12.65
Methyl *p*-hydroxybenzoate	25.29	21.74	18.04
Propyl *p*-hydroxybenzoate	88.23	77.63	72.95
Butyl *p*-hydroxybenzoate	93.17	87.98	80.90

cient was evaluated by the use of the following equation:

$$S = C_s/C_L \qquad \text{(Eq. 1)}$$

where S is the solubility coefficient relating the quantity of solute in the solid phase to the quantity of solute in the liquid phase; C_s is the equilibrium concentration of solute in the solid phase (nylon) in moles/Kg.; and C_L is the equilibrium concentration of solute in the liquid phase in moles/L. By plotting values of C_s against the corresponding values for C_L, a linear relation resulted for all the compounds at the three temperatures. From the slopes, the solubility coefficients were deduced. Table II presents the calculated C_s/C_L or solubility coefficients for the six compounds at each of the temperatures studied.

Standard Affinity, Heat of Sorption, and Entropy of Sorption.—To gain an insight into the attraction forces between the various compounds and the polyamide, standard affinities ($-\Delta\mu°$) were calculated by the use of the expression:

$$-\Delta\mu° = RT \ln S \qquad \text{(Eq. 2)}$$

where S is the equilibrium sorption constant and is equal to the solubility coefficient depicted in Eq. 1 while R and T are the usual gas constant and absolute temperatures, respectively. Table III includes the standard affinities for all the compounds at each of the three temperatures.

In a relatively narrow temperature range, the standard affinity is related to the standard heat of sorption ($\Delta H°$) through the equation shown below:

$$\Delta\mu°/T = \Delta H°/T + C \qquad \text{(Eq. 3)}$$

where all the terms are as previously defined, while C stands for a constant of integration. From the slope of the line, the heat of sorption may be calculated. Calculated values for the heats of sorption for each of the compounds are found in Table III.

Since the standard affinity, $\Delta\mu°$, may be considered as a change in free energy as the solute migrates from the solution to the solid phase (nylon), and $\Delta H°$ represents the heat of sorption or enthalpy, it follows that the entropy ($\Delta S°$) of sorption may be evaluated through the thermodynamic relationship:

$$\Delta\mu° = \Delta H° - T\Delta S° \qquad \text{(Eq. 4)}$$

The entropy values have also been included in Table III.

Permeation and Diffusion Studies.—Since the sorption process of dyes and other agents in nylon is governed primarily by the diffusion step in the substrate, experiments were conducted to evaluate the apparent diffusion coefficient and permeability constant for each compound at the three temperatures given earlier and at a number of original concentrations.

Solutions of benzoic, salicylic, and *p*-hydroxy-

TABLE III.—STANDARD AFFINITIES ($\Delta\mu°$), STANDARD HEATS OF SORPTION ($\Delta H°$), AND STANDARD ENTROPIES OF SORPTION ($\Delta S°$) FOR A GROUP OF COMPOUNDS *Via* EQUILIBRIUM SORPTION STUDIES

Temp.	$-\Delta\mu°$ (Kcal./mole)	$\Delta H°$ (Kcal./mole)	$\Delta S°$ (cal./mole °)
	Benzoic Acid		
50	1.89		
60	1.81		
70	1.75		
		−4.26	−7.33
	Salicylic Acid		
50	2.25		
60	2.18		
70	2.04		
		−5.60	−10.3
	p-Hydroxybenzoic Acid		
50	1.90		
60	1.83		
70	1.73		
		−4.74	−8.77
	Methyl p-Hydroxybenzoate		
50	2.07		
60	2.04		
70	1.97		
		−3.72	−5.08
	Propyl p-Hydroxybenzoate		
50	2.88		
60	2.88		
70	2.92		
		−2.11	+2.36
	Butyl p-Hydroxybenzoate		
50	2.91		
60	2.96		
70	2.99		
		−1.56	+4.19

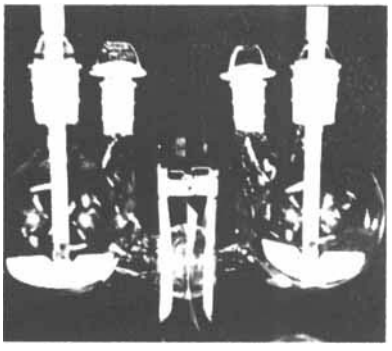

Fig. 1.—Photograph showing permeation cell with plastic film dividing the two chambers.

benzoic acids and methyl *p*-hydroxybenzoate were prepared in three concentrations ranging from 0.10 to 0.30%.

Through the use of a specially designed permeation cell, as shown in Fig. 1, permeation and diffusion studies were conducted in the following manner. Since the cell was composed of two separate flasks, divided by a square of polyamide (approximately 4 in. square with a thickness of 0.010 in.), exactly 500 ml. of the solution under investigation was added to one flask, simultaneously with the addition of an equal volume of distilled water to the other flask. The cell was then lowered into the water bath, and the stirring sleeves connected to an external power source to apply a constant rate of agitation in each flask. At periodic time intervals, aliquots of equal volume were removed from each flask, and set aside until they had reached room temperature. Spectrophotometric assays were made on those samples taken from the flask originally containing only distilled water, to determine the quantity of solute transversing the film barrier. Since the amount of solute passing through the film during the initial portions of the experiment was insignificant in comparison to the amount originally present in the high-concentration side, no assays were made of samples taken from this side of the cell. Sample withdrawal was continued until a steady state transmission was well established, with equal quantities of solute entering the low concentration flask per unit time.

Figure 2 demonstrates the permeation of *p*-hydroxybenzoic acid through the polyamide as a function of time. By plotting the total amount of solute permeating the nylon barrier against time, it is noted that following a short build-up period, a steady state of transmission is reached. The slope of the line representing the attainment of the steady state was then calculated by the method of least squares, and converted into units of moles of solute entering the flask/sec., so that the permeability constant could be obtained through the use of:

$$P = C/t \cdot LV/CA \qquad \text{(Eq. 5)}$$

where C/t is the slope of the linear portion of the permeation plot in moles/sec.; L is the thickness of the film barrier in cm.; V is the total volume under consideration in cm.^{3}; C is the initial concentration of the solution in moles per total volume; and A is the surface area of the nylon barrier through which the solute is permeating in cm.^{2}. Through the cancellation of terms, P, the permeability constant is reduced to the units of cm.^{2}/sec.

From the same figure it is possible to evaluate the apparent diffusion coefficient by use of the time-lag equation of Barrer (6), given as:

$$D = L^2/6\tau \qquad \text{(Eq. 6)}$$

where D is the apparent diffusion coefficient in cm.^{2}/sec., L is the thickness of the film in cm., and τ is the time-lag intercept obtained by extrapolation of the steady state slope to the time axis of the permeation plot, converted into seconds.

Table IV presents the values for the apparent diffusion coefficients and the permeability coefficients for all six compounds investigated in the study, at each of the three concentrations and temperatures involved.

Activation Energies of Diffusion and Permeation.—In all compounds studied, it was noted that the rates of diffusion and permeation were temperature dependent. This temperature dependence may be quantitatively expressed by determining the activation energies of diffusion and permeation and can be evaluated through the following equation:

$$\log D = \log D_0 - \Delta E_D/2.303\ RT \qquad \text{(Eq. 7)}$$

where D is the diffusion coefficient, ΔE_D the activation energy, and R and T the usual constants. A similar expression for permeation may be used to evaluate the activation energy of permeation (ΔE_P). Plots of log D *versus* $1/T$ or log P *versus* $1/T$ for each of the compounds gave linear relationships from which the activation energies were calculated. These values are shown in Table V.

Thermodynamic Constants from Kinetic Experiments.—Barrer (6) was able to show that the permeability constant and the apparent diffusion coefficient are related by the following expression:

$$S = P/D \qquad \text{(Eq. 8)}$$

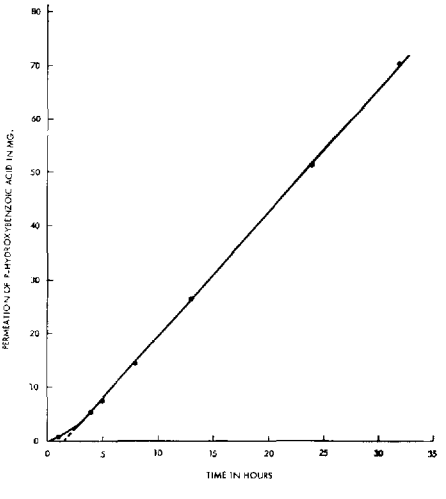

Fig. 2.—A plot of the concentration of *p*-hydroxybenzoic acid permeating through polyamide film *vs.* time.

where P is the permeability constant expressed in cm.²/sec.; D is the apparent diffusion coefficient in similar units; and S is defined as the solubility coefficient.

TABLE IV.—DIFFUSION COEFFICIENTS AND PERMEABILITY CONSTANTS FOR A GROUP OF WEAK ORGANIC ACIDS AND PHENOLS AT THREE TEMPERATURES (POLYAMIDE)

Temp., °C.	Original % Concn.	$D \times 10^8$, cm.²/sec.	$P \times 10^7$, cm.²/sec.
	Benzoic Acid		
50	0.30	0.546	1.08
60	0.30	1.18	1.83
70	0.30	2.03	2.64
50	0.20	0.689	1.15
60	0.20	1.19	1.65
70	0.20	2.20	2.64
50	0.10	0.750	1.00
60	0.10	1.29	1.45
70	0.10	2.27	2.21
	Salicylic Acid		
50	0.30	0.520	1.54
60	0.30	0.961	2.29
70	0.30	1.76	3.44
50	0.20	0.374	1.25
60	0.20	0.771	1.94
70	0.20	1.58	3.22
50	0.10	0.264	1.02
60	0.10	0.618	1.65
70	0.10	1.43	3.02
	p-Hydroxybenzoic Acid		
50	0.30	0.452	0.933
60	0.30	0.855	1.35
70	0.30	1.87	2.46
50	0.20	0.483	0.919
60	0.20	1.03	1.48
70	0.20	2.01	2.31
50	0.10	0.522	0.906
60	0.10	1.15	1.43
70	0.10	2.30	2.15
	Methyl p-Hydroxybenzoate		
50	0.30	0.579	1.38
60	0.30	1.12	2.31
70	0.30	2.00	3.45
50	0.20	0.551	1.35
60	0.20	1.07	2.29
70	0.20	1.89	3.40
50	0.10	0.521	1.33
60	0.10	1.01	2.26
70	0.10	1.81	3.35
	Propyl p-Hydroxybenzoate		
50	0.03	0.371	3.12
60	0.03	0.756	5.61
70	0.03	1.40	9.34
50	0.02	0.362	3.12
60	0.02	0.718	5.56
70	0.02	1.30	9.16
50	0.01	0.354	3.09
60	0.01	0.703	5.48
70	0.01	1.21	8.98
	Butyl p-Hydroxybenzoate		
50	0.03	0.304	2.66
60	0.03	0.648	5.40
70	0.03	1.31	9.19
50	0.02	0.298	2.64
60	0.02	0.616	5.35
70	0.02	1.22	8.99
50	0.01	0.288	2.63
60	0.01	0.586	5.10
70	0.01	1.12	8.84

TABLE V.—ACTIVATION ENERGIES OF DIFFUSION AND PERMEATION FOR A GROUP OF WEAK ORGANIC ACIDS AND PHENOLS IN THE POLYAMIDE FILM

Compd.	ΔE_D (Kcal./mole)	ΔE_P (Kcal./mole)
Benzoic acid	13.2	9.22
Salicylic acid	16.3	10.4
p-Hydroxybenzoic acid	15.9	10.1
Methyl *p*-hydroxybenzoate	13.6	10.2
Propyl *p*-hydroxybenzoate	14.1	11.9
Butyl *p*-hydroxybenzoate	15.5	13.5

Values for the solubility coefficient, S, were obtained using Eq. 8, and were seen to closely approximate values for this constant obtained by equilibrium sorption studies and calculated by Eq. 1. Using solubility coefficients obtained through permeation and diffusion studies, it was then possible to calculate the standard affinity through Eq. 2, the standard heat of sorption *via* Eq. 3, and with Eq. 4 the standard entropy of sorption.

Table VI presents these thermodynamic constants derived from permeation and diffusion studies; generally, close correlation is seen to those in Table III obtained by equilibrium sorption studies.

DISCUSSION

Equilibrium Sorption Studies.—Examination of Table II in which the solubility coefficients for the six compounds in the polyamide are given, reveals that benzoic acid, salicylic acid, and *p*-hydroxybenzoic acid have similar values with salicylic acid being somewhat more soluble in the polyamide. Upon esterification of the carboxyl group of *p*-hydroxybenzoic acid, solubility of the compounds in the nylon phase is seen to increase markedly as the hydrophobic nature of the ester is increased.

With all compounds, and more prominently with the weak organic acids, it was noted as the temperature increased, the solubility coefficients were reduced. This would seem to suggest that the binding process between the nylon and these solutes was temperature dependent, with increased temperature serving to hinder solute-plastic binding.

Standard Affinity, Heat of Sorption, and Entropy of Sorption.—Standard affinity may be considered as being the ability of a particular substrate to attract and hold solute molecules, and is mathematically dependent upon the distribution of solute in the solid and liquid phases at equilibrium, as depicted in Eq. 2.

As seen in Table III, in most cases the standard affinity decreases with increased temperature, thus paralleling what has been shown for dyes and other weak organic acids (7). With the higher molecular weight esters of *p*-hydroxybenzoic acid, however, this effect is not noted, since the mechanism of interaction of these solutes is not so temperature dependent as with the weak organic acids.

An examination of the standard heats of sorption and standard entropies of sorption for these solutes sorbed by the polyamide (Table III) leads to several suggestions concerning the mechanisms of interaction.

In regard to benzoic, salicylic, and *p*-hydroxy-

benzoic acids, and methyl *p*-hydroxybenzoate, it is seen that the standard heats of sorption range from −3.7 to −5.6 Kcal./mole. Since the energy requirement for hydrogen-bond formation is considered to be from −4 to −6 Kcal./mole, these experimental values indicate, at least on an energy requirement basis, the formation of a hydrogen bond between these solutes and the polyamide. Presumably, bonding in the case of the weak organic acids would occur between the carboxyl group of the acid and the amide linkages in the nylon chains. Due to the presence of a phenolic hydroxyl group in methyl *p*-hydroxybenzoate, bonding would likely occur between this moiety and the amide linkages in the nylon. Heats of sorption for the propyl and butyl esters of *p*-hydroxybenzoic acid are −2.1 and −1.6 Kcal./mole, respectively, thus leading to the assumption of weak secondary valence forces as being responsible for the binding process between these compounds and the polyamide.

A consideration of the experimental values for the standard entropies of sorption for these compounds may shed further light upon the binding processes.

The concept of entropy as related to drug–plastic interactions encompasses two realms of reasoning. One trend of thought is to consider entropy as being indicative of the probability of a combination occurring between solute and substrate. The greater the increase in entropy, or entropy of sorption, the greater will be the probability of combination between the interactants. Table III reveals that the entropy of sorption is increasing in the cases of the propyl and butyl esters of *p*-hydroxybenzoic acid, while this increase is not noted with the weak organic acids and methyl *p*-hydroxybenzoate. Based upon changes in entropy, as the solute migrates from one phase to another, there would be a greater probability of combination between the higher molecular weight esters and the polyamide than with the weak organic acids and the same substrate. This viewpoint seems to be well taken, in that the propyl and butyl esters show greater solubility coefficients and higher standard affinities for the polyamide.

The second method of interpreting entropy calculations is to relate them to the degree of randomness and disorder of a given system. Classically, it is well accepted that the more natural system will have a completely randomized distribution of components within the system. As randomness and disorder increases, a corresponding increase in the entropy of the system is also seen. Thus, the system with the higher entropy value will be the one in which the greater degree of random distribution is present. Therefore, should entropy calculations for a particular drug–plastic interaction indicate an increase in entropy, then the solute is traveling into a more random distribution. Conversely, should calculations result in a decrease in the entropy of the system, then the solute is being firmly bound at fixed specific sites in the plastic.

Table III shows that a decrease in entropy occurs for the binding of the weak organic acids and methyl *p*-hydroxybenzoate by the polyamide. When one considers both the entropy change and the standard heats of sorption for these compounds sorbed by the polyamide, the assumption of hydrogen-bond formation at the amide linkages of the polymer appears rational. Reduction of the entropy of these systems indicates that the solutes are being held in fixed, specific sites in the plastic. The higher entropy of sorption values for propyl and butyl *p*-hydroxybenzoate indicate that these compounds are being more randomly distributed throughout the polymer, *via* the formation of weak secondary valence forces at various nonspecific sites along the polymer chain. It is felt that the phenolic hydroxyl group present in these two compounds serves as a primary attractive force, bringing the molecules into the proximity of the amide linkages. However, as the size of the ester moiety is increased, the solute is pulled away from these amide linkages, and becomes more randomly distributed throughout the chains.

Table VI.—Thermodynamic Constants from Permeation and Diffusion Studies

Temp., °C.	S^a	$-\Delta\mu^{\circ b}$	$\Delta H^{\circ c}$	$\Delta S^{\circ d}$
		Benzoic Acid		
70	11.58	1.66		
60	13.51	1.72	−3.93	−6.61
50	16.63	1.80		
		Salicylic Acid		
70	20.33	2.05		
60	25.21	2.14	−5.60	−10.3
50	33.93	2.26		
		p-Hydroxybenzoic Acid		
70	11.33	1.65		
60	14.17	1.75	−4.78	−9.06
50	19.00	1.89		
		Methyl p-Hydroxybenzoate		
70	17.95	1.97		
60	21.48	2.03	−3.46	−4.32
50	24.56	2.05		
		Propyl p-Hydroxybenzoate		
70	70.47	2.90		
60	76.56	2.87	−2.20	+2.04
50	85.88	2.86		
		Butyl p-Hydroxybenzoate		
70	74.35	2.94		
60	85.71	2.95	−1.99	+2.78
50	89.07	2.88		

[a] S, solubility coefficient. [b] $-\Delta\mu^{\circ}$, standard affinity (Kcal./mole). [c] ΔH°, standard heat of sorption (Kcal./mole). [d] ΔS°, standard entropy of sorption (cal./mole °).

Permeation and Diffusion Studies.—The data presented in Table IV, listing the permeability constants and apparent diffusion coefficients for these compounds in the polyamide, allow certain relationships to be drawn.

It is noted that in general the rate of permeation is directly dependent upon the quantity of solute originally present in the high-concentration side of the cell. Thus, as the initial concentration of a given solute is lowered, there will be a decrease in the permeation rate for that compound through the polyamide. Since permeation follows a steady state relationship, the concentration of solute within the film remains constant, and as much solute escapes from one side of the film as enters the other side. As the amount of solute entering the film is increased, more solute appears in the low concentration side per unit time, and the rate of permeation increases.

Where the permeation process is concerned with the rate of escape of solute molecules from the film into another phase, the actual travel of these molecules through the matrix of the plastic is considered to be the diffusion process. The time between initial contact at the one side and the final escape from the opposite side is the time during which the diffusion process occurs.

It is also noted that the original concentration of solute will affect the diffusion rate, but in a different manner than with permeation. Diffusion concerns itself only with the amount of solute within the matrix of the film, and not with the concentration surrounding it. Thus, diffusion is a function of the number of solute molecules capable of entering the polymer matrix, which may not be dependent upon the external environment.

Table IV shows that as the initial concentration of benzoic and *p*-hydroxybenzoic acids is increased, the diffusion rate is lowered. Due to the probability of greater degrees of dimerization in higher concentrations, through random movement and collision of molecules in solution, there would result the formation of complexes too large and bulky to enter the polymer matrix. As concentration is reduced, more individual molecules are available to diffuse into the film, and the diffusion rate increases. This effect has also been noted by Barrer and Barrie in the diffusion of water through ethyl cellulose (8). Vasenin (9) reported on the formation of clusters with increased concentrations of various paraffins and olefins in studies on their diffusion through certain polymers, while Peters (10) encountered similar phenomena with the diffusion of dye molecules through polymer films and fibers.

This effect is not noted with salicylic acid, probably due to the formation of intramolecular hydrogen bonds between the *ortho*-hydroxyl group and the carboxyl group, thus reducing the possibility of dimer formation. Since the esters have no carboxyl groups, dimerization is eliminated, and diffusion rates increase directly with increased original concentrations.

As the size of the solute is increased in a homologous series, (as with the esters of *p*-hydroxybenzoic acid), a decrease in the rates of permeation and diffusion is noted.

Activation Energies of Diffusion and Permeation.—Activation energy refers to the amount of energy a given molecule must acquire in order for it to be capable of overcoming the restraint of its surroundings. This energy is obtained through random exchange with neighboring molecules, and is temperature dependent in that molecular movement is directly related to increased temperature.

Table V presents the activation energies of diffusion and permeation for the compounds studied in this investigation. As in the cases of permeation and diffusion, increases in the molecular weight of a homologous series of compounds will affect the activation energy for the process. Thus, more energy is needed for the movement of the bulkier and heavier butyl *p*-hydroxybenzoate than for methyl *p*-hydroxybenzoate.

Kinetic Studies Versus Equilibrium Sorption Studies.—Table VI lists the thermodynamic constants calculated from permeation and diffusion studies, and shows the close agreement with similar constants derived from equilibrium sorption studies for each compound studied (Tables II and III).

Since sorption studies require a time period of approximately 14 days to complete, and permeation studies may be completed in less than 48 hr., it is apparent that the latter method is of extreme value. Permeation and diffusion studies reveal data of a kinetic nature as well as valid approximations of thermodynamic constants leading to possible suggested mechanisms of interaction.

SUMMARY

Studies were conducted on the interaction of six weak organic acids and phenols with a polyamide, at a number of concentrations and three temperatures. From these experiments, it was possible to evaluate several constants for each individual interaction, such as (*a*) the solubility coefficient of the solute in the polyamide, (*b*) the standard affinity, (*c*) the standard heat of sorption, (*d*) the standard entropy of sorption, (*e*) the apparent diffusion coefficient, (*f*) the permeability constant, and (*g*) the activation energies of permeation and diffusion.

Values obtained for the standard heats of sorption and standard entropies of sorption for the interaction of the polyamide with benzoic, salicylic, and *p*-hydroxybenzoic acids and methyl *p*-hydroxybenzoate, indicate the formation of hydrogen bonds between these compounds and the polyamide at the amide linkages of the nylon chains. Constants calculated for the propyl and butyl esters of *p*-hydroxybenzoic acid show that these compounds are bound to the polyamide through weak secondary valence forces, and are randomly distributed throughout the polymer chains.

Apparent diffusion coefficients for the diffusion of benzoic acid and *p*-hydroxybenzoic acid in the polyamide reveal that increased initial concentrations lead to lower apparent diffusion coefficients, due to the formation of dimers incapable of entering the polymer matrix.

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Rate Studies on the Anaerobic Degradation of Ascorbic Acid IV

Catalytic Effect of Metal Ions

By PER FINHOLT, HARALD KRISTIANSEN, LESZEK KRÓWCZYŃSKI*, and TAKERU HIGUCHI†

The effect of different metal ions on the rate of the anaerobic degradation of ascorbic acid in aqueous solution has been studied. Among the bivalent metal ions tested, Pb^{2+} was the most powerful catalyst, followed by Zn^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+}, Ni^{2+}, Ca^{2+}, and Mg^{2+}. Also the trivalent metal ions Al^{3+} and Cr^{3+} catalyzed the process, Al^{3+} being more active than Cr^{3+}. The experimental results seem to indicate that the metal ions form complexes with ascorbic acid.

ALTHOUGH the catalytic effect of metals on the oxidative degradation of ascorbic acid has been well studied (1–6), no papers have appeared dealing with the influence of metals on the anaerobic degradation. Since the degradation of ascorbic acid in many liquid pharmaceutical preparations appears to follow largely the non-oxidative route, and since these preparations may contain or may be contaminated by metals, a study of the effect of metal ions on the rate of the anaerobic degradation of ascorbic acid in aqueous solution was felt necessary.

Several authors (7–11) have shown that ascorbic acid is decarboxylated when heated in aqueous solution under anaerobic conditions. It is known from other papers that metal ions may catalyze the decarboxylation of keto-acids (12, 13). By the decarboxylation of dimethyloxaloacetic acid in the presence of heavy metals a complex between the metal ion and the diion of the acid is formed and this complex undergoes a rapid decarboxylation (12).

EXPERIMENTAL

Materials.—The following metal salts were used in this study: $Ca(NO_3)_2$, $MgSO_4$, $NiSO_4$, $MnSO_4$, $Mn(NO_3)_2$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, $Zn(NO_3)_2$, $Pb(CH_3COO)_2$, $Pb(NO_3)_2$, $CuSO_4$, $AlCl_3$, and $CrCl_3$. Solutions of the metal salts were standardized when necessary with sodium ethylenediaminetetraacetate.

The ascorbic acid used was of P. Nord. (Pharmacopoea Nordica) quality. The metal salts and all reagents were of analytical grade. The water used was distilled water redistilled from a neutral glass still, boiled, and cooled under oxygen-free nitrogen.

Assay.—The residual ascorbic acid concentration of the heated solutions was determined iodometrically. An aliquot part of the sample, usually 5.00 ml., was acidified with diluted sulfuric acid and titrated under nitrogen with 0.01 *N* iodine using a few drops of starch T.S. as indicator. The metal salts added did not interfere.

Kinetic Studies.—Quantities of 0.01 *M* ascorbic acid solutions, buffered or unbuffered, containing the actual metal salt, and a sufficient amount of sodium chloride or potassium nitrate[1] to give the solutions an ionic strength of 0.5, were prepared. The solutions were filled into 5-ml. ampuls, and the air in the ampuls was replaced by nitrogen. The ampuls were sealed and heated at 96° in a constant-temperature bath. At appropriate intervals ampuls were taken out from the bath, cooled on ice, and the solution analyzed.

RESULTS AND DISCUSSION

Order of Reaction with Respect to Ascorbic Acid.—The anaerobic degradation of ascorbic acid in the presence of each of the metal salts tested was found to be strictly first order with respect to ascorbic acid.

Catalytic Effect of Bivalent Metal Ions on the Anaerobic Degradation of Ascorbic Acid.—Figure 1 shows the effect of Mn^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, and Pb^{2+} on the rate of the anaerobic degradation of ascorbic acid at pH 2–6. (Amount of metal salt added, 0.05 moles/L.; buffer, 0.3 *M* acetate.) The effect of Ni^{2+}, Ca^{2+}, and Mg^{2+} was also tested, but the results for these cations are omitted in Fig. 1. Ni^{2+} showed very close to the same effect as Mn^{2+}. Ca^{2+} and Mg^{2+} had virtually no effect.

No rate studies were carried out at pH >6, because most metals caused precipitations in the ascorbic acid solutions at pH >6.

Experiments with Cu^{2+} as catalyst showed that ascorbic acid at pH >2 was oxidized by this metal even in the absence of air. At pH 0.4 addition of 0.01 moles/L. of $CuSO_4$ had a slight catalytic effect on the anaerobic degradation of ascorbic acid.

Figure 1 shows that all active metals have the highest effect at pH 4–6. Because of this all further studies of the catalytic effect of the different metals were made at pH values within this region.

The effect of addition of different amounts of

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[1] Potassium nitrate was used in experiments involving lead because of the low solubility of lead chloride. In experiments involving the other metals tested sodium chloride was used, if not otherwise stated.

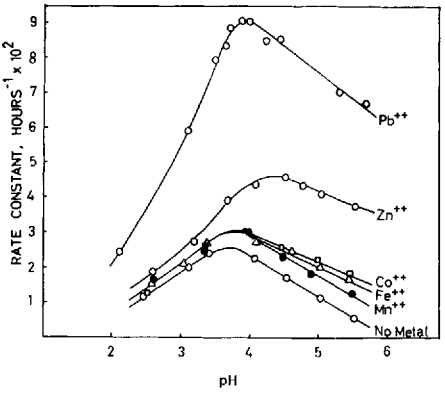

Fig. 1.—Effect of bivalent metal ions on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at different pH values and 96°. Metal salts used: $MnSO_4$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, and $Pb(CH_3COO)_2$. Amount of metal salt added, 0.05 moles/L.; buffer, 0.3 *M* acetate; ionic strength, 0.50.

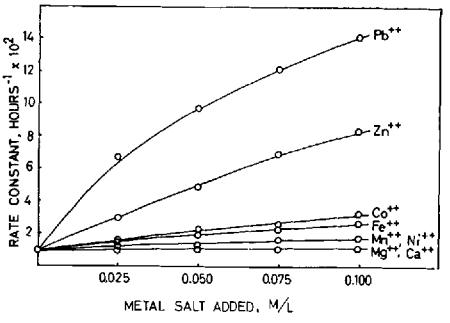

Fig. 2.—Effect of bivalent metal ions on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at pH 5 and 96°. Metal salts used: $Ca(NO_3)_2$, $MgSO_4$, $NiSO_4$, $MnSO_4$, $FeSO_4$, $CoCl_2$, $ZnSO_4$, and $Pb(CH_3COO)_2$. Buffer, 0.1 *M* acetate; ionic strength, 0.50.

bivalent metal salts on the rate of the anaerobic degradation of ascorbic acid at pH 5 (0.1 *M* acetate as buffer) is shown in Fig. 2. The results given for Zn^{2+} in Fig. 2 are from two parallel series of runs, one series made with NaCl, the other with KNO_3 for correction of ionic strength. There was no difference in the results from the two series of runs.

Since acetate may form complexes with metal ions, the two most active metals, lead and zinc, and one of the less active metals, manganese, were also tested in buffer-free solutions. The nitrates of the metals were used because nitrate ions have a very low tendency to form complexes with metal ions. For comparison lead was also tested as acetate and zinc as sulfate. The studies were made at pH 4. This pH value was chosen because ascorbic acid has such a buffer capacity at pH 4 that no addition of buffer was needed to keep the pH of the ascorbic acid solutions constant during the experiments.

The solutions were made up to an ionic strength of 0.5 with KNO_3. The results are found in Fig. 3. It will be seen that the nitrate of lead is a little more active than the acetate. The reason for this must

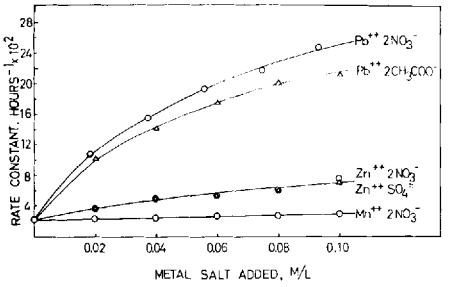

Fig. 3.—Effect of different salts of bivalent metals on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at pH 4 and 96°. Ionic strength, 0.50; no buffer added.

be that the lead ions in the acetate are partly bound as complex. The nitrate and the sulfate of zinc have the same effect. Manganese nitrate is nearly noncatalytic.

Most of the lines in Figs. 2 and 3 are slightly curved. This may be explained by assuming a weak complex formation between ascorbic acid (HA) and metal ions (Me^{2+}) and a subsequent degradation of ascorbic acid in these complexes.

The following reactions may be assumed to take place at pH 2–6:

$$HA + Me^{2+} \rightleftharpoons AMe^+ + H^+ \quad \text{Reaction } 1$$

$$HA \xrightarrow{k_1} \text{products}$$

$$A^- \xrightarrow{k_2} \text{products}$$

$$AMe^+ \xrightarrow{k_3} \text{products}$$

The following equations are valid:

$$-\frac{d[A_T]}{dt} = k_1[HA] + k_2[A^-] + k_3[AMe^+] \qquad \text{(Eq. 1)}$$

$$A_T = [HA] + [A^-] + [AMe^+] \qquad \text{(Eq. 2)}$$

$$-\frac{d[A_T]}{dt} = k[A_T] \qquad \text{(Eq. 3)}$$

$$\frac{[AMe^+][H^+]}{[HA][Me^{2+}]} = k' \qquad \text{(Eq. 4)}$$

$$\frac{[A^-][H^+]}{[HA]} = k_a \qquad \text{(Eq. 5)}$$

$$[Me^{+2}] + [AMe^+] = b \qquad \text{(Eq. 6)}$$

In the experiments the amount (b) of metal salt added (in moles per liter) is 2 to 10 times the total ascorbic acid concentration. Since the lines in Figs. 2 and 3 are but *slightly* curved, only a small part of the ascorbic acid has formed complex with the metal ions even at the highest metal salt concentrations. For these reasons it is evident that $[Me^{2+}] \gg [AMe^+]$ in all the experiments. We may, therefore, without introducing any great error, put $[Me^{2+}] = b$ in Eq. 4. Doing so and combining Eq. 4 with Eqs. 1, 2, 3, and 5 give

$$k = \frac{k_1[H^+] + k_2k_a + k_3k'b}{[H^+] + k_a + k'b} \qquad \text{(Eq. 7)}$$

TABLE I.—STABILITY CONSTANTS (k') OF METAL ION ASCORBIC ACID COMPLEXES AND RATE CONSTANTS (k_3) FOR THE DEGRADATION OF ASCORBIC ACID IN THESE COMPLEXES

Metal Ion	pH	$k' \times 10^3$	k_3 hr.$^{-1}$ × 10
Pb^{2+}	4	3.5	4
Zn^{2+}	4	3.8	1.1
Zn^{2+}	6	0.9	1.9

The experiments, the results of which are given in Figs. 2 and 3, are made at constant pH and ionic strength. $[H^+]$ is therefore a constant and Eq. 7 becomes the equation of a hyperbola with k and b as the two variables. Consequently, plots of rate constants found *versus* amount of metal salt added have to yield curved lines.

From the experiments with $Pb(NO_3)_2$ at pH 4 (Fig. 3) k_3 and k' in Eq. 7 could be calculated. The earlier found values for k_1 and k_2 were used. ($k_1 = 8.9 \times 10^{-3}$ hr.$^{-1}$, $k_2 = 5 \times 10^{-3}$ hr.$^{-1}$) (14). The calculation gave $k_3 = 4 \times 10^{-1}$ hr.$^{-1}$ and $k' = 3.5 \times 10^{-3}$. According to this calculation, the rate of degradation of ascorbic acid in the lead complex is about 50 times faster than the rate of degradation of the free ascorbic acid. (Table I.)

Similar calculations based on the experiments with $Zn(NO_3)_2$ at pH 4 (Fig. 3) gave $k_3 = 1.1 \times 10^{-1}$ hr.$^{-1}$ and $k' = 3.8 \times 10^{-3}$.

The stability constant (k') is nearly the same for the zinc–ascorbic acid complex as for the lead–ascorbic acid complex. The rate of degradation of ascorbic acid, however, is considerably faster in the lead complex than in the zinc complex.

In Fig. 4 the relation between amount of $ZnSO_4$ added and rate constant found at pH 6 is given. Addition of buffer proved not to be necessary to keep pH constant during the runs. At pH 6 the term $k_1[H^+]$ in the numerator of Eq. 7 becomes negligible compared to the other terms in the numerator and may be omitted, thus giving

$$k = \frac{k_2 k_a + k_3 k' b}{[H^+] + k_a + k' b} \qquad \text{(Eq. 8)}$$

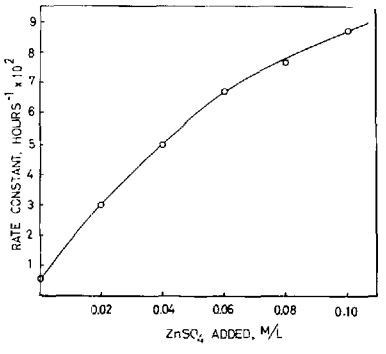

Fig. 4.—Effect of $ZnSO_4$ on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at pH 6 and 96°. Ionic strength, 0.50; no buffer added.

At pH 6 $[H^+] \ll ka_1$, and $[H^+]$ may be omitted:

$$k = \frac{k_2 k_a + k_3 k' b}{k_a + k' b} \qquad \text{(Eq. 9)}$$

Equation 9 may also be written

$$\frac{k - k_2}{k_3 - k} = \frac{k'}{k_a} \cdot b \qquad \text{(Eq. 10)}$$

Inserting the results from the experiments with 0.04 moles/L. and 0.10 moles/L. of $ZnSO_4$ added, gave $k_3 = 1.9 \times 10^{-1}$ hr.$^{-1}$ and $k' = 9 \times 10^{-4}$. These values for k_3 and k' deviate somewhat from the values found at pH 4 ($k_3 = 1.1 \times 10^{-1}$ hr.$^{-1}$ and $k' = 3.8 \times 10^{-3}$). There may be several reasons for these deviations: other reactions than those postulated may occur. Zinc may form basic compounds at pH 6; k_3 may be pH dependent.

In Fig. 5 $k - k_2/k_3 - k$ is plotted *versus* b. In accordance with Eq. 10, a straight line is obtained. The slope of this line is $k'/k_a = 8$.

Experiments with lead similar to those made with

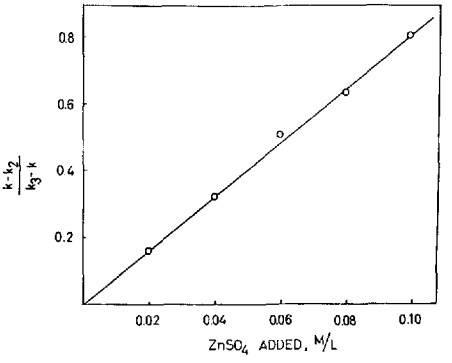

Fig. 5.—Relationship between amount of $ZnSO_4$ added and $k - k_2/k_3 - k$ (*cf.* Eq. 10) by the anaerobic degradation of ascorbic acid at pH 6 and 96° Ionic strength, 0.50; no buffer added.

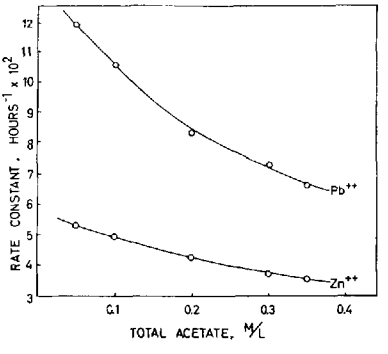

Fig. 6.—Effect of acetate buffer concentration on the pseudo first-order rate constant of the lead and zinc catalyzed anaerobic degradation of ascorbic acid at pH 5 and 96°. A quantity of 0.05 moles of $Pb(CH_3COO)_2$ or $ZnSO_4$ added per liter. Ionic strength, 0.50.

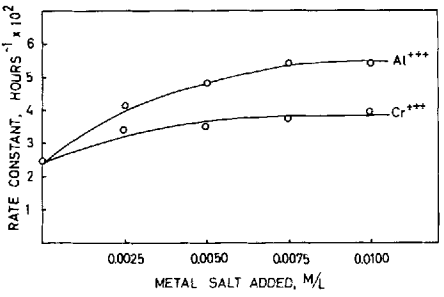

Fig. 7.—Effect of trivalent metal ions on the pseudo first-order rate constant of the anaerobic degradation of ascorbic acid at pH 4 and 96°. Metal salts used, $AlCl_3$ and $CrCl_3$; buffer, 0.3 *M* acetate; ionic strength, 0.50.

zinc (that is at pH 6 without buffer), were impossible because of the formation of precipitate.

Effect of Acetate on the Lead and Zinc Catalyzed Degradation of Ascorbic Acid.—A series of runs were made keeping pH, ionic strength, and lead concentration constant, but varying the acetate buffer concentration. The results of the runs are found in Fig. 6. There is a decrease in rate with increasing buffer concentration, probably due to an increasing complexation of lead by the buffer. Similar runs made with zinc instead of lead gave similar results (Fig. 6).

Since acetate has such a strong effect on the rates of the lead and zinc-catalyzed degradation of ascorbic acid, Eq. 7, which is applicable only to the process in buffer free solution, cannot be used for calculation of the rate constant at different pH values in solutions containing acetate. It is thus not possible to use Eq. 7 for calculation of a theoretical line fitting the experimental points in Fig. 1.

Attempts to Prove the Formation of Ascorbic Acid Metal Complexes.—According to Reaction *1*, the complex formation between ascorbic acid and metal ions in aqueous solution should cause a decrease in pH. Some experiments were made to test if this was the case.

It became necessary to use more concentrated ascorbic acid solutions than 0.01 *M* to get any measurable change in pH. A 25.00 ml. quantity of 0.1 *M* ascorbic acid solution made up to an ionic strength of 1.5 with KNO_3 and having an initial pH of 2.65, was titrated with 0.5 *M* $Pb(NO_3)_2$ solution to which nitric acid had been added to pH 2.65. The pH of the mixture decreased during the titration and reached a value of 2.29 after addition of 25.00 ml. of 0.5 *M* $Pb(NO_3)_2$ solution. A similar experiment carried out with zinc nitrate instead of lead nitrate gave a decrease in pH from 2.54 to 2.44.

The pH decreases observed may be an indication of complex formation between ascorbic acid and lead and zinc. It is of course not possible from these experiments, which are made at 25° and at an ionic strength of 1.5, to make any calculation of the stability constants of the metal–ascorbic acid complex at 96° and ionic strength of 0.5.

Catalytic Effect of Trivalent Metal Ions on the Anaerobic Degradation of Ascorbic Acid.—The evaluation of the catalytic effect of the trivalent ions Al^{3+} and Cr^{3+} presented some problems. If the aluminum concentration was 0.01 moles/L. and pH >4.1 a precipitate was formed in the ascorbic acid solutions during the runs. At pH 4 (0.3 *M* acetate as buffer) precipitations occurred at aluminum concentrations >0.015 moles/L. At the same time pH dropped 0.2–0.3 units. At aluminum concentrations $\leq$0.01 moles/L. there was no precipitation and the pH drop was only about 0.1 unit. The results of experiments at these low metal salt concentrations are given in Fig. 7. It will be seen that aluminum is a powerful catalyst at pH 4. An addition of 0.005 moles of $AlCl_3$ per liter doubles the rate of the anaerobic degradation of ascorbic acid. The shape of the curve may indicate that the major part of the ascorbic acid exists as a complex at an aluminum concentration of 0.01 moles/L.

At pH 3 (0.3 *M* acetate as buffer) aluminum showed nearly no catalytic effect.

With chromium as catalyst it turned out to be impossible to keep the pH of the ascorbic acid solutions constant during the runs when the chromium concentration was more than 0.02 moles/L. At pH 4 (0.3 acetate as buffer) and chromium concentrations $\leq$0.01 moles/L. there was only a slight decrease in pH (about 0.05 units). The results of runs at this pH are given in Fig. 7. It will be seen that the catalytic effect of Cr^{3+} is less than that of Al^{3+}.

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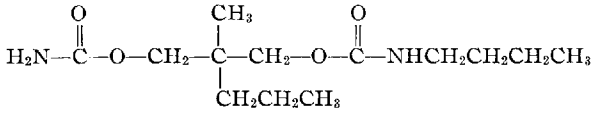
Qualitative and Quantitative Tests for Tybamate

By EDWARD F. SALIM*, JEROME I. BODIN†, HARRY B. ZIMMERMAN†, and PHILIP REISBERG†

Provisional, unofficial monographs are developed by the Drug Standards Laboratory, in cooperation with the manufacturers of the drug concerned, for publication in the *Journal of Pharmaceutical Sciences*. The ready availability of this information affords discriminating medical and pharmaceutical practitioners with an added basis for confidence in the quality of new drug products generally, and of those covered by the monographs particularly. Such monographs will appear on drugs representing new chemical entities for which suitable identity tests and assay procedures are not available in the published literature. The purity and assay limits reported for the drugs and their dosage forms are based on observations made on samples representative of commercial production and are considered to be reasonable within expected analytical and manufacturing variation.

2-METHYL-2-PROPYLTRIMETHYLENE BUTYLCARBAMATE CARBAMATE; $C_{13}H_{26}N_2O_4$; mol. wt. 274.36. The structural formula of tybamate may be represented as



Physical Properties.—Tybamate occurs as a white crystalline powder or a clear viscous liquid which may congeal to a solid form on standing. It has a mild characteristic odor and a bitter taste. The dried powder melts within a range of 2° between 49° and 54°, U.S.P. class I. It is very slightly soluble in water, very soluble in alcohol and in acetone, and freely soluble in ether.

Identity Tests.—Transfer 1 ml. of a 0.04% chloroform solution of tybamate into a glass-stoppered test tube and add 1 ml. of a mixture of acetone–glacial acetic acid (3:1), 1 ml. of a 1 in 100 solution of *p*-dimethylaminobenzaldehyde in benzene, and 5 ml. of antimony trichloride solution (dissolve 3.6 Gm. of antimony trichloride in 20 ml. of chloroform and add 5 ml. of acetic anhydride). Heat the solution at about 55° for 15 min.: a red color is produced.

Dissolve 1 Gm. of tybamate and 0.8 Gm. of xanthydrol in a mixture of 10 ml. of dehydrated alcohol and 10 ml. of glacial acetic acid. Heat the solution on a steam bath for 90 min., taking care to avoid excessive evaporation. Cool and add cold water: an oil, which may crystallize on standing, separates from solution. Filter the mixture through paper, wash the residue with water until free from acetic acid odor, and dry at 105° for 2 hr. The dried residue when recrystallized from a mixture of trichloroethylene–hexane (1:2) melts between 127° and 130°.

The infrared spectrum of a 0.5% dispersion of tybamate in potassium bromide, in a disk of about 0.82 mm. thickness, is shown in Fig. 1.

Purity Tests.—Dry about 1 Gm. of tybamate, accurately weighed, in vacuum at 30–35° for 4 hr.; it loses not more than 0.5% of its weight.

Char about 1 Gm. of tybamate, accurately weighed, cool the residue, add 1 ml. of sulfuric acid, heat cautiously until evolution of sulfur trioxide ceases, ignite, cool, and weigh: the residue does not exceed 0.1%. Retain the residue for the heavy metals test.

Dissolve the sulfated ash obtained from 1 Gm. of tybamate in a small volume of hot nitric acid and evaporate to dryness on a steam bath. Dissolve the residue in 2 ml. of diluted acetic acid, dilute to 25 ml. with water, and determine the heavy metals content of this solution by the U.S.P. heavy metals test, method 1: the heavy metals limit for tybamate is 10 p.p.m.

Assay.—Transfer about 750 mg. of tybamate, accurately weighed, into a conical flask, add 20 ml. of pyridine, and neutralize the solution with 0.1 *N* sodium methoxide to the first pink color of phenolphthalein T.S. Add 50.0 ml. of 0.1 *N* sodium methoxide, a few boiling chips, and reflux with an air condenser on a steam bath for 30 min. Cool, add 40 ml. of neutralized alcohol, and titrate with 0.1 *N* hydrochloric acid to the absence of a pink color. Perform a blank determination. The difference between the two titrations represents the volume of 0.1 *N* sodium methoxide equivalent to

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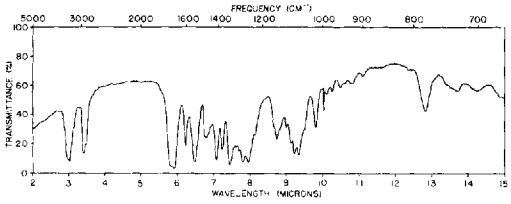


Fig. 1.—Infrared spectrum of tybamate in potassium bromide disk (0.5%); Perkin-Elmer model 21 spectrophotometer, sodium chloride prism.

the weight of $C_{13}H_{26}N_2O_4$ in the sample. Each milliliter of 0.1 *N* sodium methoxide is equivalent to 27.44 mg. of $C_{13}H_{26}N_2O_4$. The amount of tybamate found is not less than 98% and not more than 102% of the weight of the sample taken.

DOSAGE FORMS OF TYBAMATE

Tybamate Capsules

Identity Tests.—Transfer a sample of the capsule content, equivalent to about 10 mg. of tybamate, into a separator containing 75 ml. of water. Shake the aqueous solution with 25 ml. of chloroform and filter a portion of the chloroform extract. Transfer 1 ml. of the filtrate into a glass-stoppered test tube and add 1 ml. of a mixture of acetone–glacial acetic acid (3:1), 1 ml. of a 1 in 100 solution of *p*-dimethylaminobenzaldehyde in benzene, and 5 ml. of antimony trichloride solution (dissolve 3.6 Gm. of antimony trichloride in 20 ml. of chloroform and add 5 ml. of acetic anhydride). Heat the solution at about 55° for 15 min.: a red color is produced.

Transfer a sample of the capsule content, equivalent to about 1 Gm. of tybamate, into a separator containing 100 ml. of cold water. Shake vigorously for 2 min. and discard the aqueous layer. To the oily layer in the separator add 25 ml. of chloroform, 75 ml. of cold water, and shake vigorously. Allow the layers to separate, transfer the chloroform extract into a beaker, and add anhydrous sodium sulfate. The chloroform solution filtered through paper exhibits infrared absorption maxima only at the same wavelengths as that of a similar preparation of tybamate standard.

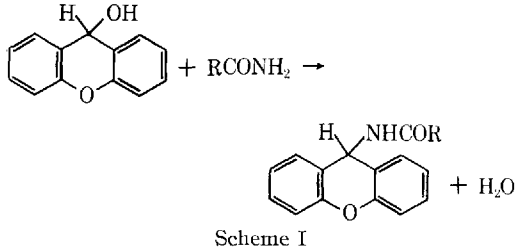
Assay.—Transfer an accurately weighed sample of the capsule content, equivalent to about 750 mg. of tybamate, into a conical flask, add 20 ml. of pyridine, and neutralize the solution with 0.1 *N* sodium methoxide to the first pink color of phenolphthalein T.S. Add 50.0 ml. of 0.1 *N* sodium methoxide, a few boiling chips, and reflux with an air condenser on a steam bath for 30 min. Cool, add 40 ml. of neutralized alcohol, and titrate with 0.1 *N* hydrochloric acid to the absence of a pink color. Perform a blank determination. The difference between the two titrations represents the volume of 0.1 *N* sodium methoxide equivalent to the weight of $C_{13}H_{26}N_2O_4$ in the sample. Each milliliter of 0.1 *N* sodium methoxide is equivalent to 27.44 mg. of $C_{13}H_{26}N_2O_4$. The amount of tybamate found is not less than 95% and not more than 105% of the labeled amount.

DISCUSSION

U.S.P. and N.F. terminology for solubility, melting range, reagents, etc., has been used wherever feasible.

Tybamate,[1] synthesized by Berger and Ludwig (1), is a tranquilizing agent which affords symptomatic improvement in a variety of psychoneurotic disorders, especially in the treatment of the anxiety and tension components of psychoneuroses.

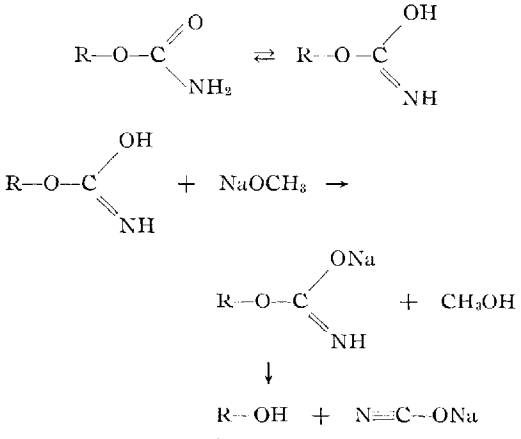
Identity Tests.—Crystalline derivatives of primary amides can be prepared with xanthydrol in glacial acetic acid. (Scheme I.)



Scheme I

The reaction is useful in characterizing aliphatic amides or carbamates (xanthylamides) and sulfonamides (*N*-xanthylsulfonamides).

Quantitative Tests.—Analysis for bulk tybamate and tybamate capsules is based on the reaction of alkali on carbamates of the general type $ROOCNH_2$ in nonaqueous media. Cerri *et al.* (2) have postulated the reaction sequence shown in Scheme II.



Scheme II

In anhydrous pyridine, the equilibrium shown in the first equation is shifted strongly to the right and the enolic species is reacted with the sodium methoxide. In aqueous systems the equilibrium favors the keto form and the reaction with alkali is no longer quantitative. The presence of small quantities of water in the solvents can induce errors in the procedure, but this effect can be nullified by increasing the methanol content in the reaction mixture by about 5% and increasing the reflux time to 55 min. (3).

The assay of bulk tybamate by the volumetric procedure gave an average recovery of 100.2 ±

[1] Marketed as Solacen by Wallace Laboratories, Cranbury, N. J.

1.2%.[2] The titration can be conducted with 0.1 *N* benzoic acid in benzene using thymol blue T.S. eliminating the addition of neutralized alcohol. In this instance, care must be exercised to prevent absorption of atmospheric carbon dioxide during the titration. Analysis of commercial tybamate capsules gave an average value of 97.2 ± 1.2%[2] of the labeled amount of tybamate.

[2] Maximum deviation from the mean value.

REFERENCES

(1) Berger, F. M., and Ludwig, B. J., U.S. pat. 2,937,119 (May 17, 1960); through *Chem. Abstr.*, **54**, 18367(1960).
(2) Cerri, O., Spialtini, A., and Gallo, U., *Pharm. Acta Helv.*, **34**, 13(1959).
(3) Wright, F., Wallace Laboratories, private communication.

Technical Articles

Particle Size Distribution and Hopper Flow Rates

By EDWARD D. SUMNER*, HERMAN O. THOMPSON, WILLIAM K. POOLE, and JAMES E. GRIZZLE

The rate of flow of varying ratios of four sieve size fractions of rock salt through the hopper of a rotary press was represented by a second degree polynomial equation. This fitted polynomial accounted for 90 per cent of the variation in the mean flow rates. No correlation was found between the tangent of the angle of repose and flow rate. High positive correlation was found between flow rate and bulk density, while high negative correlation between flow rate and porosity was observed. There was no significant increase or decrease in flow rates with regard to the quantity of rock salt remaining in the hopper.

One of the most important considerations in pharmaceutical manufacturing is the flow of particulate solids through hoppers and feeders. Capsules, divided powders, and tablets are examples of solid dosage forms which require measured filling for the production of each unit. Thus, the uniformity of the final product requires a uniform flow rate of these solid mixtures. Modern tablet machines are capable of compressing from 5000 (1) to 22,000 (2) tablets per minute. Uniformity of flow rate and flow rate of particulate solids must be considered in order to meet the requirements of these high speed units.

The laws which govern static pressures of fluids do not apply to particulate masses. Some properties of liquids in containers are: pressure is identical throughout at the same depth, pressure exerted on any point is transmitted undiminished to every portion of the enclosed liquid, and pressure exerted by a liquid on the walls of the container is always at right angles to the surface of the container.

Unlike liquids, pressures of bulk solids contained in hoppers varies with direction at different points, pressure is dependent on the shape and size of the receptacle, and the weight of a solid is transferred to the walls by shearing between particles in addition to pressure.

The principal factors affecting flow rates of particulate solids are: particle size, particle size distribution, particle shape, density, surface characteristics, and relative size and geometry of the hopper (3).

A review of the literature (3–15) reveals that the majority of the investigations and research on flow of particulate solids have been conducted on monodisperse systems and hoppers of much larger or smaller dimensions than those ordinarily used in some pharmaceutical manufacturing processes. This work was conducted to ascertain what relationship existed between particle size distribution, bulk density, porosity, and tangent of angle of repose in regard to flow rates and uniformity of flow rates.

MATERIALS AND METHODS

Material.—The particulate solid used was rock salt in the following sieve size fractions: 8/10, 10/20, 20/40, 40/60.

Received April 25, 1966, from the School of Pharmacy and Department of Biostatistics, University of North Carolina, Chapel Hill.
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Abstracted in part from a thesis submitted by E. D. Sumner to the Graduate College, University of North Carolina, Chapel Hill, in partial fulfillment of Doctor of Philosophy degree requirements.
* Present address: School of Pharmacy, University of Georgia, Athens 30601.

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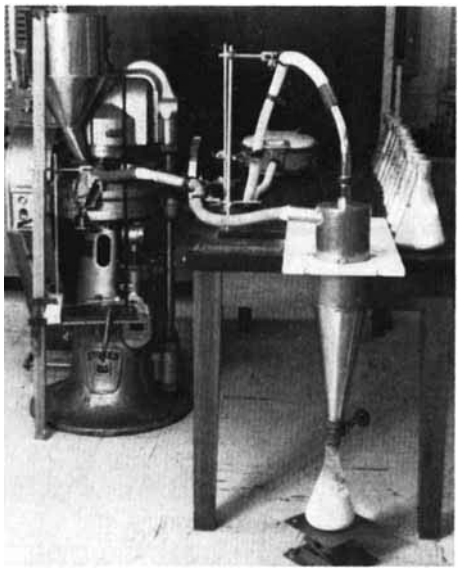

Fig. 1.—Pictorial view of flow apparatus.

Preliminary Investigation.—Since the distance between the hopper orifice and die table of the Stokes rotary press, model D-3, has some regulatory effect on flow rates of particulate solids, the position of the hopper must be stabilized in order to eliminate sagging when the hopper is charged. Preliminary studies revealed that if the hopper height was not controlled, the flow rates gradually increased as the hopper empties due to an increase in the hopper orifice–die table height. Classification studies of rock salt flowing from the rotary press hopper could not be carried out because of fracturing and attrition in the feed frame and cyclone separator.

Flow Rate.—An over-all view of the experimental layout for measuring the rate of flow is shown in Fig. 1. The rotary press, model D-3, was run at a speed of 13.3 r.p.m. throughout the study. The upper punches were left out and the lower punches were adjusted to a depth of 2 cm. The height of the hopper orifice was set at a distance of 0.7 cm. from the die plate.

The hopper supplied with the machine was charged with 22 Kg. of rock salt for each run. The main vacuum nozzle and the auxiliary nozzle were placed in such a position to make collections of all material falling on the die plate as well as material filling the dies. The vacuum for the cyclone separator was furnished by a vacuum cleaner equipped with 1.5-hp. motor.

After placing a 2000-ml. conical flask in position under the cyclone separator, the vacuum system and machine were started. The initial 1-min. collection was discarded and not used in the data. This was done in order to allow the machine and flow to become stabilized.

At 1 min., the shut-off valve on the separator was closed, the flask removed, an empty flask was again placed beneath the separator, and the shut-off valve was opened. The samples were numbered 1, 2, etc., in the order collected. This procedure was continued until the hopper was nearly empty or as long as 1-min. collections could be removed. The rock salt was used over again after subjecting it to sieving to remove fractured material.

Bulk Density.—The bulk density was determined by the procedure set forth by Butler and Ramsey (16). The reported value is an average of three determinations.

Porosity.—Porosity was calculated from the following formula (17):

$$\%e = V_b - V_p/V_b \times 100$$

where V_b and V_p represent the bulk and true volumes, respectively.

Moisture Determination.—All moisture determinations were made on the Ohaus moisture balance, model 6000. The heater was set at a distance of 1.25 in. from the pan, and the samples were dried for a period of 1 hr. One sample was taken initially and the second was taken after total discharge of rock salt from the hopper. The percentage of moisture never exceeded 0.3%, and the initial and final samples seldom varied.

Angle of Repose Measurements.—Instrumentation for measuring the angle of repose is shown in Fig. 2. The glass cylinder was charged with 1000 Gm. of the respective rock salt combination. The funnel on top of the cylinder permitted uniform loading from batch to batch. The plug in the center of the base was removed, and the material flowed out of the orifice. The height of the cone remaining on the platform was measured to the nearest ±0.05 cm. A total of three determinations was made and the reported value is an average of these. The tangent, ϕ, was calculated from the formula (18): tangent ϕ = H/R, where H is the height of the cone, and R is the radius of the platform.

Determination of Hydrostatic Pressures.—Although some relationships have been determined between height of particulate solids and unit pressure in hoppers (5), it seemed desirable to conduct some work utilizing the rotary press hopper.

The rotary press hopper was leveled and clamped in such a manner that the orifice rested on the table

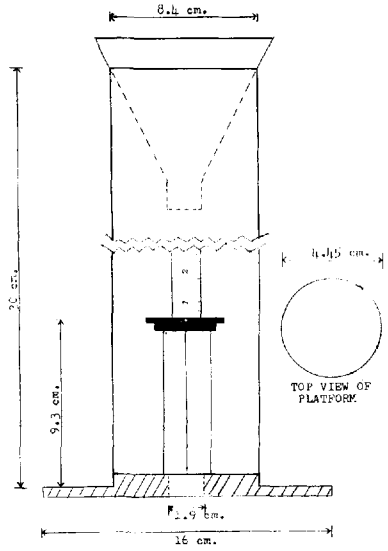

Fig. 2.—Instrumentation for determining angle of repose.

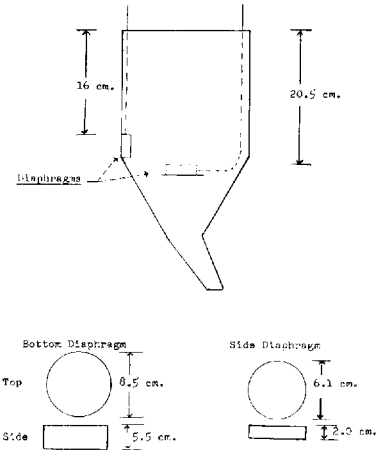

Fig. 3.—Apparatus for determining hydrostatic pressures.

surface. At first, the hopper was partially filled with rock salt. This was done to determine pressures in the larger section of the hopper.

The diaphragms were made from cylindrical metal containers which were covered on the top surface with a sheet of thin rubber. A combination of copper and glass tubing was used for retaining the water from the diaphragms. The diaphragms and tubing were filled to an arbitrary point.

The bottom diaphragm rested on a metal plate above the stationary level of material. The positions of the diaphragms are shown in Fig. 3. Fifteen kilograms of the respective rock salt combination was loaded into the hopper and lightly leveled on the surface. Under pressure of the loaded material, the rubber covering was compressed which resulted in an increase of the water level in the tubing. The water levels of the vertical and lateral diaphragms were measured from the initial markings within 1 min. after the hopper was charged. The experiment was carried out at 27 ±1°.

STATISTICAL ASPECTS OF THE INVESTIGATION

Design of the Experiment.—If the assumption is made that conditions in the machine such as speed, particle size distribution of a given mixture, hopper height, etc., remain constant throughout the entire experimentation, it is reasonable to assume that the response (rate) may be represented by some mathematical function of the constituents being used. This gives rise to what is commonly called a response surface. In this experiment, it was decided that a second degree equation should be tried, and then unneeded terms of this equation could be eliminated. The proposed model (*A*) is shown below.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j + E \quad \text{(Model } A\text{)}$$

where

Y = flow rate for a given set of X's (*i.e*, a given mixture),
β = regression coefficients (unknown),
X_i = proportions of each sieve size rock salt,
E = random uncontrollable error.

The substance at any filling may be represented by

$$X_1 + X_2 + X_3 + X_4 = 1$$

where X_1 = % of 8/10, X_2 = % of 10/20, X_3 = % of 20/40, X_4 = % of 40/60. Percentages are expressed in decimals.

Much work has been done in setting up designs in response surfaces, and in these works optimum properties are achieved by properly placing the design points (*i.e.*, X_1, X_2, X_3, X_4). However, no one seems to have considered the case where the independent variables are restricted by inequalities as in this experiment. This being the case, it was felt that the best that could be done would be to choose a design which (*a*) permits estimation of all regression coefficients of the equation (β's) and (*b*) permits analysis of the variability in response at different combinations of the X_i's.

The design of the experiment chosen was one in which (*a*) each sieve size was run separately, (*b*) sieve sizes were run in pairs, (*c*) sieve sizes were run in triplets, and (*d*) all sieve sizes were run at the same time. These various combinations are shown in Table I. Note that some of the mixtures were run twice, (*i.e.*, *7* and *24* are the same, *15* and *25*, etc.).

RESULTS AND DISCUSSION

A summary of the average flow rates and standard errors, tangent of the angle of repose, bulk density,

TABLE I.—COMBINATIONS OF ROCK SALT USED FOR FLOW STUDIES

Combination	Proportions X_1 8/10	X_2 10/20	X_3 20/40	X_4 40/60
1	1.000	...	...	...
2	...	1.000	...	...
3	...	...	1.000	...
4	...	...	...	1.000
5	0.500	0.500	...	...
6	0.500	...	0.500	...
7	0.500	...	...	0.500
8	...	0.500	0.500	...
9	...	0.500	...	0.500
10	...	...	0.500	0.500
11	0.333	0.333	0.333	...
12	0.333	0.333	...	0.333
13	0.333	...	0.333	0.333
14	...	0.333	0.333	0.333
15	0.250	0.250	0.250	0.250
16	0.718	0.094	0.094	0.094
17	0.094	0.718	0.094	0.094
18	0.094	0.094	0.718	0.094
19	0.094	0.094	0.094	0.718
20[a]	0.100	...	...	0.900
21[a]	0.179	...	...	0.821
22[a]	0.250	...	...	0.750
23[a]	0.400	...	...	0.600
24[a]	0.500	...	...	0.500
25[a]	0.250	0.250	0.250	0.250
26[a]	0.333	...	0.333	0.333

[a] Indicates that these combinations were run after the experimental data were processed for the first 19 combinations.

TABLE II.—SUMMARY OF DATA OBTAINED FOR MEAN FLOW RATE, TANGENT ANGLE OF REPOSE, BULK DENSITY, POROSITY, AND S.D. OF MEAN FLOW RATE

Combination	Av. Flow Rate, Gm./min.	Tangent Angle of Repose	Bulk Density, Gm./ml.	Porosity, %	S.E.,[a] Gm./min.
1	1167.843	0.7870	1.10	49.00	13.8786
2	1675.500	0.7110	1.17	46.00	3.6703
3	1768.958	0.7100	1.16	46.20	8.9768
4	1903.550	1.1310	1.14	47.25	6.1750
5	1542.428	0.7940	1.17	46.00	8.4208
6	1832.136	0.8170	1.24	42.80	4.8683
7	2064.666	0.9137	1.31	39.38	11.4560
8	1788.458	0.7492	1.21	44.12	7.9189
9	1955.550	0.8616	1.28	40.53	4.7476
10	1883.850	0.8166	1.21	44.02	10.4109
11	1844.750	0.8616	1.23	43.19	6.4506
12	1908.450	0.8912	1.25	42.08	8.1443
13	1969.850	0.7640	1.29	40.01	2.1633
14	1937.722	0.7043	1.27	41.03	8.3917
15	1981.950	0.7865	1.29	40.25	4.5640
16	1827.333	0.9213	1.24	42.61	6.4707
17	1794.850	0.8840	1.21	43.89	2.7821
18	1861.150	0.7115	1.24	42.80	3.4569
19	1952.277	0.8013	1.27	41.36	3.6083
20	1890.600	1.0920	1.20	44.34	6.3443
21	1942.400	0.8220	1.25	42.00	3.7881
22	1944.800	0.7560	1.30	40.00	4.9649
23	1991.833	0.7560	1.32	39.10	9.0576
24	2054.389	0.7640	1.32	38.75	6.9340
25	1960.150	0.7870	1.28	40.60	5.7280
26	1957.600	0.7330	1.29	40.10	7.6883

[a] Standard deviation of mean flow rate.

and porosity is shown in Table II. Table III shows the results of the hydrostatic pressure measurements.

Statistical Analysis.—In the discussion, the following conventions have been adopted: *observation*, a single determination for a specific combination; *sample*, the aggregate of observations for a specific combination; *experiment*, the totality of samples for all combinations. After running the first 19 combinations shown in Table I, plots of each sample were made on a time scale and on separate pieces of graph paper. The main purpose of this was to determine if any trend existed in flow rate from the first observation taken in each sample to the last. It is noted that the model proposed assumes no such trend (*i.e.*, it assumes each observation in a sample to be about like any other observation, apart from random error). The plots suggested that in some instances there appeared to be trends. Statistical tests showed, however, that the "apparent trends" could be attributed to chance variation in the data, and thus, model A was considered adequate.

As a by-product of this investigation mentioned above, a preliminary fit[1] of a modified form of the proposed model was obtained. This modified model is shown as model B.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + E \quad \text{(Model } B\text{)}$$

This fitted equation provided an estimate (perhaps rough) of the design point which would produce the greatest flow rate. This was:

[1] The term "fit" refers to the estimation of the unknown β's from the data. The least squares technique was used in estimating these coefficients.

TABLE III.—HYDROSTATIC PRESSURES OF ROCK SALT IN ROTARY PRESS HOPPER

Sieve Size	Pressure in cm. Water[a] Vertical	Lateral	Increase of Lateral Above 8/10 Fraction, %
8/10	6.35	1.45	...
40/60	8.65	1.85	27.6
8/10 40/60[b]	6.60	2.03	40.0

[a] Glass tubing with inside diameter of 0.3 cm. [b] 50% of each mixed together.

$$\hat{X}_1 = 0.1794 \ (\%\ 8/10 \text{ fraction})$$
$$\hat{X}_2 = 0.0000 \ (\%\ 10/20 \text{ fraction})$$
$$\hat{X}_3 = 0.0000 \ (\%\ 20/40 \text{ fraction})$$
$$\hat{X}_4 = 0.8206 \ (\%\ 40/60 \text{ fraction})$$

It is noted that the design point tested producing the maximum flow rate in the experiment was

$$X_1 = 0.5000 \ (\%\ 8/10 \text{ fraction})$$
$$X_2 = 0.0000 \ (\%\ 10/20 \text{ fraction})$$
$$X_3 = 0.0000 \ (\%\ 20/40 \text{ fraction})$$
$$X_4 = 0.5000 \ (\%\ 40/60 \text{ fraction})$$

At this point in the investigation it seemed advisable to take a few more samples in order to get a good fit of model A in the region of the suspected maximum flow rate (*i.e.*, the predicted maximum) and so that differences in flow rate and variation could be examined for repeated sampling of the same mixture. Since no trend existed with regard to the time an observation was taken, the means of the flow rates were thought to be indicative of the performance of a particular combination. Also, in using the means of the flow rates a source of varia-

tion was eliminated in fitting the model (*i.e.*, within combination variation).

With this in mind, model A was fitted to the means arising from samples *1* through *26*. Tests were carried out to determine whether any of the regression coefficients could be eliminated, and the results indicated that β_3 and β_{33} could be considered as zero. If these coefficients are set equal to zero in model A, the resulting model may be called model A'.

Model A' then is the one that should be used for this experiment. The data were fitted to model A' (*i.e.*, model A with $\beta_3 = \beta_{33} = 0$), and the resulting fitted equation along with the standard deviation of the estimated parameters (in parentheses) are given below.

$$\hat{Y} = \hat{\beta}_0 + \sum_{i=1}^{2} \hat{\beta}_i X_i + \sum_{i=1}^{2} \hat{\beta}_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \hat{\beta}_{ij} X_i X_j$$

$$\begin{aligned}
\hat{\beta}_0 &= 1812.7531 \quad (32.3132) \\
\hat{\beta}_1 &= 1311.3557 \quad (187.9531) \\
\hat{\beta}_2 &= 778.7229 \quad (248.3857) \\
\hat{\beta}_{11} &= -1878.3369 \quad (194.3749) \\
\hat{\beta}_{22} &= -935.3288 \quad (257.8770) \\
\hat{\beta}_{12} &= -2261.2628 \quad (408.3098) \\
\hat{\beta}_{13} &= -510.4626 \quad (262.2961) \\
\hat{\beta}_{23} &= -589.9348 \quad (340.8922)
\end{aligned}$$

The estimates of the X_i's from the above equation which would produce the maximum flow rate were calculated to be:

$$\begin{aligned}
\hat{X}_1 &= 0.3491 \ (\%\ 8/10 \text{ fraction}) \\
\hat{X}_2 &= 0.0000 \ (\%\ 10/20 \text{ fraction}) \\
\hat{X}_3 &= 0.0000 \ (\%\ 20/40 \text{ fraction}) \\
\hat{X}_4 &= 0.6509 \ (\%\ 40/60 \text{ fraction})
\end{aligned}$$

Since model A' has fewer parameters than model A, it is natural to inquire as to what is lost, if anything, by choosing the simpler model. This question is generally answered by using the multiple correlation coefficient, M, associated with each fitted model. The square of M is actually the percentage of the total variation in the sample means which is accounted for by the fitted model. The M corresponding to the fitting of A was found to be 0.9552, while that for A' was 0.9501; hence, the fitted A' is just about as good a predictor of flow rate as is the fitted A. In fact, it was found that if all regression coefficients with a three in the subscript were set equal to zero (*i.e.*, $\beta_3 = \beta_{33} = \beta_{13} = \beta_{23} = 0$), then the fitted model had an M of 0.9270. This means that knowledge of X_3 helps very little in predicting mean flow rates. In other words, the absence or presence of the $^{21}/_{40}$ fraction in the mixture has little effect on the mean of the flow rates.

Linear Correlation of Flow Rates with Angle of Repose, Bulk Density, and Porosity.—The sample correlation coefficient between flow rate and various other measurements is defined as

$$r = \frac{\sum_{i=1}^{n} (X_i - \bar{X})(Y_i - \bar{Y})}{\left[\sum_{i=1}^{n} (X_i - \bar{X})^2 \cdot \sum_{i=1}^{n} (Y_i - \bar{Y})^2\right]^{1/2}}$$

where $X_1, X_2, \ldots, X_n$ are the mean flow rates of the 26 samples and $Y_1, Y_2, \ldots, Y_n$ are the values corresponding to the variable whose linear correlation with flow rate is to be investigated.

Tangent of Angle of Repose.—$r_1 = +0.1022$. A statistical test showed that the true correlation could be considered zero, and the conclusion was made that the tangent angle of repose is uncorrelated with flow rate.

Bulk Density.—$r_2 = +0.8123$. This suggests a positive correlation and hence the conclusion was made that flow rate is positively correlated with bulk density.

Porosity.—$r_3 = -0.8160$. This indicates a negative correlation with flow rate.

Variation of Flow Rates.—The usual standard deviation of the mean was computed for each combination and used as a measure of variation of flow rate (see Table II). A statistical comparison revealed that not only are there significant differences in the variation for different mixtures (*i.e.*, combinations *1* and *13*), but there are also significant differences in the variation of flow rate of the same mixture run two different times (*i.e.*, combinations *13* and *26*). The differences in the first case were not so surprising as those in the second case due to changing bulk density, porosity, and particle size in the former. No explanation can be offered at this time as to why combinations *1*, *7*, and *10* had the greatest standard deviation. The variation of the standard error in the second case (*i.e.*, combination *13* and *26*) was probably due primarily to differences in the particle size distribution at different times. This, however, was not verified.

SUMMARY

Flow rates were obtained from constantly flowing material which approached tableting operations. There was a maximum point at which the further addition of 40/60 fraction of rock salt to the 8/10 fraction decreased the flow rate. This maximum point, among those tested, was when 50% each were mixed. The fitted model places the maximum at 35% of the 8/10 fraction and 65% of the 40/60 fraction. No correlation was found to exist between the angle of repose and flow rate.

Other factors had an effect on the flow rate. Bulk density increased with a decrease in porosity when different particle size systems were mixed. Hence, flow rate was increased due to more weight per unit volume being delivered from the hopper orifice. The flow rate was increased by the addition of smaller particles to larger particles which appeared to act as a lubricant when the voids were partially filled. Lateral hydrostatic pressure measurements on 8/10, 40/60, and 50% of 8/10 and 40/60 fractions of rock salt showed that the flow rate increased with increasing pressures, though the same relationship with vertical hydrostatic pressure does not seem to exist.

The standard error varied from a low of 2.1633 to a high of 13.8786 for various combinations of rock salt. There is an apparent difference in the uniformity of flow rate from one combination to another. However, no obvious simple trend exists since the highest variability was associated with the lowest flow rate, while the second highest variability was associated with the highest flow rate.

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Notes

Direct Titrimetric Determination of Aromatic Quaternary Ammonium Salts and Long Chain Aliphatic Sulfates and Sulfonates

By JOYE A. BILLOW and H. WELDON BAKER

Aromatic quaternary ammonium salts and long chain aliphatic sulfates and sulfonates can be assayed by a rapid, single phase aqueous titration. Sodium tetraphenylboron serves as a direct standard in the assay of the quaternaries and benzalkonium chloride as the titrant for anionic detergents. Bromophenol blue indicator gives a purple to blue color change when the anionic substances are titrated in strongly alkaline solution. The results of this method show excellent agreement with the standard chloroform extraction titration.

THE aromatic quaternary ammonium salts official in U.S.P. XVII and N.F. XII are assayed by many different methods. Sodium lauryl sulfate, the only official long chain aliphatic sulfate, is not directly assayed. It was believed that a single method type, direct titration of large anions with large cations, could be used for all aromatic quaternary ammonium salts and for the long chain aliphatic sulfates and sulfonates.

A direct titration of sodium tetraphenylboron with cetyltrimethylammonium bromide using bromophenol blue indicator was used by Schall (1) in the determination of potassium. This suggested that aromatic quaternary ammonium compounds could be assayed by direct titration of sodium tetraphenylboron, and, conversely, that large anions could be titrated directly with quaternary ammonium compounds.

Cetylpyridinium chloride is officially assayed (2) by titration with standard sodium tetraphenylboron using bromophenol blue indicator. The official method differs from that proposed by Schall in that the end point depends upon the extraction of the indicator from a chloroform layer.

A similar chloroform extraction titration, using tetrabutylammonium iodide as the standard, is official (3) for the assay of the aliphatic sulfonate dioctyl sodium sulfosuccinate. This method is adapted in method *A* as the general chloroform extraction method against which the proposed method is compared.

REAGENTS

Tetrabutylammonium Iodide (Primary Standard Cation).—A 0.01000 *M* solution prepared considering the material to be 100% pure. Titration in triplicate against sodium tetraphenylboron by method *A* gave 0.01000 *M* ± 0.00005 *M*.

Sodium Tetraphenylboron (Primary Standard Anion).—A 0.01000 *M* solution prepared on the basis of labeled 99.6% assay. This should be freshly prepared before each use because of decomposition on standing in solution.

Benzalkonium Chloride (Secondary Standard Cation).—Approximately 0.01 *M* solution prepared by diluting about 30 ml. of 12.8% benzalkonium chloride[1] to 1 L. and standardized against sodium tetraphenylboron by N.F. XII procedure for cetylpyridinium chloride (2).

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[1] Marketed as Zephirin Chloride by Winthrop Laboratories, New York, N. Y.

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TABLE I.—COMPARISON OF METHODS *A* AND *B*

Soln.	Method *A* No. Assays	Method *A* Av. *M*	Method *A* S. D.	Method *B* No. Assays	Method *B* Av. *M*	Method *B* S. D.
Benzalkonium chloride I	6	0.00902	0.00001	6	0.00903	0.00001
Benzalkonium chloride II	6	0.01030	0.00001	6	0.01029	0.00001
Dioctyl sodium sulfosuccinate	6	0.01159	0.00001	6	0.01159	0.00001
Sodium lauryl sulfate I	5	0.00906	0.00004	6	0.00910	0.00005
Sodium lauryl sulfate II	3	0.01000	0.00006	5	0.01016	0.00004
Cetylpyridinium bromide	3	0.01135	0.00001	3	0.01136	0.00001

Sodium Octylphenoxyethoxypolyether Sulfate (Secondary Standard Anion).—Dilute approximately 20 ml. of alkyl phenoxyethanesulfonate[2] concentrate to 1 L. Standardize against tetrabutylammonium iodide using the N.F. XII procedure for dioctyl sodium sulfosuccinate (3).

Bromophenol Blue T.S.—A 0.1% quantity bromophenol blue in a mixture of equal volumes of water and alcohol.

Salt Solution.—Prepared according to N.F. XII directions for assay of dioctyl sodium sulfosuccinate (3).

Chloroform.—Reagent grade.

Sodium Hydroxide Solution.—Twenty per cent.

METHODS

Method A.—*Adapted from N.F. XII Method for Dioctyl Sodium Sulfosuccinate.*—Place a sample containing about 25 mmoles cation such as tetrabutylammonium iodide in 25 ml. of water, 50 ml. of salt solution, 25 ml. of chloroform, and 0.4 ml. of bromophenol blue T.S. in a flask. Titrate with an approximately 0.01 *M* anion solution, using a magnetic stirring device to insure thorough mixing of the layers to replace the shaking procedures in the official method. Add the anion dropwise in the vicinity of the end point, allowing sufficient time for mixing and separation. Continue the titration until the blue color is completely removed from the chloroform layer.

Method B.—*Proposed Direct Method.*—This procedure is the same whether the anionic or cationic component is to be determined. Pipet 25 ml. of an approximately 0.01 *M* anion solution into a flask, add 50 ml. of water, 2 ml. of 20% NaOH, and 0.4 ml. of bromophenol blue T.S. The solution will be purple. Using a magnetic stirrer, titrate with the cation, adding it dropwise near the end point, which is a color change to a distinct, bright blue.

Two benzalkonium chloride solutions were prepared as indicated under *Reagents*. These were standardized by titration against the secondary standard anion using method *A* and the proposed method *B*. Results are given in Table I.

Dioctyl sodium sulfosuccinate was assayed by titration against standardized benzalkonium chloride solution by both methods. The dioctyl sodium sulfosuccinate solution was prepared by diluting approximately 7.5 ml. of a type of dioctyl sodium sulfosuccinate[3] to 1 L. and allowing to stand with occasional shaking until complete dissolution was obtained. Results are given in Table I.

Sodium lauryl sulfate was assayed by titration against standard benzalkonium chloride solution, using both methods *A* and *B*. Results are given in Table I.

Cetylpyridinium bromide was assayed by titration against standard sodium tetraphenylboron solution, using both methods *A* and *B*. Results are given in Table I.

DISCUSSION

Tetrabutylammonium iodide can only be used in method *A*; attempts to use it in method *B* gave no color change at the end point. Sodium tetraphenylboron can be used in both methods *A* and *B*.

As previously stated, the color change in method *B* is from purple to blue. A trial is necessary to obtain a correct end point, so that a purple-tinged blue may not be mistaken for the true end point by those not thoroughly familiar with the color change.

In method *B* the formation of a greasy precipitate in the vicinity of the end point has not been found to interfere with end point detection.

The following sulfonphthalein indicators were investigated for use in method *B* and were found unsatisfactory: thymol blue, bromocresol purple, cresol red, phenol red, dibromopyrogallol sulfonphthalein, pyrogallol sulfonphthalein, pyrocatechol sulfonphthalein.

CONCLUSIONS

The data show that the proposed direct titration (method *B*) yields results in excellent agreement with the chloroform extraction titration (method *A*) for the compounds studied, which were U.S.P., N.F., or closely related materials. No investigation of interferences was made in this study; the scope was limited to the method types presented in the official compendia. The advantages of method *B* include: reliable primary standards, inexpensive secondary standards, faster, easier titration. This method also eliminates the use of chloroform, which is both expensive and toxic.

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[2] Marketed as Triton W-30 by Rohm & Haas Co., Philadelphia, Pa.

[3] Marketed as Triton GR-5 by Rohm & Haas Co., Philadelphia, Pa.

Sodium Chloride Equivalents, Cryoscopic Properties, and Hemolytic Effects of Certain Medicinals in Aqueous Solution

By E. ROY HAMMARLUND and GERALD L. VAN PEVENAGE*

A supplemental table of NaCl equivalents and freezing point depressions at various concentrations for 21 different medicinal substances in aqueous solution is presented. Also given in the table is the isosmotic concentration of each of the materials which can form such a solution. The degree of hemolysis of human erythrocytes was determined in 45 different isosmotic solutions and the data are presented in a table to supplement the previously published values. Freezing point depression graphs for five substances—bromodiphenhydramine HCl, dexchlorpheniramine maleate, hydroxyzine HCl, sodium nafcillin, and valethamate bromide—are presented because they showed one or more discontinuities caused by some type of association or aggregation in their solutions.

THE NaCl equivalents and freezing point depressions for various aqueous medicinal solutions have been determined experimentally and reported (1, 2). Likewise, the amount of hemolysis of fresh human erythrocytes in certain aqueous isosmotic solutions was determined using the hemolytic method and has been reported previously (3).

The objective of the present investigation was to study in a similar manner a number of additional substances not included in the earlier cryoscopic and hemolytic investigations and to present the data in suitable tables to supplement the previous data. Furthermore, since it has been reported by Hammarlund and Pedersen-Bjergaard (4) and by Johnson, Goyan, and Tuck (5) and others that certain amine salts and other substances tend to aggregate in aqueous solution, data are presented for five such substances which have been found to show this behavior.

EXPERIMENTAL

Freezing Point Measurements.—The method used for most of the measurements of the freezing points of the solutions was the same as that reported previously in detail (1). The freezing point depression measurements were made directly on aqueous solutions of the compounds at selected concentrations by means of a Beckman differential thermometer. For several of the compounds used in the latter portion of the study, a cryoscopic osmometer[1] was employed for the freezing point depression measurements. Several substances were determined by both instruments and the data correlated almost exactly.

The freezing point measurements were corrected for the amount of disengaged ice, and −0.52° was used as the comparative freezing point for aqueous 0.9% NaCl solution which is isotonic with blood and tears. The materials used were of official grade of purity or better, and for those nonofficial substances, the grade of purity of each complied with the manufacturer's specifications. The NaCl equivalents and isosmotic concentrations are reported to the nearest 0.01.

Table I lists the NaCl equivalents at various concentrations for all of the currently studied substances. To use these data, one should employ the NaCl equivalent which represents the concentration nearest to the desired concentration of medicinal substance used.

Because of interest in the colligative properties of some medicinal solutions, the freezing point depressions and NaCl equivalents are included for several preparations which are not used necessarily as isotonic solutions. The term "isotonic solution" is used in its customary sense in this report—meaning that the solution freezes at the same temperature as normal saline solution, blood, and tears.

Hemolysis of Human Erythrocytes.—The method used was essentially the same as that employed by Husa and co-workers (6, 7) and is a modification of the method by Hunter (8). The principal deviations from Husa's method were that only isosmotic concentrations were employed, and an aqueous saponin solution, 100 mg./L., was used as the 100% hemolyzing solution for the erythrocytes. A fresh sample of human venous blood was used daily, and each investigation was completed within 3 hr. from the time the blood was drawn. The method employed in this study was identical with that previously described by Hammarlund and Pedersen-Bjergaard (3) except that the absorbance of each centrifuged supernatant liquid was determined in a Spectronic[2] 20 photoelectric colorimeter at 540 mμ instead of the previously used instrument employing a standard green filter.

The per cent of hemolysis found for the 45 compounds studied is listed in Table II including the isosmotic concentration used for each and its approximate pH. The few experimental solutions which were colored before the addition of blood were handled in the same manner as described previously (3). For those solutions which developed a color or cloudiness upon the addition of blood, the proportional decrease in volume of the packed, unhemolyzed, centrifuged erythrocytes was estimated visually. Any change in appearance of the erythrocytes or the solution was referred to in the footnotes for Table II.

Cryoscopic Behavior of Certain Aggregate-Formers.—When the freezing point depression data were plotted for the various substances, it was noted that the graphs for five of them had a definite discontinuity at certain concentrations. This observation suggested that there could be some type of association of the solute or aggregation taking place above a

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* National Science Foundation undergraduate research participant, 1964–1965. Present address: 326 Oregon Way, Longview, Wash.

[1] Advanced Instruments, Inc., Newton Highlands, Mass.

[2] Bausch & Lomb, Rochester, N. Y.

TABLE I.—SODIUM CHLORIDE EQUIVALENTS AND FREEZING POINT DEPRESSIONS

Chemical	Concn. of Soln., NaCl Equivalents 0.5%	1%	2%	3%	5%	At "Isotonicity"	
Amantadine HCl	0.31[a]	0.31[a]	0.31[a]	...	...	0.31[a]	(2.95)[c]
	0.090°[b]	*0.180°*[b]	*0.354°*[b]	...	...	*0.52°*[b]	(2.95)[c]
Chloroprocaine HCl U.S.P.	0.20	0.20	0.18	...	...	...	...
	0.054°	*0.108°*	*0.210°*	...	...	...	...
Diethanolamine	0.31	0.31	0.31	...	...	0.31	(2.90)
	0.089°	*0.177°*	*0.358°*	...	...	*0.52°*	(2.90)
Dimethylsulfoxide	0.42	0.42	0.42	...	...	0.42	(2.16)
	0.122°	*0.245°*	*0.480°*	...	...	*0.52°*	(2.16)
Echothiophate iodide U.S.P.	0.16	0.16	0.16	...	...	...	...
	0.045°	*0.090°*	*0.179°*	...	...	...	...
Gold sodium thiomalate U.S.P.	0.10	0.10	0.10	0.09	0.09	...	...
	0.032°	*0.061°*	*0.111°*	*0.159°*	*0.250°*	...	...
Lincomycin HCl	0.16	0.16	0.15	0.14	0.14	0.14	(6.60)
	0.045°	*0.090°*	*0.170°*	*0.247°*	*0.400°*	*0.52°*	(6.60)
Monoethanolamine N.F.	0.53	0.53	...	...	...	0.53	(1.70)
	0.154°	*0.306°*	...	...	...	*0.52°*	(1.70)
Oxymetazoline HCl	0.22	0.22	0.20	0.19	...	0.18	(4.92)
	0.063°	*0.124°*	*0.232°*	*0.335°*	...	*0.52°*	(4.92)
Polyethylene glycol 300 N.F.	0.12	0.12	0.12	0.12	0.13	0.13	(6.73)
	0.034°	*0.069°*	*0.141°*	*0.216°*	*0.378°*	*0.52°*	(6.73)
Polyethylene glycol 400 U.S.P.	0.08	0.08	0.09	0.09	0.09	0.11	(8.50)
	0.022°	*0.047°*	*0.098°*	*0.153°*	*0.272°*	*0.52°*	(8.50)
Polyethylene glycol 1500	0.06	0.06	0.07	0.07	0.07	0.09	(10.00)
	0.015°	*0.036°*	*0.078°*	*0.120°*	*0.215°*	*0.52°*	(10.00)
Polyethylene glycol 1540 N.F.	0.02	0.02	0.02	0.03	0.03	...	...
	0.005°	*0.012°*	*0.028°*	*0.047°*	*0.094°*	...	...
Polyethylene glycol 4000 U.S.P.	0.02	0.02	0.02	0.02	0.02	...	...
	0.004°	*0.008°*	*0.020°*	*0.033°*	*0.067°*	...	...
Polyvinyl alcohol (99% hydrol.)	0.02	0.02	0.02	0.02	0.03	...	...
	0.004°	*0.008°*	*0.020°*	*0.035°*	*0.075°*	...	...
Polyvinylpyrrolidone	0.01	0.01	0.01	0.01	0.01	...	...
	0.003°	*0.006°*	*0.010°*	*0.017°*	*0.035°*	...	...
Sodium cephalothin	0.18	0.17	0.16	0.15	0.14	0.13	(6.80)
	0.050°	*0.095°*	*0.179°*	*0.259°*	*0.400°*	*0.52°*	(6.80)
Sodium methicillin U.S.P.	0.18	0.18	0.17	0.16	0.15	0.15	(6.00)
	0.050°	*0.099°*	*0.192°*	*0.281°*	*0.445°*	*0.52°*	(6.00)
Sodium succinate	0.32	0.32	0.31	...	...	0.31	(2.90)
	0.092°	*0.184°*	*0.361°*	...	...	*0.52°*	(2.90)
Sodium tartrate	0.33	0.33	0.33	...	...	0.33	(2.72)
	0.098°	*0.193°*	*0.385°*	...	...	*0.52°*	(2.72)
Triethanolamine U.S.P.	0.20	0.21	0.22	0.22	...	0.22	(4.05)
	0.058°	*0.121°*	*0.252°*	*0.383°*	...	*0.52°*	(4.05)

[a] The values first listed for the chemical substances are NaCl equivalents. [b] The second values, in *italics*, are freezing point depression values in ° C. [c] The percentage concentration (w/v) at isotonicity (isosmotic) is given in parentheses in the last column.

particular concentration of the solute at the freezing temperature of the solution. In order to obtain a more definite estimation of the various points of discontinuity, the freezing point depression graphs for the five substances were plotted as the log of the freezing point depression *versus* the log of the molar concentration of the solutions. These are shown in Figs. 1 and 2 and the concentrations of the substances at their points of discontinuity in the figures were found to be as follows: bromodiphenhydramine HCl, 0.058 *M* (2.15% w/v) and 0.248 *M* (9.19% w/v); dexchlorpheniramine maleate, 0.088 *M* (3.44% w/v); hydroxyzine HCl, 0.055 *M* (2.48% w/v); sodium nafcillin, 0.062 *M* (2.52% w/v); and valethamate bromide, 0.098 *M* (3.80% w/v).

DISCUSSION

Isosmotic solutions of 20 substances prevented hemolysis of erythrocytes and solutions of 25 other substances failed to prevent hemolysis, the degree of hemolysis varying from slight to complete. This type of result has been discussed previously in considerable detail (3).

Figures 1 and 2 show that five of the substances studied undergo a solute association or aggregation similar to that found for dibucaine HCl, tetracaine HCl, and pramoxine HCl and which was discussed previously by Hammarlund and Pedersen-Bjergaard (4) and by Johnson, Goyan, and Tuck (5).

The freezing point depression data for bromodiphenhydramine HCl (Fig. 2) presented an anomaly as at 0.248 *M* concentration the freezing point curve broke sharply back to exactly the same slope that it had prior to its point of the initial aggregation at 0.058 *M*.

One objection to the use of the freezing point depression method for the sole determination of aggregation is that it cannot reveal any temperature dependence of the aggregation. This is so, since, for a given concentration, observations are restricted to only one temperature, the freezing temperature. A substance might exhibit a freezing point depression curve similar to that of any other aggregate-forming substance; however, in this instance a break in its curve would not indicate some critical concentration, but would indicate rather a particular temperature

below which some other change takes place in the system such as the occurrence of a solute–solvent interaction. In order to determine whether aggregation depends significantly upon temperature, it is necessary to resort to other analytical techniques in which measurements are made on corresponding solutions over a range of temperatures. Johnson, Goyan, and Tuck (5) have done this for various substituted amine salts by employing a thermoelectric vapor-phase osmometer at 25°, and they found evidence of aggregation at this temperature for several substances. Likewise, Farhadieh (9) in a study of the aggregation of dibucaine HCl, tetracaine HCl, diphenhydramine HCl, procaine HCl, tripelennamine HCl, pyrilamine HCl, and bromodiphenhydramine HCl employed both a vapor pressure osmometer and a Dike-Jones[3] con-

TABLE II.—HEMOLYSIS OF ERYTHROCYTES IN ISOSMOTIC SOLUTIONS

Substance	Isosmotic Concn., % w/v	Hemolysis, %	Approx. pH
Amantadine HCl	2.95	91	5.7
Aminocaproic acid	3.52	0	7.2
Bethanechol chloride U.S.P.	3.05	0	6.0
Calcium disodium edetate U.S.P.	4.50	0	6.1
Chloramphenicol sodium succinate U.S.P.	6.83	Partial[a]	6.1
Chlordiazepoxide HCl N.F.	5.50	66[b]	2.7
Dexamethasone sodium phosphate N.F.	6.75	0[c]	8.9
Diethanolamine	2.90	100	11.3
Dimethylsulfoxide	2.16	100	7.6
Edathamil disodium	4.44	0	4.7
Furtrethonium iodide	4.44	0	5.4
Hydroxyzine HCl N.F.	6.32	100[d]	1.3
Lincomycin HCl	6.60	0	4.5
Mafenide HCl	3.55	0	5.0
Mepivacaine HCl N.F.	4.60	45	4.5
Metaraminol bitartrate U.S.P.	5.17	59[d]	3.8
Methapyrilene HCl N.F.	6.00	99	6.2
Methitural sodium	3.85	78[e]	9.8
Methoxyphenamine HCl	3.47	96	5.4
p-Methylaminoethanol-phenol tartrate	5.83	0	6.2
Methyldopate HCl	4.28	Partial[f]	3.0
N-Methylglucamine	5.02	4	11.3
Methylphenidate HCl N.F.	4.07	66	4.3
Monoethanolamine N.F.	1.70	100[g]	11.7
Nalorphine HCl U.S.P.	6.36	63	4.1
Oxymetazoline HCl	4.92	86[f]	5.7
d-Pantothenyl alcohol	5.60	92	6.8
Pargyline HCl	3.18	91	3.8
Phentolamine mesylate U.S.P.	8.23	83[e]	3.5
Polyethylene glycol 300 N.F.	6.73	53	3.8
Polyethylene glycol 400 U.S.P.	8.50	0	4.4
Polyethylene glycol 1500	10.00	4	4.1
Potassium acetate N.F.	1.53	0	7.6
Pralidoxime chloride	2.87	0	4.6
Sodium bismuth thioglycollate	5.29	0	8.3
Sodium cephalothin	6.80	Partial[a]	8.5
Sodium colistimethate U.S.P.	6.85	0[h]	8.4
Sodium methicillin U.S.P.	6.00	0	5.8
Sodium oxacillin U.S.P.	6.64	0[i]	6.0
Sodium succinate	2.90	0	8.5
Sodium tartrate	2.72	0	7.3
Sodium warfarin U.S.P.	6.10	0	8.1
Theophylline sodium glycinate N.F.	2.94	0	8.9
Triethanolamine U.S.P.	4.05	100	10.7
Xylometazoline HCl	4.68	88[j]	5.0

[a] Solution becomes very cloudy. [b] Solution darkens and brown sediment forms; solution foams when shaken. [c] Solution turns light yellow—*no* hemolysis. [d] Solution turns dark brown. [e] Solution turns light brown. [f] Solution darkens and brown sediment forms. [g] Solution turns red-brown. [h] Solution foams readily. [i] Cells turn purple and solution foams readily. [j] Solution turns light brown and foams readily.

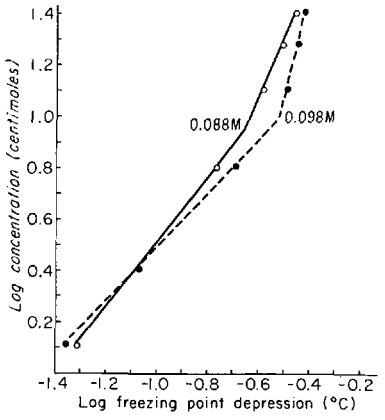

Fig. 1.—Cryoscopic behavior of certain aggregate-formers. Key: O, dexchlorpheniramine maleate; ●, valethamate bromide.

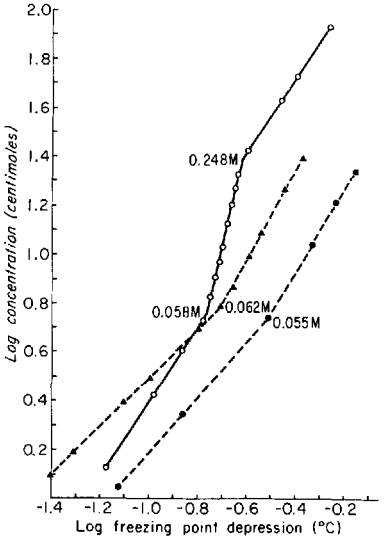

Fig. 2.—Cryscopic behavior of certain aggregate-formers. Key: O, bromodiphenhydramine hydrochloride; ▲, sodium nafcillin; ●, hydroxyzine hydrochloride.

[3] Leeds and Northrup Co., Philadelphia, Pa.

ductivity bridge at 25°. The data from both of the analytical procedures demonstrated that there was some aggregation at 25° for each substance which had shown aggregation previously at its freezing temperature, except for pyrilamine HCl which did not aggregate at 25° in the concentration range studied. Moreover, with both the conductivity and vapor pressure methods bromodiphenhydramine HCl data showed an initial aggregation point at 0.052 *M* which agreed quite well with the freezing depression data of the initial aggregation concentration (0.058 *M*), but it did not show the second break in the curve at 0.248 *M* which was disclosed by the freezing point method. The fact that this second break was not found at 25° could mean that it was temperature dependent or resulted from some other condition not controlled in the study. This point needs further investigation.

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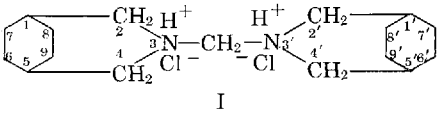
All attempts to prepare Mannich bases of indanedione failed. In every instance the end product was an amorphous solid with a high melting point and insoluble in most organic solvents. In one instance, while using 3-azabicyclo (3.2.2)nonane as the secondary amine component, a small amount of white crystalline substance was isolated from the mother liquor and identified as methylenebis-3-azabicyclo (3.2.2)nonane dihydrochloride with the help of infrared and NMR spectra. The structure of this compound was confirmed by unambiguous synthesis.

THE CONDENSATION between an amine (primary or secondary) or its salt with formaldehyde and a compound having an active hydrogen is known as the Mannich reaction.

Numerous Mannich bases are recorded in the literature (1–9); these have been prepared for pharmacological screening as antispasmodics, analgesics, chemotherapeutics, and local anesthetics.

In an effort to prepare Mannich bases of indanedione-1,3 for pharmacological testing, the condensation reaction was attempted several times utilizing dimethylamine, diethylamine, morpholine, piperidine, and 3-azabicyclo (3.2.2)nonane (AZBN) as the secondary amine component. Formaldehyde was used either as its aqueous solution or paraformaldehyde. Each synthesis resulted in a high yield of amorphous solid which was insoluble in most organic solvents. This solid material was washed several times with ether and ethanol, dried, and analyzed for elemental content. The analytical data did not correspond with the desired Mannich base. In one instance while using AZBN the mother liquor was refrigerated after adding acetone. This gave a white crystalline solid in small amounts. A pure sample was prepared after three recrystallizations from ethanol. The analytical values corresponded with methylenebis-3-azabicyclo(3.2.2)-nonane dihydrochloride (I). The infrared spectrum showed no carbonyl absorption. This ruled out the possibility of its being an indanedione Mannich base. The infrared spectrum was somewhat similar to that of AZBN.



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The NMR spectrum was consistent with the proposed structure (I). Bands at δ = 1.95 (16H, singlet) are due to methylene protons at 6,7,8,9,-6′,7′,8′,9′ and those at δ = 2.17 (6H, multiplet) correspond to protons at 1,5,1′,5′ and the methylene group between the two nitrogens. Bands at δ = 3.36 (8H, triplet) may be assigned to protons at 2,4 and 2′,4′. Bands at δ = 7.47 (2H, singlet) represent protons at 3 and 3′. When D_2O was added, the peak at δ = 7.47 disappeared due to the exchange of the protons on the nitrogen atoms.

Methylenebis-3-azabicyclo(3.2.2)nonane dihydrochloride was synthesized by utilizing another route. The melting point and infrared spectrum of the resulting product were identical in every respect to those relative to the Mannich (AZBN) mother liquor product. The mixed melting point showed no depression. On the basis of the above evidence the structure (I) of methylenebis-3-azabicyclo(3.2.2)-nonane dihydrochloride is assigned to the product isolated from the mother liquor. Isolation of similar types of by-products are recorded in the literature (10, 11). Thus *N,N′*-tetraethylmethylenediamine (10) and methylenedipiperidine (11) have been obtained when using diethylamine and piperidine, respectively.

The mechanism of the Mannich reaction has been investigated by Hellmann and Opitz (12) and Cummings and Shelton (13). It is proposed (14) that the reaction is initiated by a condensation between the amine and formaldehyde to yield an amino-

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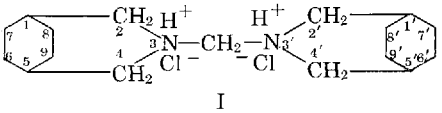

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Methylenebis-3-azabicyclo(3.2.2)nonane Dihydrochloride (I).—AZBN (6.25 Gm., 0.05 mole) dissolved in 25 ml. of ethanol was acidified with concentrated hydrochloric acid to pH 4. Paraformaldehyde (1.11 Gm., 0.037 mole) was added and the reaction mixture refluxed on a boiling water bath for 3 hr. At the end of this period the contents were cooled and 100 ml. of acetone was added. The solution was refrigerated overnight. The desired product which was obtained as white needles, was recrystallized from ethanol or ethanol–acetone, m.p. 301° dec. Yield, 5.5 Gm. (65%). The mixed melting point obtained with the product isolated from the Mannich condensation (AZBN) showed no depression. Infrared spectra of both products were identical.

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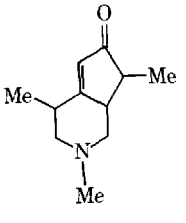
Antidiabetic Effect of Tecomine and Tecostanine

By YOUSSEF HAMMOUDA and M. SAMIR AMER*

The hypoglycemic properties of tecomine citrate and tecostanine hydrochloride on fasting blood sugar, glucose tolerance, depancreatized, and alloxan-diabetic rabbits is described. The two drugs proved to be effective antidiabetic agents only in the presence of the pancreas.

TECOMINE (I) and tecostanine (II) are two alkaloids isolated by Hammouda and Motawi (1) and Hammouda *et al.* (2) from the leaves of *Tecoma stans* (Juss.). The leaves of the various species of *Tecoma* have long been used by the natives in Mexico for the control of diabetes (3, 4). Since the structure of the two alkaloids isolated therefrom was elucidated (5–7), it was of interest to determine whether the two alkaloids are responsible for the long known antidiabetic properties of the leaves. The present study was initiated to determine the hypoglycemic properties of the two alkaloids and to determine the possible mechanism by which they produce this effect. A short note was previously published on this subject (8).



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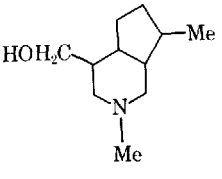

II

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Attempted Mannich Condensation of Indanedione-1,3 with AZBN.—AZBN (12.51 Gm., 0.10 mole) dissolved in 50 ml. of ethanol was acidified to pH 4 by dropwise addition of concentrated hydrochloric acid. Indanedione (14.6 Gm., 0.10 mole) was added followed by 4.5 Gm. (0.15 mole) of paraformaldehyde. The reaction mixture was heated on a water bath for 4 hr. The reaction mixture was cooled and filtered. This gave a product weighing 11.5 Gm. which could not be recrystallized because of its insolubility in most organic solvents. The mother liquor was diluted with 100 ml. of acetone and refrigerated overnight. A crystalline solid (6.5 Gm.) was obtained; this was recrystallized from ethanol, m.p. 301° dec. NMR data, δ = 1.95 (16H, singlet); 2.17 (6H, multiplet); 3.36 (8H, triplet); 7.47 (2H, singlet).

Anal.—Calcd. for $C_{17}H_{32}Cl_2N_2$: C, 60.90; H, 9.59; Cl, 21.74; N, 8.35. Found: C, 60.13; H, 9.77; Cl, 21.81; N, 8.55.

Methylenebis-3-azabicyclo(3.2.2)nonane Dihydrochloride (I).—AZBN (6.25 Gm., 0.05 mole) dissolved in 25 ml. of ethanol was acidified with concentrated hydrochloric acid to pH 4. Paraformaldehyde (1.11 Gm., 0.037 mole) was added and the reaction mixture refluxed on a boiling water bath for 3 hr. At the end of this period the contents were cooled and 100 ml. of acetone was added. The solution was refrigerated overnight. The desired product which was obtained as white needles, was recrystallized from ethanol or ethanol–acetone, m.p. 301° dec. Yield, 5.5 Gm. (65%). The mixed melting point obtained with the product isolated from the Mannich condensation (AZBN) showed no depression. Infrared spectra of both products were identical.

Anal.—Calcd. for $C_{17}H_{32}Cl_2N_2$: N, 8.35; Found: N, 8.53.

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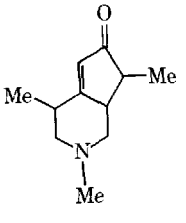
Antidiabetic Effect of Tecomine and Tecostanine

By YOUSSEF HAMMOUDA and M. SAMIR AMER*

The hypoglycemic properties of tecomine citrate and tecostanine hydrochloride on fasting blood sugar, glucose tolerance, depancreatized, and alloxan-diabetic rabbits is described. The two drugs proved to be effective antidiabetic agents only in the presence of the pancreas.

TECOMINE (I) and tecostanine (II) are two alkaloids isolated by Hammouda and Motawi (1) and Hammouda *et al.* (2) from the leaves of *Tecoma stans* (Juss.). The leaves of the various species of *Tecoma* have long been used by the natives in Mexico for the control of diabetes (3, 4). Since the structure of the two alkaloids isolated therefrom was elucidated (5–7), it was of interest to determine whether the two alkaloids are responsible for the long known antidiabetic properties of the leaves. The present study was initiated to determine the hypoglycemic properties of the two alkaloids and to determine the possible mechanism by which they produce this effect. A short note was previously published on this subject (8).



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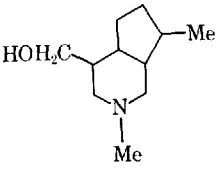

II

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TABLE 1.—EFFECT OF TECOMINE CITRATE AND TECOSTANINE HYDROCHLORIDE ON THE FASTING SUGAR LEVEL IN RABBITS

Time After Injection, hr.	Blood Sugar Level, mg./100 ml. Blood[a]				
	Tecomine Citrate		Tecostanine HCl		Tolbutamide
	Oral, 50 mg.[b]/Kg.	i.v., 20 mg.[b]/Kg.	Oral, 50 mg.[b]/Kg.	i.v., 20 mg.[b]/Kg.	Oral, 250 mg./Kg.
0	107.3	98.3	110.0	104.9	100.1
1	100.1	74.3	111.0	83.4	99.1
2	101.3	61.5	102.6	67.7	86.3
3	83.6	55.3	75.3	50.3	79.1
4	64.5	60.7	54.3	59.5	78.6
5	49.7	73.3	59.6	70.1	83.3
6	53.6	80.2	81.1	79.7	89.5
10	92.7	99.3	97.3	101.9	93.3
24	100.7	90.1	95.5	102.3	105.1

[a] Average of four to six experiments. [b] Calculated as the free base.

MATERIALS AND METHODS

The animals used in the present study were healthy male rabbits weighing 1.5–2.5 Kg. fed *ad libitum* on a balanced diet. Blood samples for the assay of blood sugar were obtained by bleeding the ear vein into a heparinized pipet. Blood sugar was determined by the method of Nelson (9). Alloxan diabetes was produced by injecting alloxan monohydrate[1] intravenously in a dose of 200 mg./Kg. in 4 divided doses over a period of 24 hr. Alloxan was dissolved in isotonic saline to make a 3% solution immediately before injection. The animals were allowed to drink a 5% glucose solution for the entire period of the alloxan diabetes. Hyperglycemia developed slowly and reached a maximum after 2 weeks. The animals were used only after producing glucosuria continuously for 3 consecutive days. Tecomine citrate and tecostanine hydrochloride were prepared from the dried fresh leaves of *T. stans* (1) and were tested for purity before use.

RESULTS

Fasting Animals.—The rabbits used in this study were fasted for 12–18 hr. before the experiments were started. The drugs were dissolved in physiological saline and given either intravenously or orally *via* a stomach tube. The results are given in Table I.

It is clear from the table that both alkaloids are potent hypoglycemic agents producing severe reductions in the blood sugar of fasting rabbits when given in a dose of 20 mg./Kg. i.v. or 50 mg./Kg. orally. When given intravenously the maximum hypoglycemic effect is reached earlier than when given orally even in higher doses. This is to be expected since the drugs have to be absorbed from the alimentary tract. The absorption from the alimentary tract would account for the 1–2-hr. lag when the drug is given orally. When given intravenously, the effect on the blood sugar reaches maximum (179% of tolbutamide) after 3 hr. and 14 min. for tecomine citrate and after 3 hr. and 23 min. for tecostanine hydrochloride (186% of tolbutamide). Some of the animals experienced hypoglycemic coma at the height of the hypoglycemic effect.

Glucose Tolerance.—The rabbits used in this series were fasted for 12 hr. before the start of the experiment. The drugs, dissolved in isotonic saline, were injected intravenously, followed 40 min. later with 3 Gm. of glucose as a 5% solution. The results are shown in Fig. 1. It is clear from the figure that both tecomine citrate and tecostanine hydrochloride in the dose used, *i.e.*, 20 mg./Kg., reduced the time needed for the blood sugar to return to normal from about 3.5 to 2.5 hr. There was a statistically significant difference between the control values and the values obtained with either drug at 3.5 hr. after the administration of glucose.

Depancreatized Rabbits.—After complete depancreatization, the rabbits developed hyperglycemia in 24 hr. After the hyperglycemia was established and glucosuria produced, tecomine citrate (20 mg./Kg.) and tecostanine hydrochloride (20 mg./Kg.) were injected i.v. Blood samples were collected 3 hr. later, since the alkaloidal salts exhibited maximum hypoglycemic effect at this time when given intravenously (see Table I), and assayed for sugar content. The results obtained are shown in Table II.

A Student *t* test was carried out on the values obtained before and after treatment, and the probability ratios (*P*) are included in the table. From the table it could be seen that the two alkaloids

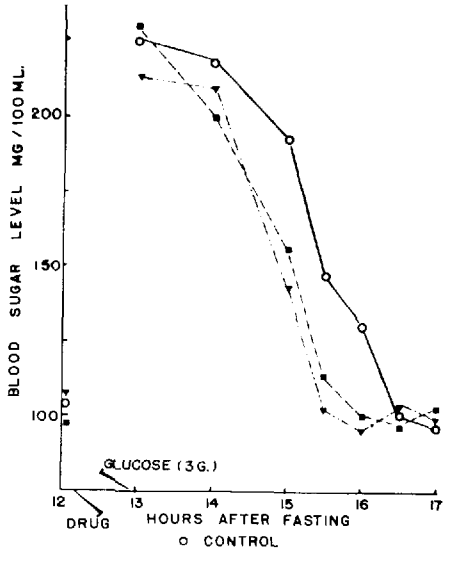

Fig. 1.—Results of glucose tolerance. Key: O, control; ▼, tecomine citrate, 20 mg./Kg.; ■, tecostanine hydrochloride, 20 mg./Kg.

[1] La Roche and Co., Ltd., Basle, Switzerland.

TABLE II.—EFFECT OF INTRAVENOUS ADMINISTRATION OF TECOMINE CITRATE AND TECOSTANINE HYDROCHLORIDE ON THE BLOOD SUGAR OF DEPANCREATIZED RABBITS

Substance Administered	Dose,[a] mg./Kg.	Rt.	Blood Sugar, mg./100 ml. Blood[b] Initial	Maximal[c]	p
Tecomine citrate	20	i.v.	257	238	0.2–0.5
Tecostanine hydrochloride	20	i.v.	301	264	0.1–0.2

[a] Calculated as the free base. [b] Average of four to six experiments. [c] Three hours after the injection of the alkaloidal salts

TABLE III.—EFFECT OF TECOMINE CITRATE AND TECOSTANINE HYDROCHLORIDE ON THE BLOOD SUGAR LEVEL IN ALLOXAN DIABETIC RABBITS

Substance Administered	Rabbit No.	Blood Sugar Level, mg./100 ml. Fasting Before Alloxan	Fasting After Alloxan[a]	Days After Alloxan[d] 16	22	45
Saline (control)	1–4[b]	103.7	275.6	289.7	310.0	
Tecomine citrate, 20 mg.[c]/Kg.	5	105.3	270.4	115.0	124.0	131.3
	6	110.3	320.1	107.3	113.4	123.3
Tecostanine hydrochloride, 20 mg.[c]/Kg.	7	92.7	303.7	100.1	97.2	101.6
	8	99.0	256.4	123.3	139.3	143.1

[a] Before drug treatment was started. [b] Average of four rabbits. [c] Calculated as the free base. [d] Treatment instituted

are not significantly effective in reducing the blood sugar of depancreatized rabbits since the difference between the treated and control is not statistically significant ($P > 0.05$). Doses of 50 mg./Kg. were also given and were unable to produce statistically significant reduction in the blood sugar of the depancreatized animals used.

Alloxan Diabetes.—Alloxan diabetic rabbits with blood sugar above 250 mg. % and exhibiting glucosuria were prepared for this study. The two alkaloidal salts were injected intravenously in isotonic saline. Three hours later, blood samples were collected and assayed for reducing sugar. Only eight animals were used in this study, *viz.*, four controls given saline and four experimental.[2] The results are shown in Table III.

It is clear from Table III that both tecomine citrate and tecostanine hydrochloride in the daily dose of 20 mg./Kg. were effective in reducing the hyperglycemia produced by alloxan to near normal levels. It should be noted that the four controls died on the 23rd, 27th, 29th, and 33rd day after alloxan administration while the experimental animals were living until the 45th day when the experiment was terminated.

DISCUSSION

From the results obtained in this study, it is clear that the salts of tecomine and tecostanine, the two alkaloids isolated from the leaves of *T. stans*, have valuable properties as antidiabetic agents. They are effective both orally and intravenously with a high margin of safety (LD_{50} 300 mg./Kg. in mice) (7), in the doses used in the present study. The two alkaloids present an entirely new nucleus for hypoglycemic activity which provides new possibilities toward the production of hypoglycemic agents. Structure-activity relationships of the new nucleus are presently under study in this laboratory to determine the active site on the two molecules.

From the mechanistic point of view, the two alkaloids seem to need a minimum of active β-cells of the pancreas for action, and in this respect resemble other orally active antidiabetic drugs, *i.e.*, sulfonylureas (10). The two alkaloids were inactive in depancreatized animals. The beneficial effects observed with the two alkaloids in alloxan diabetes are in favor of introducing them for clinical trial. The two alkaloids are without noticeable toxic effects on the rabbits used in the doses applied.

[2] The limited quantities of the alkaloidal salts available prevented the use of a larger number of animals.

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Utility of Two Convulsant Techniques as Indicators of CNS Excitability

By HAROLD H. WOLF and GERARD A. STOCK, JR.*

A study was undertaken to evaluate the relative usefulness of pentylenetetrazol (PTZ) *versus* hexafluorodiethyl ether (HFE) as chemoconvulsive agents which could be employed to quantitate CNS excitability in mice. The PTZ was given by constant i.v. infusion, whereas the HFE was administered by inhalation. Levels of excitability were altered with several doses of (+)-amphetamine and pentobarbital. The results indicate that the PTZ infusion procedure is considerably more sensitive and less variable than the HFE inhalation technique for the determination of drug-induced fluctuations in central activity.

THE OBSERVATION made by Krantz and his coworkers (1) that hexafluorodiethyl ether (HFE) caused convulsions when inhaled has led to the usage of this agent as a convulsant in psychiatric treatment. In recent years, a number of authors have commented on the utility of this procedure as a tool for the laboratory evaluation of central nervous system activity. For example, Davis and Webb (2) have employed the drug to quantitate a circadian rhythm of chemoconvulsive response and also to demonstrate that, in mice, susceptibilities to auditory and chemical convulsive stimuli are not necessarily correlated in a positive manner (3). Other workers have found the technique valuable for predicting the activity of anticonvulsants (4) and for demonstrating increased sensitivity of the central nervous system following cortical ablations (5).

Several of these studies (4, 5) have shown that the type of seizures produced by the inhalation of HFE are similar to those seen following the intravenous administration of pentylenetetrazol (PTZ). Moreover, the HFE technique has been shown to be considerably easier to employ. However, whereas much evidence is available concerning the sensitivity and reliability of the i.v. PTZ technique as a means of evaluating drug-induced fluctuations in central activity (6–9), comparatively little similar information is available for the inhalation of HFE. Therefore, a series of experiments were designed to compare the sensitivity and variability of the two techniques when used to quantitate changes in CNS activity induced by drugs.

EXPERIMENTAL

The experimental animals employed were 20 to 25 Gm., adult, male, albino mice of a random bred Swiss strain obtained from Maxfield Animal Supply. All experiments were conducted during the same time of day and room temperature was maintained between 23–26°.

PTZ was administered by timed intravenous infusion, employing a modification of a technique originally described by Orloff (10). In this procedure, the animal is briefly restrained while a 0.5% solution of the convulsant in saline is infused into a tail vein at a constant rate of 0.247 ml./min. The amount of time which elapses from onset of infusion until the animal displays 3 sec. of continuous clonic activity is recorded. From this information and the body weight of the mouse, the number of mg./Kg. of PTZ required to induce seizure can be calculated.

HFE[1] was administered as a 10% solution in 95% ethyl alcohol by means of a timed inhalation technique. The apparatus employed was a modified version of that described by Adler (5). With this procedure, the convulsant solution is infused at a constant rate of 0.136 ml./min. onto a gauze wick fixed near the top of a 300-ml. bell jar. The jar, whose open bottom edge had been coated with stopcock grease, is first placed over a mouse on a glass plate containing a filter paper mat, thus providing a relatively airtight container. A wire mesh screen dividing the chamber approximately in half effectively prevents the mouse from rising on its hind legs in an attempt to avoid the vapors of HFE which sink to the bottom of the jar. As with the PTZ technique, the amount of time which elapses from onset of infusion to 3 sec. of continuous clonic activity is recorded and the mg./Kg. of HFE required to induce seizure are calculated. Following each convulsive episode, the vapors are removed by means of a vacuum water pump which is connected to a side arm of the bell jar.

Employing these two techniques, several experiments were conducted to quantitate the fluctuations in central activity induced by pentobarbital and (+)-amphetamine. Aqueous solutions of soluble salts of these drugs, *i.e.*, sodium pentobarbital and (+)-amphetamine sulfate were administered intraperitoneally in a constant volume of 1 ml./100 Gm. body weight. Preliminary investigations, employing the chemoshock threshold procedures, revealed that the times of peak effect for pentobarbital and (+)-amphetamine were 10 and 15 min., respectively.

In order to compare the sensitivity and variability of the two convulsant techniques, groups of 14 mice each were given graded doses of pentobarbital, (+)-amphetamine, or requisite volumes of saline (controls) and evaluated for seizure susceptibility at the appropriate times of peak effect. From the accumulated data, a series of threshold ratios, *i.e.*, the mean mg./Kg. of convulsant required to produce clonic seizure in drug-treated animals divided by the mean mg./Kg. of convulsant required to reach the same end point in controls was computed for both PTZ and HFE. The 95% confidence intervals for the individual threshold ratios were calculated by a method described by Goldstein (11).

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* National Science Foundation Undergraduate Research Participant, 1965.

[1] Kindly supplied as Indoklon by Dr. A. H. Neeley, Ohio Chemical and Surgical Equipment Co., Murray Hill, N. J.

RESULTS AND DISCUSSION

The results obtained with five doses of pentobarbital, *i.e.*, 2, 4, 8, 16, and 32 mg./Kg., are shown in Fig. 1. The vertical bracketed lines represent 95% confidence intervals for the calculated ratios and any line which does not cross 1.0 indicates that the dose of pentobarbital under consideration had a significant ($p < .05$) effect on convulsive threshold.

It is obvious from the results presented that a decrease in CNS excitability, as expressed by an increase in threshold ratio, can be demonstrated for pentobarbital using either convulsant technique. However, it is also quite clear that the HFE procedure is considerably less sensitive than that employing PTZ. Thus, within the dose range examined, twice as much pentobarbital (16 *versus* 8 mg./Kg.) was required to demonstrate a significant elevation in threshold by the inhalation *versus* i.v. infusion technique. Moreover, in all instances the amount of CNS depression induced by equivalent doses of pentobarbital was more readily apparent by the PTZ procedure. The magnitude of this difference is statistically significant at doses of 8, 16, and 32 mg./Kg.

In this study the amount of data variability, as portrayed by the length of the vertical bracketed lines, was essentially the same for both convulsant procedures at four of the five doses of barbiturate tested. At the highest dose of pentobarbital the PTZ technique manifested more variability than inhaled HFE, but the former procedure was, as indicated, 1.6 times more sensitive.

The results of a similarly designed experiment employing (+)-amphetamine to alter level of CNS excitability are presented in Fig. 2. Here it can be seen that the PTZ technique is also considerably more sensitive to the effects of a central stimulant. In fact, with HFE, the authors were not able to demonstrate a significant decrease in threshold ratio with any dose of (+)-amphetamine tested. On the other hand, with PTZ, significant CNS excitability was evident at both 8 and 16 mg./Kg. of (+)-amphetamine.

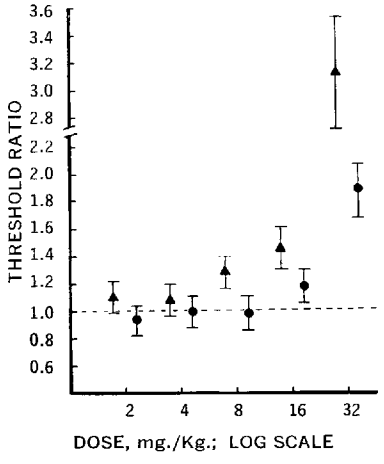

Fig. 1.—Alterations in CNS activity induced by pentobarbital. Key: ▲, PTZ technique; ●, HFE technique.

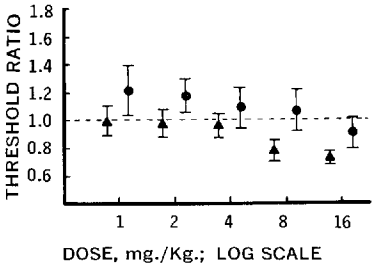

Fig. 2.—Alterations in CNS activity induced by (+)-amphetamine. Key: ▲, PTZ technique; ●, HFE technique.

It will be noted that small doses of (+)-amphetamine (1 and 2 mg./Kg.) induced a slight, but statistically significant, elevation in convulsive threshold as determined by the HFE technique. This seemingly paradoxical effect of (+)-amphetamine has been observed by other workers (12, 13) employing electroshock procedures and has been attributed to amphetamine's desynchronizing action in the frontal cortex (14) and its inhibitory action at the level of the reticular formation (15). The reasons for the inability of the PTZ technique to detect this phenomenon remain, as yet, unclear.

An examination of the 95% confidence intervals in Fig. 2 reveal that, in all instances, the inhalation of HFE provided more variable data than did the i.v. administration of PTZ. The maximum difference in variability between the two techniques amounted to 19% and was observed following the administration of 16 mg./Kg. of (+)-amphetamine.

The results obtained in this study are essentially in agreement with those reported by Truitt *et al.* (4), who observed that PTZ provided the most sensitive index of a change in convulsive threshold produced by phenobarbital. On the other hand, our results differ somewhat from those observed by Adler (5), who reported that, although an increased sensitivity to both PTZ and HFE results from ablations of either the frontal or posterior cortex, the data obtained with HFE manifest less variability. This apparent discrepancy in findings may result from species differences (mice *versus* rats) or from the methods employed to produce changes in level of central activity (drugs *versus* cortical ablations).

In summary, it would appear that although the HFE technique has the obvious advantage of simplicity of operation and lack of animal restraint, the utility of the procedure is hampered by a relative lack of sensitivity. Moreover, when the technique is employed to evaluate drug-induced changes in CNS excitability, the data collected manifest somewhat more variability than similar information obtained using timed intravenous infusion of PTZ.

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Determination of Carbonyl Compounds by Sodium Nitrite Titration of Excess 2,4-Dinitrophenylhydrazine in the Presence of Hydrazone

By LEON KURLANSIK and EDWARD F. SALIM

Numerous methods have been developed for the quantitative determination of carbonyl compounds with 2,4-dinitrophenylhydrazine. Generally, it is necessary to separate the prepared hydrazone from excess reagent before final measurement. A simplified method for the determination of aldehydes and ketones is presented in which excess 2,4-dinitrophenylhydrazine in the presence of hydrazone is titrated with standard sodium nitrite solution.

Since Mathewson (1) prepared hydrazones of water-soluble carbonyl compounds with 2,4-dinitrophenylhydrazine, this reagent has been used extensively for qualitative characterization and quantitative estimation of carbonyl compounds. Applications of gravimetric (2–4), spectrophotometric (5–7), and titrimetric procedures for quantitative determinations of aldehydes and ketones have been reported. Among the titrimetric methods developed are solution of the hydrazone in standard base and determination of excess sodium hydroxide (8), nonaqueous titration of hydrazone in pyridine with tetrabutylammonium hydroxide (9), determination of reduced nitro-groups of the hydrazone (10) or excess hydrazine (11), and direct amperometric titration of the carbonyl with 2,4-dinitrophenylhydrazine solution (12).

A common feature of most published methods is the separation of prepared hydrazone from excess reagent before the final measurement is conducted. The preponderance of procedures deals with the hydrazone and relatively few involve the determination of excess reagent. Isolation of hydrazones which are slightly soluble in the reaction media generally results in low recoveries.

Vulterin and Zyka (13) have described the potentiometric titration of 2,4-dinitrophenylhydrazine with 0.1 *M* sodium nitrite and have postulated the reaction to proceed by formation of the 2,4-dinitrophenylnitrosohydrazine. Since the β nitrogen of the hydrazone is substituted, corresponding nitroso addition presumably does not occur. Baldinus and Rothberg have reported the titration of hydrazones with sodium nitrite but only after vigorous treatment with sulfuric acid and tetrahydrofuran (14). Based on this information a simplified method for the determination of carbonyl compounds has been developed by an initial reaction with 2,4-dinitrophenylhydrazine and subsequent titration of excess reagent in the presence of prepared hydrazone using standard sodium nitrite.

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EXPERIMENTAL

All titrations were conducted potentiometrically using a Beckman Expandomatic pH meter equipped with a calomel and platinum electrode system.

Reagents.—*2,4-Dinitrophenylhydrazine Reagent Solution.*—Add 9 Gm. of finely powdered 2,4-dinitrophenylhydrazine (2,4-DNPH) to a stirred mixture of 100 ml. of 85% phosphoric acid, 100 ml. of ethanol, and 20 ml. of sulfuric acid. Stir for 30 min., add 100 ml. of ethanol, and continue mixing for an additional hour. Cool and filter prior to use. (The reagent is approximately 0.1 *M* and is stable for at least 3 weeks.)

Sodium Nitrite, 0.1 M.—Dissolve 7.5 Gm. of sodium nitrite in sufficient water to make 1000 ml. The solution was standardized against U.S.P. sulfanilamide reference standard, previously dried at 105° for 3 hr., by potentiometric titration using calomel *versus* platinum electrodes.

General Procedure.—Transfer about 1 meq. of test compound to a glass-stoppered, 125-ml. conical flask and dissolve or suspend in 20 ml. of ethanol. Add 25.0 ml. of 2,4-DNPH reagent, insert the stopper, and place the flask in a constant-temperature bath maintained at 50–55° for 1 hr. (*Note:* aldehydes are allowed to react at room temperature for the prescribed 1 hr.) Cool and transfer the contents of the flask to a 400-ml. beaker with the aid of 100 ml. of water. Add 10 ml. of hydrochloric acid, 5 Gm. of potassium bromide, and titrate slowly

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Determination of Carbonyl Compounds by Sodium Nitrite Titration of Excess 2,4-Dinitrophenylhydrazine in the Presence of Hydrazone

By LEON KURLANSIK and EDWARD F. SALIM

Numerous methods have been developed for the quantitative determination of carbonyl compounds with 2,4-dinitrophenylhydrazine. Generally, it is necessary to separate the prepared hydrazone from excess reagent before final measurement. A simplified method for the determination of aldehydes and ketones is presented in which excess 2,4-dinitrophenylhydrazine in the presence of hydrazone is titrated with standard sodium nitrite solution.

Since Mathewson (1) prepared hydrazones of water-soluble carbonyl compounds with 2,4-dinitrophenylhydrazine, this reagent has been used extensively for qualitative characterization and quantitative estimation of carbonyl compounds. Applications of gravimetric (2–4), spectrophotometric (5–7), and titrimetric procedures for quantitative determinations of aldehydes and ketones have been reported. Among the titrimetric methods developed are solution of the hydrazone in standard base and determination of excess sodium hydroxide (8), nonaqueous titration of hydrazone in pyridine with tetrabutylammonium hydroxide (9), determination of reduced nitro-groups of the hydrazone (10) or excess hydrazine (11), and direct amperometric titration of the carbonyl with 2,4-dinitrophenylhydrazine solution (12).

A common feature of most published methods is the separation of prepared hydrazone from excess reagent before the final measurement is conducted. The preponderance of procedures deals with the hydrazone and relatively few involve the determination of excess reagent. Isolation of hydrazones which are slightly soluble in the reaction media generally results in low recoveries.

Vulterin and Zyka (13) have described the potentiometric titration of 2,4-dinitrophenylhydrazine with 0.1 *M* sodium nitrite and have postulated the reaction to proceed by formation of the 2,4-dinitrophenylnitrosohydrazine. Since the β nitrogen of the hydrazone is substituted, corresponding nitroso addition presumably does not occur. Baldinus and Rothberg have reported the titration of hydrazones with sodium nitrite but only after vigorous treatment with sulfuric acid and tetrahydrofuran (14). Based on this information a simplified method for the determination of carbonyl compounds has been developed by an initial reaction with 2,4-dinitrophenylhydrazine and subsequent titration of excess reagent in the presence of prepared hydrazone using standard sodium nitrite.

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Sodium Nitrite, 0.1 M.—Dissolve 7.5 Gm. of sodium nitrite in sufficient water to make 1000 ml. The solution was standardized against U.S.P. sulfanilamide reference standard, previously dried at 105° for 3 hr., by potentiometric titration using calomel *versus* platinum electrodes.

General Procedure.—Transfer about 1 meq. of test compound to a glass-stoppered, 125-ml. conical flask and dissolve or suspend in 20 ml. of ethanol. Add 25.0 ml. of 2,4-DNPH reagent, insert the stopper, and place the flask in a constant-temperature bath maintained at 50–55° for 1 hr. (*Note:* aldehydes are allowed to react at room temperature for the prescribed 1 hr.) Cool and transfer the contents of the flask to a 400-ml. beaker with the aid of 100 ml. of water. Add 10 ml. of hydrochloric acid, 5 Gm. of potassium bromide, and titrate slowly

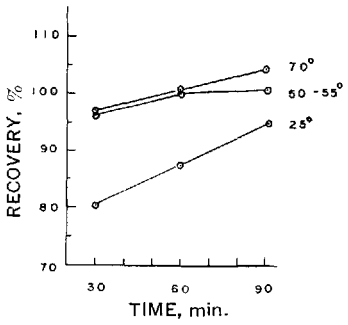

Fig. 1.—Reactivity of methylprednisolone with 2,4-dinitrophenylhydrazine reagent at various temperatures.

with moderate stirring, adding 0.1 *M* sodium nitrite in 0.1-ml. increments near the end point. Perform a blank determination. The difference between blank and sample titrations represents the volume of 0.1 *M* sodium nitrite equivalent to the carbonyl content in the sample.

The end point was determined by means of differential curves ($\Delta E/\Delta$ ml. *versus* ml.). It was noted that the volume of titrant added at the point of maximum rise in potential was the equivalent volume, thereby eliminating the necessity to construct a differential curve for each titration.

RESULTS AND DISCUSSION

Initial studies were conducted using standard 2,4-DNPH reagents. Johnson's reagent (15) consisting of ethanolic phosphoric acid was considered too viscous to allow the uniform additions necessary in the proposed method and was noted to yield quantitative hydrazone formation only after prolonged reaction. The reagent developed by Brady (16) utilizing ethanolic sulfuric acid was observed to accelerate formation of hydrazone but produced undesirable side reactions as demonstrated by high recoveries with methylprednisolone. Experiments using various combinations of phosphoric and sulfuric acids to resolve the difficulties associated with each reagent led to the formulated solution used throughout subsequent investigations.

TABLE I.—DETERMINATION OF ALDEHYDES AS 2,4-DINITROPHENYLHYDRAZONES

	Recovery, %
Benzaldehyde[b]	98.8, 98.4
Cinnamaldehyde[b]	99.4, 99.7
p-Dimethylaminobenzaldehyde[a]	99.4, 99.6
Ethyl vanillin[a]	100.4, 100.6
Formaldehyde solution U.S.P.	100.1[c]
Heptaldehyde	98.5
α-Naphthaldehyde[b]	99.8[d]
p-Nitrobenzaldehyde[a]	98.7
2-Pyridinecarboxaldehyde	99.1
Salicylaldehyde[b]	100.4, 99.5
Vanillin[a]	99.6

[a] Recrystallized before use. [b] Redistilled before use. [c] Calculated on basis of formaldehyde content found by U.S.P. assay. [d] Average of 5 determinations of S.D. ± 0.30.

A supplemental study was performed to determine the optimum time and temperature conditions for reactivity. The results shown in Fig. 1 indicate that the reaction for methylprednisolone was quantitative at 50–55° after 1 hr. The conditions established for methylprednisolone were believed to express the parameters by which sterically unhindered ketones could be readily quantitated. Analogous tests for aldehydes showed that quantitative results could be obtained at room temperature within a 1-hr. period. A summary of these data is included in Table I. For the limited number of aldehydes tested, no pronounced effects were observed for increased number of carbons in aliphatic compounds nor the addition of electron donating or withdrawing groups *ortho* or *para* to the aromatic aldehydes.

The determination of ketones was found to be influenced by certain factors so that the method was not so generally applicable as with the aldehydes. Ketones successfully determined are included in Table II. The general procedure was found to be

TABLE II.—DETERMINATION OF KETONES AS 2,4-DINITROPHENYLHYDRAZONES

	Recovery, %
Benzophenone[a]	99.8
ω-Bromoacetophenone[a]	98.8
Chalcone[a]	100.8
Cyclohexanone[b]	99.0
Dehydrocholic acid[a]	100.1[c]
3,4-Dihydro-1(2H)-naphthalenone	95.8
1,3-Diphenyl-2-propanone[a]	99.8
Estrone	100.4
Menadione[a]	99.1[d]
Methyl isobutyl ketone[b]	96.6, 96.6
Methylprednisolone	99.1, 99.7
Methyltestosterone	99.5
Prednisolone	99.1
Prednisone	100.0[c,e]
Progesterone	100.6

[a] Recrystallized before use. [b] Redistilled before use. [c] Reacted for 3 hr. [d] Calculated on basis of monohydrazone. [e] Recovery based on hydrazone formation at 3 and 20 positions only.

quantitative for mono-ketones above C_5. Lower aliphatic ketones produced the following results: acetone (84.8%), methyl ethyl ketone (89.8%), and methyl isopropyl ketone (93.7%). The low values may be explained on the basis of nitrous acid oxidation of methyl or methylene groups adjacent to a carbonyl group. In aqueous HCl solution the reaction proceeds to the formation of a 1,2-dicarbonyl compound and hydroxylamine hydrochloride (17). The hydrazones of lower molecular weight ketones contain the grouping CH_3—C=N— which is the nitrogen analog of the carbonyl group and may undergo a similar reaction during the final titration with sodium nitrite.

Several steroids were included in this general survey because of the varied positions occupied by carbonyls and in light of the wide use of such compounds as medicinal agents. Steroids containing a keto group in the 3 or 17 position were quantitative in the alloted reaction time. Dehydrocholic acid

with carbonyls in the 3, 7, and 12 positions required 3 hr. at 50–55° for quantitative reactivity. Prednisone, a 3,11,20-trione compound, gave 66.7% recovery after 3 hr. and no further reaction was evident after 17 hr. elapsed time. Since the 11 keto position is known to be extremely unreactive, the result at 3 hr. can be considered quantitative based on hydrazone formation at the 3 and 20 positions only. Progesterone, prednisolone, and methylprednisolone which represent 3,20 di-ketones yielded quantitative bis-hydrazones. Prednisolone, esterified as the acetate, gave an incomplete reaction—81.5%—after 3 hr. The presence of the bulkier substituent adjacent to carbon 20 appears to be sufficient to drastically hinder the formation of hydrazone at the C_{20} carbonyl.

SUMMARY

The titration of excess 2,4-dinitrophenylhydrazine using sodium nitrite without prior separation of the precipitated hydrazone allows a shorter analysis time for the determination of aldehydes and a substantial number of ketones. The procedure was found to be reproducible to ±0.5% and the results for ethyl vanillin, formaldehyde solution U.S.P., prednisolone, menadione, and prednisone were consistently within 0.5% of comparative values obtained by official or other recognized methods of analysis.

The method is neither applicable to certain aliphatic ketones nor to sterically hindered steroids. Carbonyl compounds which contain a functional group such as an amine or phenol capable of reacting with sodium nitrite cannot be determined by the proposed procedure.

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Amides Derived from Hepta- and Octamethyleneimine

By HEINO A. LUTS, W. A. ZUCARELLO*, W. LEWIS NOBLES†, and JEROME F. GRATTAN

Some new physiologically active amides of hepta- and octamethyleneimine moiety have been prepared. Their preparation and biological activities are given.

THE KNOWLEDGE that a number of biologically important compounds occurring in nature contain trimethoxyphenyl or trimethoxybenzoyl groups as a part of their molecule has prompted considerable investigation into the various ways which this moiety can be incorporated into molecules and elicit various pharmacological actions.

Vargha and his associates (1) have reported on the tranquilizing and analgesic effects of the simple benzamide containing the above moiety as well as a number of heterocyclic amides. In varying the amine moiety of the amide, they noted that morpholine and 2-methylmorpholine exhibited the most desirable therapeutic properties among the compounds studied. Correspondingly, Schlager (2) in a review article has reported on a large number of derivatives containing 3,4,5-trimethoxybenzoyl moiety.

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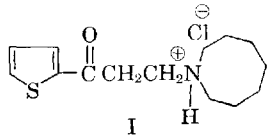

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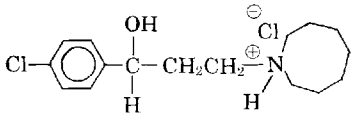

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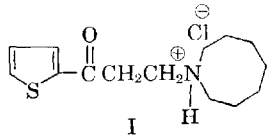

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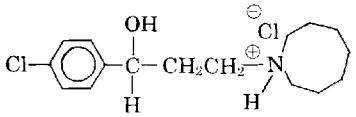

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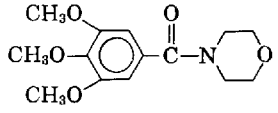
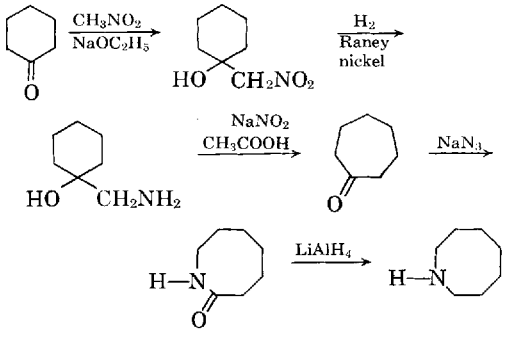
exhibited marked analgesic activity at a dosage level of 32 mg./Kg.

In addition to the work of Vargha and associates (1), the pharmacological activities of a number of different amides have been observed; these have exhibited sedative, analgetic, anticonvulsant, and cardiotonic activity (4). It is perhaps particularly noteworthy that *N*-3,4,5-trimethoxybenzoylmorpholine has elicited marked neurodepressive as well as analgetic activity (5, 6).



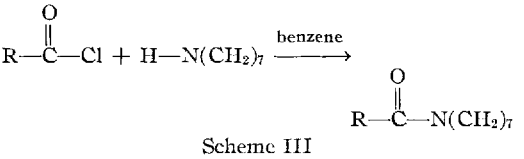
III

It is apparent in a review of the literature that the heterocyclic amine moiety has not been studied in molecules possessing seven to eight methylene groups



Scheme I

as an integral part of the structure. Hence, a concerted literature review was carried out as a prelude to the extension of the authors' studies to rings larger than pyrrolidine and piperidine, *i.e.*, heptamethyleneimine and octamethyleneimine. It was anticipated that the enlargement of the carbon



Scheme III

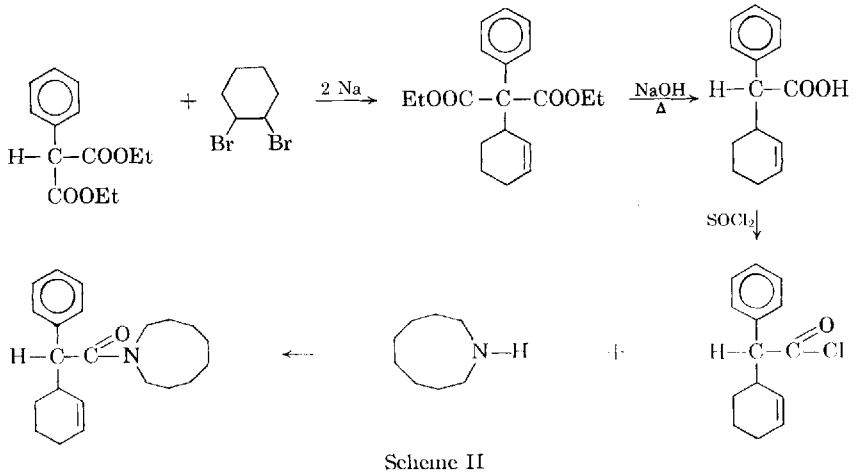
content of the amine moiety would increase the lipid solubility, and perhaps differ in enzymatic attack.

With the increasing availability of furan derivatives, considerable interest has been evidenced in the preparation of furan relatives of compounds of the benzene series which possess medicinal value; the same is true of a great number of pyridine derivatives. Thus, also drawing from previous knowledge of the activity of *N*-3,4,5-trimethoxybenzoylmorpholine, the hepta- and octamethyleneimine amides of this same acid moiety have been prepared and submitted for pharmacological study. In addition, certain heterocyclic acids have been condensed with these same amines. Furthermore, α-phenyl, α-cyclohexenyl acetic acid was utilized as an acid moiety because of the widespread use of acids of this general type in antispasmodics.

The method of preparing cyclohepta- and cyclooctanones was that of Boeseken and Derx (7); for hepta- and octamethyleneimine, the method was that of Blicke and Doorenbos (8) (Scheme I). The α-phenyl, α-cyclohexene acetic acid was by the Neesby *et al.* (9) (Scheme II) method. All the acid chlorides were prepared by the standard method of using thionyl chloride. The amides were prepared by reacting acid chlorides with heptamethyleneimine in benzene (Scheme III).

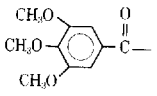
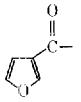
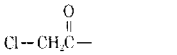
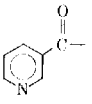
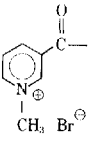
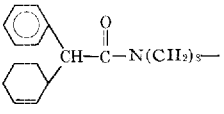
EXPERIMENTAL

General Procedure.—To a solution of 0.05 mole of appropriate acid chloride in 150 ml. of benzene solution was added dropwise 0.1 mole of appropriate amine. The reactants were then refluxed for 30 min. and formed crystals, separated by filtration. The benzene solution was then evaporated to dryness and the formed crystalline product washed twice with petrolcum ether.



Scheme II

TABLE I.—ANALYTICAL RESULTS
R—N(CH$_2$)$_7$

Compd.	R	Over-All Yield	Mol. Wt.	M.p., °C.	Formula	Anal., N Calcd.	Anal., N Found
I		78	307.37	86	$C_{17}H_{25}NO_4$	4.56	4.46
II		89	207.25	(b.p.) 141–149/ .3–.35 mm.	$C_{12}H_{17}NO_2$	6.76	6.81
III		84	189.66	62–63	$C_9H_{16}ClNO$	7.37	7.28
IV		82	218.28	(b.p.) 162–170/ .24 mm.	$C_{13}H_{18}N_2O$	12.83	12.98
V		94	313.22	173	$C_{14}H_{21}BrN_2O$	8.95 Br = 25.89	9.04 Br = 26.51
VI		87	325.47	100–101	$C_{22}H_{31}NO$	4.28	4.53

PHARMACOLOGICAL DATA

The preliminary pharmacological findings demonstrate that compound I (Table I) is a mild stimulant, and not a depressant, with a poor over-all activity. No analgesic activity was found. Compound II exhibited depressant effect; following a brief period of mild excitation, it also exhibited anticonvulsant activity and at a low dose level. Furthermore, compound II antagonized strychnine and exerted a weak analgesic effect and had local anesthetic effect at the 1% dose level when instilled repeatedly in rabbit's eye. Compound III also exhibited depressant activity and was a spasmolytic agent. It antagonized the tremors consequent on tremorine injection, although it did not antagonize the parasympathomimetic effects of the drug. Furthermore, it also exerted a mydriatic effect. Compound IV acted as a stimulant; no reserpine antagonism, but compound V was a mild depressant. Both compounds were cholinergic and peripheral vasodilators. Compound VI has shown some antispasmodic activity; the compound is still under investigation. A more detailed report will be presented in another publication.

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Determination of Codeine in Terpin Hydrate and Codeine Elixir

By MARTIN I. BLAKE and BRUCE CARLSTEDT

Ion exchange chromatography and nonaqueous titrimetry are applied to the analysis of codeine in terpin hydrate and codeine elixir. The method is simple and accurate.

ALTHOUGH terpin hydrate and codeine elixir has been official for many years, it has been only recently that an assay procedure (1) has been included in the monograph. The assay involves the extraction of the alkaloid from the elixir with chloroform, followed by titration with perchloric acid in dioxane using methanolic methyl red as the indicator. The Association of Official Agricultural Chemists (2) recognizes a spectrophotometric procedure for both the terpin hydrate and codeine in the elixir. It is based on a report by Milos (3). The codeine is determined by measuring the absorbance of an aqueous solution of the hydrochloride salt at 285 mμ. Interfering substances are removed by ether extraction.

In a recent paper (4) Milos extracted the codeine from the elixir as the *p*-toluenesulfonic acid complex on a column of chromatographic siliceous earth.[1] The complex was eluted with chloroform–acetic acid solution and passed through a second column of chromatographic siliceous earth containing $NaHCO_3$ as the stationary phase. The codeine in the eluate was determined spectrophotometrically at 278 mμ.

Ion-exchange resins have been employed in the determination of alkaloids and alkaloidal salts. These have been noted in a previous paper (5). The analysis of alkaloids and their salts by nonaqueous titration and by ion-exchange chromatography has been extensively reviewed by Higuchi and Bodin (6). Kucharsky and Safarik (7) have thoroughly reviewed the literature on the determination of alkaloids by titration in nonaqueous media.

In the present paper the techniques of ion-exchange chromatography and nonaqueous titrimetry are applied to the analysis of codeine in the elixir of terpin hydrate and codeine.

EXPERIMENTAL

Preparation of the Ion-Exchange Column.—The strongly acidic cation-exchange resin Dowex 50-×8 (200–400 mesh) was used in this study. The chromatographic column was prepared as described in an earlier paper (5). Five grams of resin was added to the tube as a slurry in water. The column was washed with 250 ml. of distilled water, 50 ml. of 2 *N* HCl, and distilled water until the eluate gave a negative test with $AgNO_3$ T.S. The column was ready for use.

Assay Procedure.—Exactly 20 ml. of terpin hydrate and codeine elixir[2] was transferred by pipet to a 150-ml. beaker. About 40 ml. of ethanol was added to reduce the viscosity of the elixir and permit a more rapid flow rate through the column. The solution was passed through the resin column. The beaker was rinsed several times with small amounts of ethanol and the washings were added to the column. The column was washed with an additional 50 to 75 ml. of ethanol.

The codeine was eluted from the column with 10% ammonia in ethanol prepared by diluting stronger ammonia water with ethanol. A total of 100 ml. of eluate was collected. The solution was evaporated to dryness by gentle aeration by directing a fine stream of air above the surface of the liquid. The residue was dissolved in ethanol and the solution was evaporated to dryness. This procedure was repeated.

The codeine content of the residue was determined by dissolving in 30 ml. of glacial acetic acid and titrating visually with 0.01 *N* perchloric acid in acetic acid. The indicator for the titration was 0.2% methyl violet in glacial acetic acid. The exact indicator color change was noted by using indicator solution in conjunction with a potentiometric titration. The color change corresponding to the graphic end point was from violet to dark blue. A blank determination was performed with each series of titrations, and any necessary corrections were made.

Comparison was made with the N.F. XII assay procedure (1).

RESULTS AND DISCUSSION

On the basis of 10 consecutive determinations the proposed procedure gave an average per cent recovery (based on labeled amount) of 99.66 with a standard deviation of ± 1.17. By the N.F. method the per cent recovery was 98.04 with a standard deviation of ± 1.64.

The proposed assay for codeine in terpin hydrate and codeine elixir is simple, rapid, and accurate. The codeine is extracted from the elixir by passage through a sulfonic acid ion-exchange resin. This obviates the tedious and time-consuming solvent extraction procedure. The codeine is readily eluted from the column by displacement with the stronger base ammonia. The codeine is recovered from the eluate by aeration which removes the ammonia, ethanol, and any water which may be present. The codeine is then titrated in glacial acetic acid with acetous perchloric acid using methyl violet as the indicator. This technique produces a much sharper and more readily detectable end point than the official method. Considerable difficulty was experienced in end point detection when the official assay procedure was performed. In addition, lower results were obtained with a higher standard deviation.

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Received July 25, 1966, from the Department of Pharmacy, University of Illinois at the Medical Center, Chicago 60612.

Accepted for publication September 8, 1966.

[1] Marketed as Celite 545 by Johns-Manville, New York, N. Y.

[2] Prepared by the Manufacturing Pharmacy Division, Department of Pharmacy, University of Illinois at the Medical Center, Chicago.

Stirring Apparatus for the Investigation of Unstable Strongly Adsorbing Chemicals

By R. J. SAUCHUK, J. M. ANDERSON, and J. G. NAIRN

An apparatus is described which is useful for the evaluation of adsorption phenomena. The mixing apparatus provides the following features: an inert atmosphere to maintain maximum stability, a smooth, all-glass surface to eliminate spurious adsorption, a convenient method of analysis to permit maximum accuracy for rapid reactions, constant temperature, and controlled, measurable mixing speeds.

IN ORDER to provide an inert atmosphere for the adsorption studies of compounds subject to oxidation by air, a tightly-stoppered flask with a magnetic stirring device was employed. This method also provided a rapid, convenient method of analysis for kinetic studies. It was found that strong adsorption took place on all stirring bars covered with either Teflon or Tygon. Over short periods of time, a Pyrex sealed stirring bar was found to be satisfactory. However, prolonged use resulted in the formation of a ground-glass surface, both on the stirring bar and on the bottom of the reaction flask. This rough surface, which acted as an adsorption site, invalidated experimental results.

A floating, magnetic stirrer was designed to overcome this problem of spurious adsorption (Fig. 1). A Pyrex-covered magnetic bar $^3/_8$ in. × $^7/_8$ in. was sealed to the bottom of a cylindrical float. The size of the float is dependent upon the following factors: the density of the solution, the height of the float above the magnetic stirrer, the speed of rotation, and the dimensions of the reaction flask. A mirror was enclosed within the float so that the speed of rotation could be determined by a photoelectric counting device. A hook was formed at the top of the float for ease of manipulation. The extent of agitation in the vessel can be controlled by varying the dimensions of the flat portion of the float.

To facilitate spectrophotometric analysis, a cell was sealed by means of a short extension onto the shoulder of a 250-ml. conical flask fitted with a standard taper $^{34}/_{45}$ stopper. The flask was placed in a constant-temperature bath, under which a magnetic stirrer was positioned. This closed system eliminated loss of volatile components and prevented exposure of the reaction mixture to atmospheric oxygen.

The speed of rotation was determined by allowing a beam of light from a 12-v. lamp to fall upon the mirror in the rotating float. The reflected light was allowed to sweep across a photoelectric cell, which by means of the electrical circuit (Fig. 2) activated an impulse counter. The magnetic stirring motor was maintained at moderate temperatures by allowing cold water to pass through Tygon tubing encircling the housing. A more precise control of the mixing speed was obtained by supplying power to the magnetic stirrer through a variable transformer.

Satisfactory performance for counting and float stability up to 400 r.p.m. was obtained.

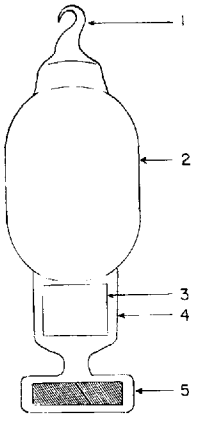

Fig. 1.—Floating magnetic stirrer. Key: 1, hook; 2, cylindrical body; 3, mirror; 4, flattened mirror casing; 5, Pyrex-covered magnetic bar.

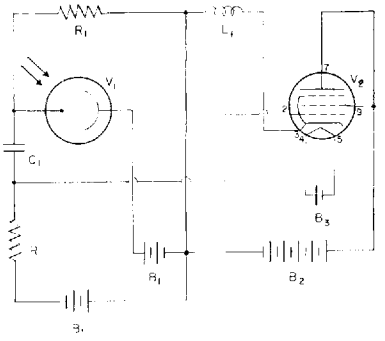

Fig. 2.—Electrical circuit for photoelectric counting device. Key: B_1, 45 v. (type B battery); B_2, 300 v. (power supply); B_3, 6 v. (filament supply); R_1, 22 megohms; C_1, 1 mf.; L_1, counter (Central Scientific Co.); V_1, photocell (Rogers 922 or equivalent); V_2, 6 CW5.

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The authors are grateful to Mr. G. L. Bragg, who constructed the photoelectric counting device.

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Reaction of Aspirin with Amines. Potential Mechanism for Aspirin Allergy

By MICHAEL A. SCHWARTZ and GORDON L. AMIDON*

A possible explanation for the lack of hypersensitivity to salicylic acid by patients known to be allergic to aspirin is offered. It is shown that while aspirin reacts with amino acids to produce salicylamide derivatives, salicylic acid does not. This may be the mechanism by which aspirin combines with protein to form antigen.

IT IS NOT yet clear whether hypersensitivity to aspirin is immunologic in origin or is a direct effect of aspirin on tissues which is not mediated by an antigen–antibody reaction. On the hypothesis that an allergic reaction is involved, several studies have been made of the immunogenicity of synthetic aspirin–protein conjugates in animals (1–4). These conjugates were prepared by treatment of protein with acetylsalicyl chloride or azide. In each case it was shown that antibodies to the aspiryl or salicyl hapten were produced, but attempts to demonstrate the presence of these antibodies in humans known to be aspirin hypersensitive were not successful.

One remarkable characteristic of aspirin hypersensitivity is the fact that individuals who react to aspirin rarely do so with salicylate (5). If it is assumed that aspirin hypersensitivity is mediated by an antigen–antibody reaction, then the antigen must be formed *in vivo* by coupling of aspirin to protein. One explanation of the lack of reactivity to salicylate may be that salicylate is unable to react with protein to form the antigenic conjugate as does aspirin.

The anhydride nature of aspirin has been noted by Davidson and Auerbach (6) who found that aspirin reacted in pyridine at 100° with *p*-toluidine to produce a small yield (0.13%) of *N*-salicyloyl-*p*-toluidide. Salicylic acid itself could not react in the same way. Surprisingly little attention has been paid to the reactions of aspirin with nucleophiles in aqueous media. Troup and Mitchner (7) have found acetylated phenylephrine resulting from reaction of aspirin with the latter in tablet formulations and Jacobs *et al.* (8) recently reported a similar reaction between codeine and aspirin. There has been no demonstration of any salicyloyl derivative being formed in these reactions.

The present work reports the results of experiments in model systems where a deliberate search was made for salicyloyl derivatives in the reaction of aspirin with amino acids.

EXPERIMENTAL

Preparation of N-Salicyloyl Derivatives of Amino Acids.—To a solution of 0.03 mole of the amino acid in 25 ml. of water was added 3.2 Gm. (0.08 mole) sodium hydroxide and 4.4 Gm. (0.02 mole) phenyl salicylate and the mixture was refluxed 2–4 hr. (until the phenyl salicylate had dissolved). The mixture was cooled, acidified, and the precipitate collected and recrystallized from an appropriate solvent. Details are listed in Table I.

Preparation of N-Acetyl Derivatives of Amino Acids.—In 4 ml. of glacial acetic acid was suspended 1.5–2.0 Gm. of the amino acid and 2.0 ml. acetic anhydride was added. The mixture was heated gently until all the amino acid had dissolved and on cooling the *N*-acetyl derivatives crystallized. Details of each compound are listed in Table I.

For both the salicyloyl and acetyl derivatives, infrared spectra showed those bands expected.

Products of Reaction of Aspirin with Amino Acids.—*ε-Aminocaproic Acid.*—Six and one-half grams of ε-aminocaproic acid was mixed with one equivalent of sodium hydroxide and 1.8 Gm. aspirin was added. The reaction was allowed to proceed 15 min. at 40° and then acidified to pH 2, the precipitate filtered off, and the filtrate evaporated to dryness. The residue was extracted with benzene and the benzene removed under reduced pressure. The resulting residue was dissolved in chloroform and subjected to thin-layer chromatography.

Glycine.—The reaction was carried out exactly as above except that methanol was substituted for benzene and acetone for chloroform.

Thin-Layer Chromatography.—Separation of the products of reaction of aspirin with glycine and ε-aminocaproic acid was accomplished by thin-layer chromatography on Silica Gel G using either benzene–acetic acid–water, 2:2:1 (I) or 4:8:3 (II), as the developing solvent. The spots were detected by exposing the plates to iodine vapor, and the R_f values are given in Table II. As a control, the experiment with glycine was repeated substituting salicylic acid for aspirin.

RESULTS AND DISCUSSION

The thin-layer chromatograms of the extracts from workup of the reaction mixtures are shown in Fig. 1. It is quite clear from these plates that the *N*-salicyloyl derivatives of both glycine and ε-aminocaproic acid are formed in the reactions of aspirin with the respective amino acids. This is the first demonstration of a salicylamide derivative resulting from reaction of aspirin with an amine. The amount formed is quite small. From the size of the spots on the thin-layer plates, it is estimated that only about 0.01–0.1% of the aspirin reacted by this route. Nevertheless, these results show the feasibility of reaction of aspirin with amino groups on proteins to produce conjugates which may be antigenic. When salicylic acid was substituted for aspirin, no salicyluric acid could be detected in the reaction mixture. The over-all reaction taking place may be depicted as in Scheme I. Direct nucleophilic attack by amine will result in aminolysis of the ester producing salicylic acid and an acet-

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* National Science Foundation undergraduate research participant.

TABLE I.—DETAILS FOR EACH COMPOUND

Compd.	Solvent for Recrystallization	M.p. Found	M.p. Lit.
N-Salicyloyl glycine	$CHCl_3$–ethyl acetate, 3:1	162–164	170.2 (164)[a]
N-Salicyloyl-ε-amino-caproic acid	$CHCl_3$–CCl_4	105–108	...
N-Acetylglycine	...	205–207	206–208[b]
N-Acetyl-ε-aminocaproic acid	$CHCl_3$	97–99	...

[a] Heilbron, I., "Dictionary of Organic Compounds," vol. 2, Oxford University Press, New York, N. Y., 1953, p. 775. [b] *Ibid.* vol. 1, p. 206.

TABLE II.—R_f VALUES FOR COMPOUNDS

Compd.	R_f Value in Solvent System I	R_f Value in Solvent System II
Salicylic acid	0.47	0.60
N-Salicyloyl glycine	...	0.16
N-Salicyloyl-ε-aminocaproic acid	0.23	...
N-Acetylglycine	...	0
N-Acetyl-ε-aminocaproic acid	0	...

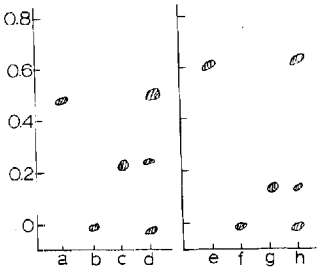
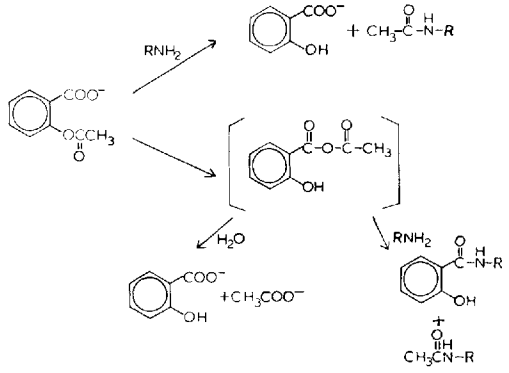


Fig. 1.—Diagram of TLC plates utilized in identification of reaction products. Key: a, salicylic acid; b, *N*-acetyl-ε-aminocaproic acid; c, *N*-salicyloyl-ε-aminocaproic acid; d, extract of products of reaction of aspirin with ε-aminocaproic acid; e, salicylic acid; f, *N*-acetylglycine; g, *N*-salicyloyl glycine; h, extract of products of reaction of aspirin with glycine.

amide derivative. Competing with this reaction is the intramolecular hydrolysis of aspirin which proceeds through an anhydride of acetic and salicylic acids (9). It seems likely that in the presence of amine both acetyl and salicyloyl derivatives might be formed depending on the relative electrophilicity of the respective carbonyl carbons. In the presence of H_2O^{18} it was found that aspirin hydrolysis produced salicylic acid containing 6% of the O^{18} which had taken part (10). Of course the amine competes with water as the nucleophile, and as a result only a very small proportion of the



Reaction of Aspirin with Amines
Scheme I

original aspirin would become salicylamide derivative.

If salicyloyl–protein is indeed proved to be the antigenic determinant in hypersensitivity to aspirin in humans, then it can be intimated that such compounds as salicyl–salicylic acid and aspirin anhydride would be potentially allergenic drugs. The product of reaction of the former, as can be seen by analogy with Scheme I, would be a salicyloyl amide by both pathways. Aspirin anhydride is quite labile (11) and probably subject to aminolysis to produce acetyl–salicyloyl amide derivatives.

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Use of 2-(4-Hydroxybenzeneazo) Benzoic Acid for Protein Determination

By RONALD G. LEONARDI and VINCENT DE PAUL LYNCH

The compound, 2-(4-hydroxybenzeneazo) benzoic acid, also known as HBABA, was evaluated for its ability to detect cell protein. While this test may be suitable for large protein concentrations, it is not sensitive enough for the stated purpose with concentration below 1.0 mg.

THE CANCER Chemotherapy National Service Center describes a technique used for the determination of cell growth or inhibition in its program using the cell culture system as a primary screen for the detection of antitumor agents (1, 2). Their protocol is based upon the Folin-Ciocalteu method for the measurement of cell protein as modified by Eagle and Oyama (3). This involves the colorimetric determination of cell protein as an indirect measurement of cell growth.

In 1954, Rutstein *et al.* suggested that blood or serum protein levels could be determined quantitatively by interaction of the protein with the anionic dye, 2-(4-hydroxybenzeneazo) benzoic acid (4). Their method was reported to be more accurate and more rapid than electrophoretic methods (5).

In this research, an attempt was made to adapt Rutstein's procedure to the determination of cell growth along the lines of the CCNSC protocol.

EXPERIMENTAL

Rutstein's original standard curve was duplicated by using the procedure he described (4). Since this work was done within the range of normal concentrations of human serum albumin (approximately 3.5 to 4.5 mg.%), the aliquots of serum needed were small but the original quantity of reagents was large. The total volume of the reaction mixture was 25 ml. The range of detection of the standard curve was from 0 to 14 mg. The curve was found to be linear up to a level of 8 mg.

In this study, using the CCNSC protocol (1, 2), cell protein concentration of HeLa cell cultures was found to be in the range of 0.005 to 0.1 mg. Initially, to determine whether HBABA would detect protein at these levels, a series of protein concentrations within these ranges were prepared. Armour's protein standard solution[1] was used as the protein source. Because of the low quantity of protein which the authors desired to detect, a modification of the volume of reagents used was required.

To determine if the dye would detect cellular protein levels, a series of protein concentrations ranging from 0.005 to 0.1 mg. was prepared. The procedure followed was the same as that described by Rutstein (4) for serum albumin determinations, but the volume of the reactants was adjusted as follows.

A sufficient quantity of acetate buffer was added to the specified quantity of bovine albumin to give a total volume of 2 ml. To this was added 1 ml. of normal saline followed by 4 ml. of the HBABA solution. Ten minutes were allowed for completion of the reaction and development of color. Per cent transmittance and absorbance was read in a colorimeter at 520 mμ. The final solution volume was maintained at 7.0 ml., and pH adjusted to 6.2.

TABLE I.—STANDARD CURVE FOR HBABA[a]

Protein Concn.	Transmittance, %[b]	Absorbance
0.0	93.8	0.0278
1.0	92.3	0.0348
2.0	91.6	0.0381
3.0	89.1	0.0501
4.0	87.4	0.0585
5.0	85.5	0.0680
6.0	84.4	0.0736
7.0	83.2	0.0799
8.0	82.4	0.0841
9.0	81.5	0.0888
10.0	81.1	0.0910

[a] 2-(4-Hydroxybenzeneazo) benzoic acid. [b] Each reading is the average of three tests.

In the determination of cell protein levels, the following procedure was applied.

Two milliliters of a suspension containing 100,000 cells/ml. was centrifuged at 2500 × g for 10 min. The supernatant liquid was removed from the packed cells by decantation. To the cells was added 2 ml. of a 0.05% trypsin solution and 5 drops of 0.01 *N* hydrochloric acid, for the purpose of lysing the cells. Two milliliters of acetate buffer solution was added to the lysate and the mixture shaken vigorously. Finally, the HBABA solution was added, 10 min. allowed for color development, and the per cent transmittance and absorbance read in a suitable colorimeter at 520 mμ.

RESULTS AND CONCLUSIONS

Using this modified procedure, it was determined that HBABA could be used to detect protein concentrations above 1.0 mg. However, this dye does not give consistent or reproducible results below a concentration of 0.3 mg. (Table I).

Similar results were found when this procedure was used to determine the protein concentration of cell cultures standardized to contain 100,000 cells/ml. of media.

It is to be concluded that while this assay may be adequate enough to be used in the clinical evaluation of serum protein levels, it is not sensitive enough to apply to the determination of cellular protein concentrations.

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[1] Protein Standard Solution supplied by Armour Pharmaceutical Co., Kankakee, Ill.

Mesoionic Ψ-Oxatriazoles as Hypotensive Agents

By LEMONT B. KIER*, A. AL-SHAMMA, R. HAHN, and A. TYE

A number of alkyl substituted mesoionic Ψ-oxatriazoles were investigated as hypotensive agents. They were found to produce rapid, deep, and sustained hypotension with no observable side effects. The potency followed a reverse order of mesomeric contribution of the alkyl substituents. Molecular orbital calculations revealed a large positive character on the N_3 and, in comparison with the corresponding 3-alkylsydnone and 4-acylsydnone nitrogen atom, appears to correlate with hypotensive potency.

Over the past several years studies in this laboratory on the mesoionic sydnones (I) have revealed a marked pharmacological activity in the form of CNS stimulation (1–3). In addition it was found that the sydnones possess a moderate diuretic and hypotensive property (4). Unfortunately, this hypotensive activity manifested itself at a dose level very close to the convulsive dose level, so that useful information concerning structure–activity relationships, mechanism of action, and the development of a potentially useful drug was denied.

With these problems in mind, the authors have studied a modified sydnone structure in the hope of finding a greater hypotensive activity and a reduced convulsive activity. The approach, in this study, was to consider a change in the ring which would not alter the over-all mesoionic character, *i.e.*, it was decided to study an isoconjugate heterocycle. An example of such a ring system is the mesoionic pseudooxatriazole (II). This compound is identical to the sydnone ring except for the replacement of the No. 4 carbon atom of the sydnone with a nitrogen atom.

The pseudooxatriazoles have been known since 1933, when Ponzio (5) prepared the phenyl derivatives by treating a phenyl diazonium salt (III), with nitroform (IV). More recently, Boyer and Canter (6) have prepared alkyl Ψ-oxatriazoles from the action of nitrous acid (V) on the appropriate semicarbazide (VI). Hashimoto and Ohta (7) have prepared the methyl derivative from the action of carbonyl chloride (VII) on *N*-nitroso-*N*-methylhydrazine (VIII). The most recent contribution to the synthesis of these compounds has been by Farrar (8), who synthesized the phenyl derivative from potassium diazomethane-disulfonate (IX), and a benzenediazonium salt (III) followed by treatment with nitrous acid. The Ψ-oxatriazoles studied in this work were previously described (9). The authors have confined their study to a few simple alkyl substituted Ψ-oxatriazoles in order to minimize partition coefficient effects and yet to study the electronic effect of substituents on the ring (Scheme I.)

In order to examine the electronic alterations produced in the ring by replacement of the —CH— of the sydnone with an —N— in the Ψ-oxatriazoles, the electronic structure was calculated using molecular orbital methods previously described for the sydnones (10). The ground state structure is shown in Fig. 1 for methyl Ψ-oxatriazole. The calculations have been tested by the use of the equation derived for the sydnones (10) relating the U.V. absorption maximum with the energy difference between the highest filled and lowest vacant molecular orbital (characteristic of an approximation of a $\pi \rightarrow \pi^*$ transition). From the authors' calculations of methyl Ψ-oxatriazole a value of 264 mμ for the predicted transition was obtained, which is quite close to the experimental value of 260 mμ obtained in a methanol solution. Thus, it was felt that the calculations have some validity in reproducing the electronic structure of the Ψ-oxatriazoles.

A comparison between the electronic structures of methyl Ψ-oxatriazole and 3-methyl sydnone (Fig. 1) is illuminating. The exocyclic oxygen of methyl Ψ-oxatriazole has a lower charge density while the No. 3 nitrogen atom has a substantially more positive character.

The two flanking nitrogen atoms both have charge densities in the negative range, while the —CH— flanking the No. 3 nitrogen of the sydnones is positive in character.

EXPERIMENTAL

In mice, the six Ψ-oxatriazoles under study, methyl, ethyl, isopropyl, 3-pentyl, *sec*-butyl, and *tert*-butyl, in doses of 200 mg./Kg. i.p. caused no apparent signs of toxicity. Doses of 600–800 mg./Kg. were required to produce death.

Administration of the liquid compounds in doses of 20 mg./Kg. i.v. in anesthetized dogs produced a prompt depressor effect in all cases. After the initial fall in blood pressure, there was, characteristically a small short-lived rise followed by a long period of hypotension which lasted for several hours in the case of the more potent compounds (Table I). During this period of hypotension, the animals remained normal with regard to respiration, EKG pattern, and gross and general appearance. The heart rate showed no important changes, being sometimes slightly increased or slightly decreased, although usually unaltered. The hypotensive effect ranged from approximately a 10% decrease for methyl Ψ-oxatriazole to over a 40% decrease for the *tert*-butyl Ψ-oxatriazole. There was a tendency to longer duration of effect with the more potent compounds (Table I). Hypotensive activity was also found in the spinal cat which indicates that in the cat the action is not centrally mediated. Pretreatment in dogs with atropine (1–2 mg./Kg.) or pyribenzamine (5 mg./Kg.) did not block the hypotensive effects, indicating that cholinergic effects or histamine release are unlikely causes of the hypotensive effects. Pressor responses in the dog to DMPP (25 mcg./Kg.) and epinephrine or norepinephrine (2–4 mcg./Kg.) were not reduced by the Ψ-oxatriazoles, indicating that ganglionic or α-receptor blockade are also unlikely causes.

When the compounds were tested on an isolated rabbit ileum and rat ileum or uterus maintained in a muscle bath, they produced a prompt inhibition of the smooth muscle activity in concentrations of 1–5 μl./ml.

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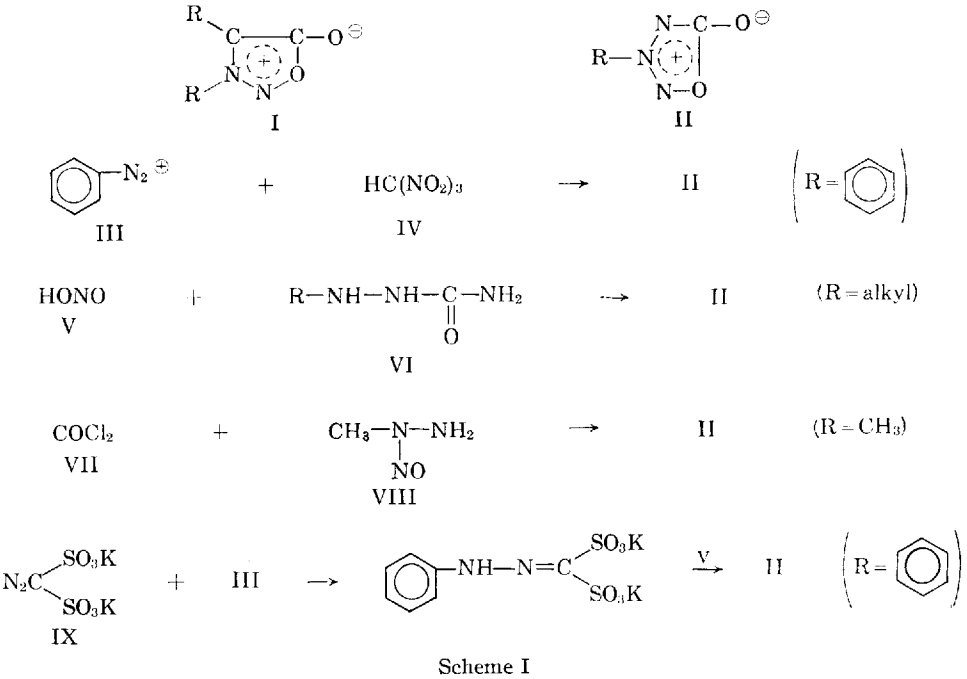

Scheme I

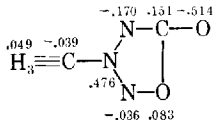

Fig. 1.—Electronic structure of methyl Ψ-oxatriazole from molecular orbital calculations.

TABLE I.—BLOOD PRESSURE FALL AT VARIOUS INTERVALS IN ANESTHETIZED DOGS

Ψ-Oxatriazole, 20 mg./Kg.	Blood Pressure Fall in % of Normal					
	T + 10 sec.	T + 5 min.	T + 10 min.	T + 15 min.	T + 30 min.	T + 60 min.
Methyl	19	9	8	10		
Ethyl	20	12	12	16		
Isopropyl	34	25	30		21	24
3-Pentyl	31	28	32		36	33
sec-Butyl	57	36	46		41	36
tert-Butyl	54	34	43		53	57

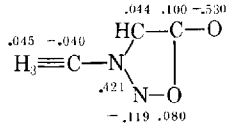

Fig. 2.—Electronic structure of methyl sydnone. (From *Reference 10.*)

The Ψ-oxatriazoles then, in doses that produce no obvious toxic effects, produce in dogs prompt, prolonged hypotensive effects which may well be due to the relaxation of smooth muscle at the periphery.

DISCUSSION

From Table I it is evident that the decreasing order of potency at the dose level studied is *tert*-butyl > *sec*-butyl ≧ 3-pentyl ≧ isopropyl > ethyl > methyl. This is in the increasing order of mesomeric contribution of the alkyl group to the aromatic ring. A minimum mesomeric contribution to ring then appears to improve the hypotensive potency.

An examination of the electronic structure of methyl Ψ-oxatriazole in Fig. 2 reveals that a major portion of the positive character of the ring resides on the trisubstituted nitrogen atom. The possible significance of the positive character of the ring, and especially the positive character of the trisubstituted nitrogen atom is revealed in a comparison of the Ψ-oxatriazoles, the 3-phenyl-4-acylsydnones, and the alkylsydnones. Greco and Kier (11) have shown that the 3-phenyl-4-acylsydnones possess a moderate hypotensive potency, while the alkylsydnones show less hypotensive activity (4). The order of potency and the corresponding charge densities of the trisubstituted nitrogen atoms for these three series of compounds are:

Ψ-oxatriazoles	>	3-phenyl-4-acylsydnones	>	3-alkylsydnones
q_n = +.476		q_n = +.448		q_n = +.421

This trend suggests an important role for the trisubstituted nitrogen atom as a partial cationic moiety in the interaction of the compound with a receptor. If this correlation is correct, the prediction follows that the as yet untested phenyl-Ψ-oxatriazole with a trisubstituted nitrogen charge density of +.484 should be a more potent hypotensive agent than the compounds reported.

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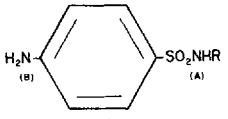
Molecular Orbital Calculations on Some Sulfanilamides

By A. CAMMARATA

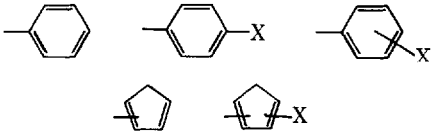
Simple HMO-calculations on three sulfanilamides have been done using semi-empirical parameters for the SO_2 group which intrinsically take into account the effects of *d*-orbital overlap. Charge variations at the sulfonamido and at the aromatic *p*-amino position are noted which are in qualitative agreement with expectations.

RECENTLY a number of quantum chemical calculations on substituted sulfanilamides have been reported (1, 2). These calculations indicated that a variation in R in structure I results in a variation



I

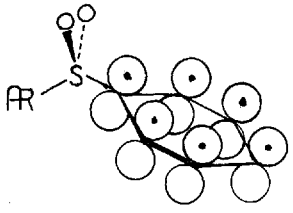
of the positive formal π-electronic charge on nitrogen A whereas the positive formal π-electronic charge on nitrogen B remains unaffected. When these compounds were classified into groups depending on the nature of R, where R was



and where any of these R may contain heteroatoms (2), it was found that the variation in formal charge on nitrogen A was consistent with their pKa's, *i.e.*, higher formal charge, lower pKa. Hence, it was concluded that (*a*) the well known relationship between bacteriostatic activity and pKa (3) has a quantum chemical foundation; and (*b*) although a *p*-amino group may be necessary for activity, the insensitivity of the formal charge on nitrogen B to variations in R did not support the view (4–6) that the bacteriostatic action of the sulfanilamides is primarily associated with the *p*-amino group.

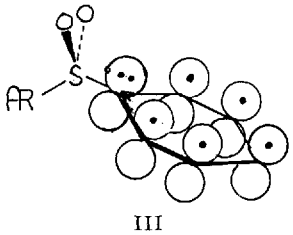
Since Seydel (5, 6) has presented evidence, based on infrared studies, indicating that substituents placed on the sulfonamido-nitrogen are able to influence the electronic environment of the *p*-amino group, it is expected that charge variations at the *p*-amino position should be calculable by making a suitable choice of Hückel parameters for the SO_2 group. Apparently, any choice of parameters which is made for the SO_2 group should somehow include the contribution by the sulfur *d*-orbitals to the overall conjugation, for neglect of such participation, as found by Martin (1, 2), does not lead to variations in charge at the *p*-amino position.

The approach taken here is to utilize semiempirical parameters derived for the SO_2 group from electron paramagnetic resonance (EPR) studies on diphenyl and *p*,*p*′-ditolyl sulfone anion radicals (7) in simple Hückel molecular orbital (HMO) calculations on substituted sulfanilamides. While objections may be raised to the application to sulfanilamides, of parameters derived from ESR studies on diarylsulfones, a rationalization for their use can be provided. Pictorially, a diarylsulfone may be represented as in II, where only one of the aromatic rings has its π-



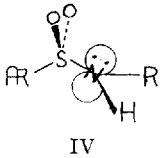
II

network described, and where the dot inside of each *p*-orbital represents the electron which may be associated with that orbital.[1] Formation of a diarylsulfone anion radical infers that an extra electron is being donated to the π-network. For conjugation to occur across the sulfur atom at some time the *p*-orbital adjacent to the sulfur atom must contain two electrons (this in keeping with the simple pictorial representation in II), one electron which was originally associated with that orbital and the other the electron which was donated to the π-network. This situation may be represented as in III, which at the atom adjacent to sulfur is



III

formally analogous to a similar representation of sulfanilamide, and presumably its derivatives (IV).



IV

Note, it is also tacitly assumed that the amido-nitrogen is in an sp^2-hybridized state to enable

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[1] This type of description appears generally accepted: "For delocalization of π-electron density along the length of a chain or the circumference of a ring, it is necessary that *each atom* comprising the chain or ring have associated with it at least one *p*-type electron that is not being employed in formation of single bonds" (11). "Each p_z-electron is paired with its neighbor, and the *p*-orbitals overlap in the π-manner around the ring" or along the length of a chain (12). The description is equivalent to one in which only molecular orbitals are considered, as in arriving at molecular orbitals one must make linear combinations of these one-electron atomic orbitals.

conjugation through the *d*-orbitals of the sulfur atom. Such conjugation would require overlap of a sulfur *d*-orbital with the *p*-orbital on nitrogen (8, 9).

Using the Hückel parameters $\alpha_{SO_2} = \alpha_C - 2.60\ \beta_{C-C}$ and $\beta_{C-SO_2} = 0.99\ \beta_{C-C}$ which were derived for the sulfone group in the above-mentioned EPR studies, and which intrinsically take into account *d*-orbital participation, the formal π-electronic charges of three sulfanilamides were calculated. As these calculations were performed without the aid of a computer the following simplifying assumptions were made for the exchange integrals involving the sulfone group: $\beta_{C-SO_2} = \beta_{C-C}$ and $\beta_{N-SO_2} = \beta_{C-C}$.[2] The Coulomb and exchange integrals used for all other atoms and bonds were those suggested by Streitwieser (10).

The compounds selected were chosen primarily to establish whether, by using this approach, charge variations could be noted at the *p*-amino position for select sulfanilamide derivatives. Thus, in Fig. 1 there are reported the calculated formal π-electronic charges for: (*a*) an electron-donating substituent, (*b*) unsubstituted sulfanilamide, and (*c*) an electron-withdrawing substituent. Inspection of the calculated formal π-electronic charges on the sulfonamido and on the *p*-amino position indicate their respective values to be in accord with expectations. The charge at the *p*-amino position appears to vary in accord with the type of substituent placed on the amido nitrogen, *i.e.*, low for an electron-donating substituent, high for an electron-withdrawing substituent, and the charge at the amido position also appears consistent for the type of group substituted on it. Furthermore, the apparent magnitudes of the charge variation at the amido position (0.07) relative to the *p*-amino position (0.02) is consistent with the observation of Bell and Roblin (3) that whereas the pKa's of a series of sulfanilamides may vary over a wide range their pK_b's (for the *p*-amino group) remain within a very narrow range. It should be emphasized that, at present, these calculations must be considered as being more qualitative than quantitative. Although the trends suggested are in reasonable accord with observation it must yet be demonstrated that these trends provide a correlation. This aspect is currently under investigation. These calculations, however, do suggest that if the effects of *d*-orbital overlap, due to the sulfur of the SO_2 group, are taken into account, variations in electronic charge at the *p*-amino position of sulfanilamides could be noted.

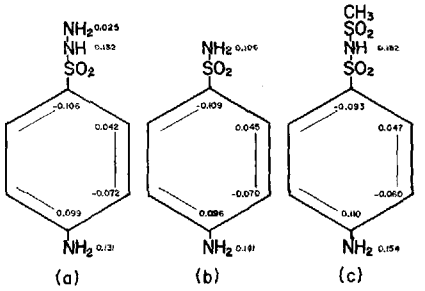

Fig. 1.—Calculated formal π-electronic charges. Key: a, an electron-donating substituent; b, unsubstituted sulfanilamide; c, an electron-withdrawing substituent.

[2] The error introduced by the approximation $\beta_{N-SO_2} = \beta_{C-C}$ is expected to be no greater than that introduced in calculations on hydrocarbons in which it is assumed that all β's are equal. This arises from the following considerations: k is generally assumed to be proportional to the overlap integral, S (13) and is linearly related to the bond distance (*Reference 10*, pp. 104–105). Aromatic double bonds have lengths 1.33–1.35 Å., while single sp^2–sp^2 bonds, *e.g.*, the central bond in butadiene, have lengths 1.48–1.50 Å. For precise work with hydrocarbons it is apparent that different β-values should be used depending upon the type of bond. This is seldom done in comparative studies. The S—N and S—C bond distances for sulfanilamides are 1.62 and 1.75 Å., respectively (14, 15). As can easily be seen, the difference in bond distance between aromatic and single sp^2–sp^2 bonds is approximately equal to the difference in bond distance between the S—N and S—C bonds in sulfanilamide. Thus, since $\beta_{C-SO_2} = \beta_{C-C}$ is a semiempirically determined quantity, it appears valid to assume $\beta_{N-SO_2} = \beta_{C-C}$. The approximation is not expected to greatly affect the calculated charge densities (*cf. Reference 10*, p. 106). The largest source of error is expected to be due to the Hückel theory itself, especially when applied to materials containing heteroatoms. However, while the absolute magnitudes of the calculated quantities cannot be considered significant, their values *relative* to those for other homologs have direct bearing on their physical interpretation.

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Microbiological Testing of Aerosol Preparations Using *Chaetomium globosum*

By J. W. BLASECKI, JR.*, and M. IANNARONE

Chaetomium globosum, **a common fungus of the soil, and a member of the class *Ascomycetes,* is a cellulolytic organism, whose growth is dependent primarily upon the degradation of cellulose material. This study explores the possibility of utilizing a modified growth and test procedure applicable to the A.O.A.C. recommended prescribed method for testing antifungal activity of aerosols. The test procedure developed brings the method to the organism under conditions that are applicable to the natural growth habits. A convenient method is described employing the inhibition of growth of *C. globosum* on sterile filter paper after exposure to certain aerosols. Sterile phenol solutions and certain blends of quaternary ammonium compounds were used as the challenging agents.**

THE USE of aerosol preparations in the treatment of fungal infestations requires appropriate microbiological testing procedures. At present, the A.O.A.C. (1) employs a method involving a *Trichophyton* sp., a pathogen, as the test object. In these laboratories, a simplified, convenient, and reliable method has been developed utilizing a nonpathogenic fungal organism, *C. globosum*. *C. globosum* (ATCC-6205) a common fungus of the soil, is a cellulolytic organism, depending primarily upon the degradation of cellulose materials for its growth (2). A sterile strip of filter paper was placed on a cooled tube-slant of a suitable medium,[1] and the strip was inoculated with spores. This was used as the stock culture after incubation for 7 days at 25–30°. Sterile Whatman No. 2 filter paper was used as the source of cellulose. This growth pattern and property was adapted to the evaluation of antifungal activity, since the inhibition of growth of this organism on sterile filter paper was easily observed after exposure to certain aerosols.

PROCEDURE

The A.O.A.C. spray test procedure (1, 3, 4) for evaluating the antifungal activity of aerosol preparations recommends that a standardized spore suspension of *Trichophyton interdigitale* (ATCC-640) be spread over an area of 1 sq. cm. Thus, it was decided that the test organism for this study would be the spores and vegetative structures of *C. globosum*. This stage of growth was produced on 1-cm. squares of filter paper at the end of 7 days at 25–30°. Spray testing (1) was performed at this stage of growth.

A 1-cm. square containing the test organism was removed from the medium, using sterile forceps, and placed on a sterile glass slide in a Petri dish, the bottom of which was layered with two disks of sterile filter paper. This was repeated for all of the remaining squares (10 per test). The above were placed in an incubator at 25–30° for 2 hr. to allow the squares to dry.

At the end of this period, the plate was supported at a 45° angle, and the respective squares were sprayed using phenol controls and test compounds (Table I) under test for the specified period of time,[2] at a distance of 8 to 10 in. from its surface. The above procedure was repeated for all plates under test. A DeVilbiss No. 251 atomizer was used to produce the spray of the phenol control solutions.

After all squares were sprayed, each one was aseptically removed, respectively, from the glass slide, and was placed on the filter paper bed Petri dish. The dishes were placed in an incubator 24 hr. for drying the sprayed filter squares.

Using aseptic technique, a 2 × 2-cm. square of sterile filter paper was placed on the medium in a Petri dish and the dried 1-cm. square was transferred on to the 2 × 2-cm. square. This was repeated for all the recommended number (ten) of 1-cm. squares. All of the above dishes were incubated for 7 days at 25–30°. Untreated 1-cm. squares containing the viable test organism, were transferred to 2 × 2-cm. squares to serve as controls. This insures the viability of the test organism on both the 1 and 2 × 2-cm. squares and serves to establish a base for the growth pattern of the organism. Growth of the test organism on to the 2 × 2-cm. square at the end of the 7 days was read as positive, while no growth was read as negative (Tables II and III).

The 1 and 2 × 2-cm. squares displaying no apparent growth 7 days after spraying were divided into two groups of equal number. Control procedures were initiated on one group immediately, while the other group was reincubated for an additional 7 days. The same control procedures were then repeated on this second group. (Those squares exhibiting no growth after the first 7 days of incubation remained so for the additional 7 days.)

The following control procedures were utilized for the negative no-growth squares.

A.—The 1-cm. square was removed from the original backup square, inverted on a new 2 × 2-cm. square on fresh medium, and reinoculated with spores from a stock culture. The above was repeated until half of the 1-cm. squares were treated.

B.—The remaining 1-cm. squares were inverted

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[1] Medium recommended by American Type Culture Collection: $NaNO_3$, 2.0 Gm., $MgSO_4$, 500 mg., KCl, 500 mg., KH_2PO_4, 140 mg., K_2HPO_4, 1.20 Gm., yeast extract, 20 mg., agar, 15.0 Gm., water, 1.0 L., $Fe_2(SO_4)_3 \cdot XH_2O$, 10 mg. The final pH of the medium is in the range 7.0–7.2.

[2] Spraying time with the DeVilbiss No. 251 atomizer was 5 sec., while spraying time with the commercial pressurized aerosols (as with the quats) was 3 sec.

TABLE I.—SOLUTIONS AND COMPOUNDS TESTED

1, Sterile phenol solutions of the following concentrations (% w/v): 0.5; 1.0; 2.0; 3.0; 4.0; 5.0; 5.25; 5.75; 6.0; 7.0.	
2, Pressurized aerosols of quaternary ammonium compounds. The two formulations used were as follows (% w/w):	
A, BTC-2125[a]	0.1
Dichlorodifluoromethane[b]	50.0
95% Ethanol	49.9
B, Cyncal-type 14[c]	0.1
Dichlorodifluoromethane[b]	50.0
95% Ethanol	49.9

[a] Onyx Chemical Corp., Jersey City, N. J. *n*-Alkyl dimethyl benzyl ammonium chlorides, 25% (C_{14}, 60%; C_{16}, 30%; C_{12}, 5%; C_{18}, 5%). *n*-Alkyl dimethyl ethylbenzyl ammonium chlorides, 25% (C_{12}, 50%; C_{14}, 30%; C_{16}, 17%; C_{18}, 3%). Inert, 50%. [b] Marketed as Freon-12 by E. I. du Pont de Nemours & Co., Wilmington, Del. [c] Sterwin Chemicals, Inc., Industrial Chemicals Division, Subsidiary Sterling Drug, Inc., New York, N. Y. Alkyl dimethyl benzyl ammonium chlorides, 80% (C_{14}, 50%; C_{12}, 40%; C_{16}, 10%). Ethanol, 20%.

TABLE II.—STANDARDIZING PHENOL ACTIVITY USING *C. globosum* AS TEST ORGANISM

% Phenol (% w/v)	Sq. Showing Growth 7 Days After Spraying (%)
Untreated controls	100
0.5	100
1.0	100
2.0	100
3.0	100
4.0	100
5.0	100
5.25	60
5.50	30
5.75	10
6.0	0
7.0	0

TABLE III.—ANTIFUNGAL ACTIVITY OF TWO PRESSURIZED AEROSOL FORMULATIONS

Formulation	Sq. Showing Growth 7 Days After Spraying (%)
A, BTC-2125[a]	0
B, Cyncal-type 14[a]	0
Untreated controls	100

[a] See Table I.

directly on fresh medium and reinoculated with spores from a stock culture.

C.—All 2 × 2-cm. backup squares, on the original medium, were inoculated directly with spores from a stock culture.

Controls were incubated for 7 days. Growth of the organism in each of the controls at the end of this period indicates antifungal activity of the test material, and could not be due to stasis caused by possible absorption of the test material by the filter paper.

In addition, an uninoculated square was sprayed,[3] according to the prescribed procedure, and placed in an incubator for 24 hr. It was transferred to a Petri dish and challenged with spores from a stock culture.

All stasis controls displayed growth in 7 days.

CONCLUSIONS

The results presented in this paper suggest the possiblity of utilizing the prescribed procedure for testing the antifungal activity of certain aerosol preparations using *C. globosum*. Similar organisms could be adapted to the test method. Since the only challenging chemical employed was phenol, and the problem of differentiating between stasis and cidal effects in any procedure of this kind was complicated by the substantive properties of the chemical and its ability to diffuse into the media and/or the back-up 2 × 2-cm. squares in this instance, it would appear that differentiation between stasis and cidal activity is valid.

The test organism, used throughout the procedure, consisted of both the spores and the entire vegetative structure. It will be recognized, however, that if the material under test was effective against spores, it should be similarly effective against the vegetative cells. It could be assumed that the use of the test material on the entire structure of the organism was a more drastic test and closely simulates the use of the test material under the natural growth conditions.

The use of the 1-cm. square of filter paper for the growth of the test organism and its use in the aerosol testing procedure was considered to be valid, since the spores inoculated on any given square were under the exact same growth conditions, with respect to time, temperature, and medium, as those on other squares. This was further substantiated by the fact that the growth of the organism was restricted to the 1 sq. cm. area of the filter paper. Thus, it could be assumed that any given 1-cm. square, at the end of 7 days, contains approximately the same amount of vegetative growth and about the same number of spores.

Experience in these laboratories indicate that the procedure described is simpler, more convenient, more reliable, and safer than the present A.O.A.C. method.

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[3] 6.0% phenol, BTC-2125, and Cyncal-type 14 were the three test compounds used in this particular control.

Sympathetic Blocking Action of Iproniazid in the Unanesthetized Dog

By J. N. SPENCER

Iproniazid, on intravenous injection in the unanesthetized dog, inhibited the increase in arterial blood pressure resulting from the intravenous injection of epinephrine, DMPP, and tyramine. Adrenergic blockade appeared to be an important factor in this action of iproniazid, although the possibility of inhibition of the release of norepinephrine from nerve terminals could not be eliminated. These results confirm *in vitro* studies of previous investigators.

THERE is abundant clinical and experimental evidence that drugs which inhibit monamine oxidase (MAO) are capable of altering sympathetic responsiveness. Patients receiving these drugs commonly exhibit orthostatic hypotension (1). On the other hand, the same patients on eating a food high in tyramine, such as cheese, may develop an alarming hypertension (2). The mechanism responsible is not clear. Goldberg and Da Costa (3) as well as Gertner (4) noted that a number of MAO inhibitors block transmission through the superior cervical ganglion of experimental animals. However, Zbinden *et al.* (5) and Brodie (6) are of the opinion that MAO inhibitors do not act at the ganglion, but at the receptor site, preventing the release of norepinephrine from the nerve endings. Either site of action could account for the above effects. Both views, however, are based on *in vitro* studies or studies conducted on animals under barbiturate anesthesia. Barbiturates are known to alter neurohormone release (7) even to the point of reversal of the response to common autonomic stimuli (8). In view of this, it was deemed of value to determine the effect of a MAO inhibitor on the autonomic responsiveness of trained unanesthetized dogs.

EXPERIMENTAL

Healthy, adult, mongrel dogs of either sex, weighing from 12 to 29 Kg., were used in the study. The animals were trained to lie quietly on their backs, with minimal restraint during the recording of arterial blood pressure from a femoral arterial puncture. Records of the blood pressure and the circulatory response to the intravenous injection of epinephrine (0.002 mg./Kg.), DMPP[1] (0.08 mg./Kg.), acetylcholine (0.006 mg./Kg.), and tyramine (0.1 mg./Kg.) were obtained every 48 to 72 hr. until the blood pressure had stabilized and a consistent circulatory response to the above agents had been obtained in at least three successive tests. The average of the values obtained in the three tests served as the experimental control.

The MAO inhibitor, iproniazid,[2] was dissolved in normal saline and injected intravenously in terms of base content in a single dose of 10 or 20 mg./Kg. or in two doses of 20 mg./Kg. each, 24 hr. apart. Records were obtained of the blood pressure and the circulatory response to the injection of epinephrine, DMPP, acetylcholine, and tyramine 24, 48, 72, 96, and 120 hr. after the injection of the iproniazid.

RESULTS

The administration of iproniazid in a single dose of 10 or 20 mg./Kg. was without significant effect on the blood pressure or the circulatory response to the injection of epinephrine, DMPP, acetylcholine, or tyramine. However, following two doses of 20 mg./Kg., each 24 hr. apart, within 24 to 48 hr. there was a marked and statistically significant inhibition of the pressor reaction to the injection of epinephrine, DMPP, and tyramine (Table I). This occurred in the absence of any significant alteration in the recumbent blood pressure or in the depressor action of acetylcholine and was evident for 96 to 120 hr. after the injection of iproniazid. The effect on the action of tyramine was the most marked, the pressor response being reversed to one of the depressor within 24 hr. (Table I and Fig. 1). The alteration in the action of epinephrine and DMPP was not as marked as that of tyramine, although reversal of the pressor action of both agents was observed.

From the above it would appear that iproniazid has an adrenergic blocking action. Adrenergic blockade would account for the inhibition of the action of epinephrine and DMPP as well as the reversal of the pressor action of tyramine. Tyramine not only releases norepinephrine from tissue stores but also acetylcholine (9). In the presence of adrenergic blockade, its cholenergic action predominates. Excitement following the administration of atropine prevented the determination of the involvement of acetylcholine in the depressor action of tyramine in the unanesthetized dog, but in animals anesthetized with chloralose 24 hr. after the injection of iproniazid, atropine completely blocked the tyramine depressor response. Thus, acetylcholine release appeared to be implicated in the reversal of the pressor action of tyramine. However, it cannot be determined from this study if adrenergic blockade was the only factor involved in the alteration of autonomic responsiveness produced by iproniazid. The fact that the pressor action of both DMPP and tyramine were blocked at a time when the inhibition of the action of epinephrine was minimal (120 hr. observation) suggests the possibility of an interference with norepinephrine release from nerve terminals. Such an action, like adrenergic blockade, would uncover the cholinergic action of tyramine (9).

Results comparable to those obtained with iproniazid were observed on occasion in unanesthetized dogs following the intravenous injection of tranylcypromine or β-phenylisopropylhydrazine.

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[1] 1:1 Dimethyl-4-phenylpiperzinium iodide.

[2] Trademarked as Marsilid by Hoffmann-LaRoche, Inc., Nutley, N. J.

TABLE I.—EFFECT OF IPRONIAZID ON BLOOD PRESSURE AND THE ACTION OF EPINEPHRINE, DMPP, ACETYLCHOLINE, AND TYRAMINE (MEAN OF 5 EXPERIMENTS)

Observation Time After Iproniazid	Blood Pressure, mm. Hg			Action of Epinephrine		Action of DMPP		Action of Acetylcholine		Action of Tyramine	
	Systolic Pressure	Diastolic Pressure	Change (Systolic/Diastolic)	mm. Hg Change in Systolic Pressure	% Decrease from Control	mm. Hg Change in Systolic Pressure	% Decrease from Control	mm. Hg Change in Systolic Pressure	% Deviation from Control	mm. Hg Change in Systolic Pressure	% Decrease from Control
0 (control)[a]	119 ± 5[b]	66 ± 3	...	34 ± 6	...	44 ± 7	...	−31 ± 7	..	22 ± 8	...
24 hr.	116	68	−3/2	11	68	27	39	−33	6	−10	145[d]
48 hr.	120	62	1/−4	21	38[c]	21	52[c]	−31	0	−10	145[d]
72 hr.	112	63	−7/−3	13	62[c]	12	73[c]	−26	16	0	100[d]
96 hr.	107	55	−12/−11	13	62[d]	13	70[c]	−38	22	−15	169[c]
120 hr.	116	61	−3/−5	28	18	16	64[c]	−35	13	−4	118[c]

[a] Mean of all control observations. [b] ± Standard error of mean. [c] P, 0.05 (Wilcoxon test of rank sums). [d] P, 0.02 (Wilcoxon test of rank sums).

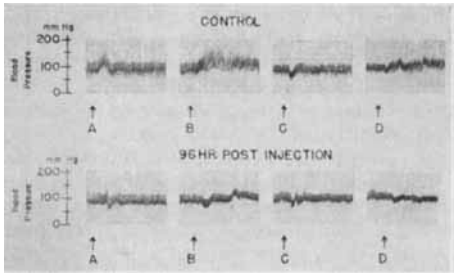

Fig. 1.—Effect of iproniazid on the response of the blood pressure to the injection of autonomic agents. Arrows indicate points of intravenous injection of autonomic agents. Key: A, 0.08 mg./Kg. DMPP; B, 0.002 mg./Kg. epinephrine; C, 0.006 mg./Kg. acetylcholine, D, 0.1 mg./Kg. tyramine. (Dog 4, 23.5 Kg. male.)

The effect, however, tended to be masked by the amphetamine-like action of these drugs (10, 11). Thus, tranylcypromine and β-phenylisopropylhydrazine as well as iproniazid inhibit autonomic responsiveness in the unanesthetized dog. Adrenergic blockade appears to play an important role in this action, although inhibition of norepinephrine release from nerve terminals may be a factor. These observations confirm previous results obtained in *in vitro* studies and in studies conducted on anesthetized animals (5, 6).

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Errata

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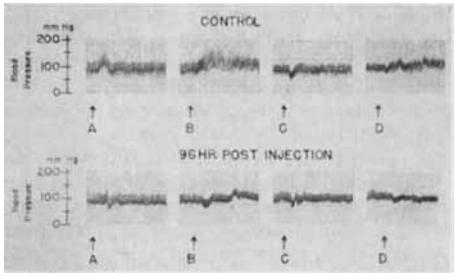

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